

**INTERNATIONAL SOCIETY  
FOR THE STUDY OF HARMFUL ALGAE**

**12th International Conference  
on Harmful Algae**

**PROCEEDINGS**



**Copenhagen, Denmark**

**Intergovernmental Oceanographic Commission of UNESCO**

**Proceedings of the 12th International Conference on  
Harmful Algae, Copenhagen, Denmark, 4-8 September  
2006**



# **Proceedings of the 12th International Conference on Harmful Algae, Copenhagen, Denmark, 4-8 September 2006**

Editor-in-Chief

**Øjvind Moestrup**, Copenhagen

Associate Editors

**Greg Doucette**, Charleston

**Henrik Enevoldsen**, Copenhagen

**Anna Godhe**, Göteborg

**Gustaaf Hallegraeff**, Hobart

**Bernd Luckas**, Jena

**Nina Lundholm**, Copenhagen

**Jane Lewis**, London

**Karin Rengefors**, Lund

**Kevin Sellner**, Edgewater

**Karen Steidinger**, St. Petersburg, USA

**Patricia Tester**, Beaufort, NC

**Adriana Zingone**, Naples

International Society for the Study of Harmful Algae

Intergovernmental Oceanographic Commission of UNESCO

2008

**For bibliographic purposes this publication should be cited as:**

Moestrup, Ø. *et al.* (eds) Proceedings of the 12th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen

**ISBN:** 978-87-990827-1-1

**Printed by:** Joint Stock Printing Company of Science and Technology (Vietnamese Academy of Science and Technology)

## INTRODUCTION

The 12th International Conference on Harmful Algae (12th ICHA) took place at DGI-Byen in central Copenhagen on 4-8 September 2006. It was the first conference of the series in which ISSHA (International Society for the Study of Harmful Algae) was directly involved, and this engagement was highly successful. The conference was discussed in a preliminary way by members of the International and the Local Committees in Cape Town immediately following the XI Conference, and the detailed programme was prepared at a joint whole-day meeting of the two committees at the Danish Institute of Fisheries Research in Charlottenlund, Denmark during the summer 2005.

540 delegates from 60 countries participated in 12th ICHA which comprised plenary talks, oral sessions, poster sessions and symposia.

The conference was opened by the rector of Copenhagen University, Dr Ralf Hemmingsen, whose welcome speech was followed by a short fairy tale of Hans Christian Andersen read by Kirsten Olesen, one of the best-known Danish actors.

A few weeks prior to the conference the Baltic Sea near Copenhagen was covered with some of the largest blooms of the toxic blue-green alga (cyanobacterium) *Nodularia spumigena*, and the blooms extended into the harbour of Copenhagen, preventing bathing and causing general concern in the city.

However, at the time of the conference the blooms had been dispersed by wind.

The city of Copenhagen invited the conference to a reception at the City Hall. Other social events included an auction, tirelessly orchestrated by Barrie Dale, and whose revenue was given ISSHA. The Conference Dinner took place at Langelinie Pavillionen, where participants were brought by boat from the conference centre.

Manuscripts submitted to these proceedings were initially handled by 12 associate editors, each of whom oversaw reviewing of the manuscripts. Accepted manuscripts were read and edited by the chief editor, assisted by Pia Haecky, Copenhagen, who was responsible for setting up the manuscripts for publication. Dr. Nguyen Ngoc Lam and Institute of Oceanography, Vietnam, kindly assisted with the printing and distribution of the proceedings.

On behalf of the organizers I wish to thank the participants, the editors, the reviewers, the auctioneer, the sponsors and not the least ISSHA, headed by President Patricia Tester, for making this conference so successful.

Copenhagen, June 2008  
Øjvind Moestrup



**ISSHA CONFERENCE COMMITTEE**

Don Anderson, USA  
Allan Cembella, Germany  
Barrie Dale, Norway  
Greg Doucette, USA  
Henrik Enevoldsen, IOC  
Gustaaf Hallegraeff, Australia  
KC Ho, Hongkong/China  
Jane Lewis, UK  
Øjvind Moestrup, Denmark  
Pat Tester, USA  
Mingjiang Zhou, China  
Adriana Zingone, Italy

**LOCAL ORGANIZING COMMITTEE 12th ICHA**

Øjvind Moestrup (Convener)  
Per Andersen  
Thyra Bjergskov  
Kirsten Christoffersen  
Gertrud Cronberg  
Niels Daugbjerg  
Lars Edler  
Marianne Ellegaard  
Henrik Enevoldsen  
Anna Godhe  
Edna Granéli  
Gert Hansen  
Per Juel Hansen  
Peter Henriksen  
Kevin Jørgensen  
Jacob Larsen  
Nina Lundholm  
Karin Rengefors  
Helge A. Thomsen

**Main sponsors of the 12th ICHA:**

Intergovernmental Oceanographic Commission of UNESCO (IOC)

The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS)

The Danish Natural Science Research Council

The European Commission

The United States National Oceanic and Atmospheric Administration, National Ocean Service, Center for Sponsored Coastal Ocean Research

The Carlsberg Foundation

University of Copenhagen

Directorate for Food, Fisheries and Agri Business, Danish Ministry of Food, Agriculture and Fisheries

Danish Veterinary and Food Administration, Ministry of Family Affairs

**Other Sponsors:**

Scandinavian Airlines

Satlantic

Danish Shellfish Center

Elsevier

Fluid Imaging Technologies

Marbef

Fjord's Mussels

City of Copenhagen

Heinz Waltz GmbH

**ISSHA Travel Award Sponsors:**

Scientific Committee on Oceanic Research

Royal Danish Ministry of Foreign Affairs (DANIDA)

Mediterranean Action Plan/UNEP

Prof. T. Yasumoto

United States National Science Foundation

## CONTENTS

### 1. ALLELOPATHY

Piumsomboon, A., Soasii, P., Sivaipram, I., Songroop, C., Rungsupa, S. & Fukami, K.: Relationship between heterotrophic bacteria and bloom-forming phytoplankton species from the coastal area of Thailand

2

---

Place, A.R., Brownlee, E.F., Nonogaki, H., Adolf, J.E., Bachvaroff, T.R., Sellner, S.G. & Sellner, K.G.: Responses of bivalve molluscs to the ichthyotoxic dinoflagellate *Karlodinium veneficum*

5

---

Spilling, K.: Diatom effect on dinoflagellate growth

9

---

Tillmann, U., John, U., Krock, B. & Cembella, A.: Allelopathic effects of bioactive compounds produced by harmful algae

12

---

Vassilakaki, M. & Pflugmacher, S.: Promotion of oxidative stress in *Synechocystis* sp. strain PCC 6803 via cyanobacterial toxins

19

---

Wiegand, C., Jarosch, A., Behrend, A. & Krause, E.: Physiological stress responses of *Daphnia magna* exposed to cyanobacterial compounds

22

---

### 2. ECOPHYSIOLOGY AND AUTECOLOGY

Baptista, M.F. & Vasconcelos, M.T.S.D.: Metal concentration in freshwater sediments seasonally subjected to toxin-producing cyanobacterial blooms

26

---

Doan-Nhu, H., Nguyen Thi, M.A. & Nguyen-Ngoc, T.G.: *Pseudo-nitzschia* in south central coastal waters of Vietnam: growth and occurrence related to temperature and salinity

29

Fensin, E. & Touchette, B.W.: Going beyond nutrients: the role of environmental factors in shaping harmful algal blooms in estuarine waters

33

---

Lekan, D.K. & Tomas, C.R.: Effects of varying salinity and N:P ratios on the growth and toxicity of *Karenia brevis*

36

---

Li, J., Glibert, P.M., Lu, S., Lu, D., Shi, X. & Zhang, C.: Nitrogen uptake rates during a dinoflagellate bloom in the East China Sea, 2005: variation with N:P ratio

40

---

Lindberg, V., Mohlin, M. & Wulff, A.: UV responses in three strains of the cyanobacterium *Nodularia spumigena*

44

---

Yoshida, T., Takahashi, Y., Ishikawa, K., Ming-Kei, W. & Hiroishi, S.: Survival of *Heterocapsa circularisquama* (Dinophyceae) as a pellicle cysts induced by low temperature in the laboratory

47

---

### 3. MOLECULAR GENETICS

Alpermann, T.J., Bezteri, B., Tillmann, U., Cembella, A.D. & John, U.: Species discrimination in the genus *Alexandrium* by Amplified Fragment Length Polymorphism

51

---

Bachvaroff, T.R., Adolf, J.E. & Place, A.R.: Phylogeography of Atlantic Coast *Karlodinium veneticum* strains: a genetic marker correlate of toxin type 55

Cho, Y., Hiramatsu, K., Ogawa, M., Omura, T., Ishimaru, T. & Oshima, T.: Genetic characteristics of non-toxic subclones obtained from toxic clonal culture of *Alexandrium tamarense* (Dinophyceae)

59

---

Elandaloussi, L., Venail, R., Quijano-Scheggia, S., Fernández-Tejedor, M., Mallat, E., Diogène, J., Garcés, E., Camp, J. & Andree, K.: Molecular tools for the identification of *Pseudo-nitzschia caliantha* and *P. delicatissima* in the Ebre Delta, Spain

62

Jaeckisch, N., Singh, R., Curtis, B., Cembella, A. & John, U.: Genomic characterization of the spirolide-producing dinoflagellate *Alexandrium ostenfeldii* with special emphasis on PKS genes  
65

Krüger, T., Oelmüller, R., Hiller, S. & Luckas, B.: Differences within the nodularin synthetase gene cluster between toxic *Nodularia spumigena* and non-toxic *Nodularia harveyana*  
68

Litaker, W., Mason, P., Jeong, H.J., Vogelbein, W., Vandersea, M. Kibler, S. & Tester, P.: Using distinct ribotype groups in recognizing dinoflagellate HAB species  
72

#### 4. HAB MONITORING

Bresnan, E., Turrell, E. & Fraser, S.: Monitoring PSP toxicity and *Alexandrium* hotspots in Scottish waters  
76

Diogène, J., Fernández, M., Cañete, E., Caillaud, A., Mallat, E., Delgado, M. & Furones, D.: The monitoring programme for harmful algal blooms in shellfish production areas in Catalonia. Long term data and impact on aquaculture  
80

Kirkpatrick, G.J. Millie, D.F., Moline, M.A. Lohrenz, S.E. & Schofield, O.M.: Phytoplankton community composition observed by autonomous underwater vehicles  
83

Licea, S., Navarrete, A., Bustillos, J. & Martínez, B.: Monitoring a bloom of *Pyrodinium bahamense* var. *compressum* in El Salvador and the southern coast of Mexico (November 2005-March 2006)  
86

Poulton, N., Nelson, H. & Sieracki, C.: Identifying and detecting harmful algal bloom species using a colour imaging flow cytometer (FlowCAM®)  
90

Steidinger, K.A., Tustison, J.A., Weisberg, R.H., Barth, A. & Heil, C.A.: Retrospective GIS analyses of the Florida red tide database

93

---

Stumpf, R.P.: Developing operational capabilities for nowcasts and forecasts of harmful algal blooms

96

---

Tomlinson, M.C., Wynne, T.T., Stumpf, R.P., Dyble, J., Fahnenstiel, G.L. & Tester, P.A.: Using remote sensing to aid in the detection and monitoring of *Microcystis aeruginosa* in western Lake Erie and Saginaw Bay, USA

99

---

Wynne, T.T., Stumpf, R.P., Tomlinson, M.C., Villareal, T.A., Wiles, K., Heideman, G., Byrd, M., Buzan, D. & Campbell, L.: Moving towards an operational harmful algal bloom forecasting system in Texas (USAS)

103

---

## 5. POPULATION DYNAMICS

Adolf, J.E., Bachvaroff, T.R. & Place, A.: Manger à trois: toxic and non-toxic *Karlodinium veneficum* strains with a predator, *Oxyrrhis marina*, and prey, *Storeatula major*

107

---

Estrada, M., Arin, L., Blasco, D., Baluw, A., Camp, J., Garcés, E., Sampedro, N. & Vila, M.: A fuzzy logic model for *Alexandrium minutum* proliferations in harbours of the Catalan coast (NW Mediterranean)

111

---

Fernández-Tejedor, M., Elandaloussi, L.M., Mallat, E., Cañete, E., Caillaud, A., Riobo, P., Paz, B., Franco, J., Ibarra, D., Cembella, A., Blasco, D. & Diogène, J.: The Ebro Delta coastal embayments, a GEOHAB pilot site for the study of HAB population dynamics

114

---

Genovesi-Giunti, B., Vaquer, A., Laabir, M., Vincent, C., Fiandrino, A., Collos, Y & Pastoureadu, A.: Bottom cell clusters as inocula for bloom initiation of *Alexandrium catenella* in a shallow lagoon (Thau, Southern France)

117

Granéli, E.: Top-down and bottom-up control of Harmful Algal Blooms (HABs)

120

---

Jenkinson, I.R. & Wyatt, T.: Rheological properties of exopolymeric secretions in HABs may be functions of length scale

126

---

Lawrence, J. & Brown, C.: Life-strategies of viruses that infect *Heterosigma akashiwo*

129

---

Lu, D., Göbel, J., Gao, Y., Qi, Y., Zou, J., Xia, P. & Du, W.: Succession pattern of HAB species before large-scale blooms of dinoflagellates in the East China Sea in spring 2004/2005

132

---

Sheng, J., Malkiel, E., Pfitch, D.W., Katz, J., Adolf, J., Belas, R. & Place, A.R.: Microscopic digital holography imaging of dinoflagellate behaviour

135

---

Sobrinho-Gonçalves, L. & Moita, M.T.: Copepods feeding on a thin-layered bloom of *Dinophysis acuta*

138

---

Touzet, N. & Raine, R.: Discrimination and dynamics of naturally occurring mixed *Alexandrium* populations using rRNA-targeted fluorescent oligonucleotide probes

141

---

Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N. & Hiroishi, S.: Temporal changes in microcystin-producing and non-microcystin-producing *Microcystis* populations of a Japanese lake

144

---

## 6. MITIGATION

Brownlee, E.F., Sellner, S.G. & Sellner, K.G.: Potential role of mitigating Chesapeake Bay algal blooms

148

Granéli, E., Esplund, C., Legrand, C., Franzén, H. & Granéli, C.: Minimizing economical losses with the help of “real-time” HAB surveillance

152

---

Gumbo, J.R. & Cloete, T.E.: Preliminary assessment of *Bacillus mycoides* as a biological control agent for *Microcystis* blooms

155

---

Sengco, M.R. & Sellner, K.G.: HABs and clay flocculation: review and discussion of studies, impacts and future directions

158

---

## 7. REGIONAL EVENTS

Ahmed, A.S., Hiller, S. & Luckas, B.: *Microcystis aeruginosa* bloom and the occurrence of microcystins in a freshwater eutrophic lake in Comilla, Bangladesh

162

---

Brown, L. & Bresnan, E.: Seasonal occurrence of *Pseudo-nitzschia* species in the west coast and Shetland Isles, Scotland

165

---

Chang, F.H., Stewart, F., Inglis, G. & Fitridge, I.: Dinoflagellate cysts from New Zealand ports and harbours with emphasis on the distribution of harmful species

168

---

Costa, P.R., Garrido, S., Rosa, R., Ferreira, M., Sequeira, M., Brotas, V. & Sampayo, M.A.M.: Accumulation and transfer of the amnesic shellfish poisoning toxin, domoic acid, in the marine food web off the Portuguese coast

171

---

Cox, F., Borchert, J. & Lona, B.: Domoic acid intrusion into Puget Sound, Washington, USA

174

---

Di, B.P., Tang, D.L., He, F.L. & Li, J.: Changes in seasonality and causative species in harmful algal blooms in the Bohai Sea area 1950-2004

177

Faust, M.A., Kibler, S.R., Litaker, R.W., Vandersea, M.W., Holland, W.C. & Tester, P.A.: Dinoflagellate dominance and blooms in Belizean mangrove embayments consistent with Margalef's Mandala

180

---

Fuentes, C., Clement, A. & Aguilera, A.: Summer *Alexandrium catenella* bloom and the impact on fish farming in the XI Aysén region, Chile

183

---

Fux, E., Bire, R. & Hess, P.: Comparison of the accumulation of lipophilic marine biotoxins in passive samplers, transplanted mussels and indigenous mussels on the west coast of Ireland

187

---

Hinzmann, M.F., Craveiro, S.C. & Calado, A.J.: Quantification of epibenthic communities, including toxic dinoflagellates, in different green macroalgal substrates in Ria de Aveiro (Portugal)

190

---

Honsell, G., Dell'Aversano, C., Vuerich, F., Sosa, S., Tartaglione, L. & Tubaro, A.: *Pseudo-nitzschia* and ASP in the Northern Adriatic Sea

193

---

Horner, R.A., Greengrove, C.L., Postel, J.R., Gawel, J.E., Davies-Vollum, K.S., Cox, A., Hoffer, S., Sorensen, K., Hubert, J., Neville, J. & Frost, B.W.: *Alexandrium* cysts in Puget Sound, Washington, USA

196

---

Kim, Y.-S. & Kim, C.-H.: Paralytic Shellfish Poisoning (PSP) toxins in *Alexandrium catenella* and *A. tamarense* isolated from southern coastal and offshore waters of Korea

200

---

Marshall, H.G., Burchardt, L., Egerton, T.A. & Laane, M.: Status of potentially harmful algae in the lower Chesapeake Bay estuarine system

203

---

Martin, J.L., LeGresley, M.M., Hanke, A.S. & Page, F.H.: *Alexandrium fundyense* – red tides, PSP shellfish toxicity, salmon mortalities and human illnesses in 2003-04 – before and after

206

Matsuyama, Y.: Red tide due to the dinoflagellate *Karenia mikimotoi* in Hiroshima Bay 2002: environmental features during the red tide and associated fisheries damages to finfish and shellfish aquaculture

209

---

Meave del Castillo, E., R. Rodríguez S. & Vargas, M.: Blooms of *Pyrodinium bahamense* var. *compressum* along the Pacific Coast of Central America and southern Mexico

212

---

Nguyen-Ngoc, L. & Larsen, J.: On the genus *Alexandrium* (Dinoflagellata) in Vietnamese waters: - two new records of *A. satoanum* and *A. tamutum*

216

---

Nikolaidis, G., Aligizaki, K., Koukaras, K. & Moschandreu, K.: Mucilage phenomena in the North Aegean Sea, Greece: another harmful effect of dinoflagellates?

219

---

Ní Rathaille, A., Touzet, N. & Raine, R.: Inter-annual variability of *Alexandrium* blooms in Cork Harbour, Ireland

223

---

Rajan, A. & Al Abdessalam, T.Z.: Harmful algal blooms and eutrophication: nutrient sources, composition and consequences in the Arabian Gulf bordering Abu Dhabi Emirate

226

---

Reger, R.N. & Tomas, C.R.: Biology and seasonal distribution of *Hermesinium adriaticum* in the New River of North Carolina

230

---

Terenko, L. & Terenko, G.: Dynamics of algal blooms in the Ukrainian coastal Black Sea

233

---

Wang, Z.H., Qi, Y.Z. & Yang, Y.F.: Distribution of dinoflagellate resting cysts in surface sediments from Changjiang River estuary before and during the spring bloom in 2004

236

Zhou, M.J., Zhu, M.Y., Wang, Y.F., Zhu, D.D., Lü, S.H., Lu, D.D., Shi, X.Y. & Zhang, C.S.: Role of short-term climate fluctuation on the outbreak of a large-scale dinoflagellate bloom along the east Chinese coast in 2005

239

---

## 8. TAXONOMY, BIOGEOGRAPHY

Akselman, R., Reguera, B. & Lion, M.: HAB-MAPS of toxic marine microalgae in coastal and shelf waters of South America

243

Al-Handal, A.Y., Karlson, B., Edler, L. & Skjevik, A.-T.: Phytoplankton distribution, diversity and nutrient variations at the West Coast of Sweden, with special reference to harmful algae

246

Cyronak, T. & Tomas, C.: Morphological and genetic description of an unusual *Amphidinium* (Dinophyceae) species

249

Hégaret, H., Shumway, S. & Wikfors, G.H.: Harmful algae can be transported via relocation of bivalve shellfish

253

Ho-Van, T., Nguyen-Ngoc, L. & Morton, S.L.: The toxic benthic dinoflagellate *Prorocentrum arabianum* Morton et Faust isolated from Phan Ri Bay, South Vietnam

256

Kloepper, S., John, U. & Cembella, A.D.: A new Mediterranean genotype of *Fibrocapsa* sp.

259

Mann, D.G. & Evans, K.M.: The species concept and cryptic diversity

262

Tester, P.A., Faust, M.A., Vandersea, M.W., Kibler, S.R., Chinain, M., Holmes, M., Holland, W.C. & Litaker, R.W.: Taxonomic uncertainties concerning *Gambierdiscus toxicus*: proposed epitype

269

**9. TOXICOLOGY**

Artigas, M.L., Amorim, A., Vale, P., Gomes, S.Sa., Botelho, M.J. & Rodrigues, S.M. Prolonged toxicity of *Scrobicularia plana* after a PSP event and its relation to *Gymnodinium catenatum* cyst consumption and toxin depuration

273

---

Caillaud, A., Cañete, E., Mallat, E., Fernández, M., Mohammad-Noor, N., Moestrup, Ø. & Franco, J.M. : Evaluation of the toxicity of *Prorocentrum* species by liquid chromatography-mass-spectrometry and cell-based assay

276

---

Cañete, E., Caillaud, A., Fernández, M., Mallat, E., Blanco, J. & Diogène, J.: *Dinophysis sacculus* from Alfacs Bay, NW Mediterranean. Toxin profiles and cytotoxic potential

279

---

Dias, E., Pereira, P., Batoreu, M.C.C., Jordan, P. & Silva, M.J.: Cytotoxic and genotoxic effects of microcystins in mammalian cell lines

282

---

Gol'din, E.: The dinoflagellate *Gyrodinium fissum*: harmful species or potential biotechnological object?

286

---

Gomes, S.S., Vale, P., Botelho, M.J., Rodrigues, S.M., Cerejo, M. & Vilarinho, M.G.: ELISA screening for yessotoxins in Portuguese shellfish

290

---

Juhel, G., O'Halloran, J., Culloty, S.C., O'Riordan, R.M., Davenport, J., O'Brien, N.M., James, K.J., Furey A. & Allis, O.: *In vivo* exposure to microcystins induced DNA damage in haemocytes of the zebra mussel, as measured with the Comet assay

293

---

Kirkpatrick, B., Bean, J.A., Fleming, L.E., Backer, L.C., Akers, R., Wanner, A., Dalpra, D., Nierenberg, K., Reich, A. & Baden, D.G.: Aerosolized red tide toxins (brevetoxins) and asthma: a 10-day follow up after 1 hour acute beach exposure

297

Kotaki, Y., Lundholm, N., Katayama, T., Furio, E.F., Romero, M.L., Relox, J. R., Yasumoto, T., Naoki, H., Hirose, M.Y., Thanh, T.D., Thuoc, C. V., Huyen, N. T. M., Thu, P. T., Takata, Y., Kodama, M. & Fukuyo, Y.: ASP toxins of pennate diatoms and bacterial effects on the variation in toxin composition

300

---

Krock, B., Alpermann, T., Tillmann, Y., Pitcher, G.C. & Cembella, A.D.: Yessotoxin profiles of the marine dinoflagellates *Protoceratium reticulatum* and *Gonyaulax spinifera*

303

---

Laurent, D., Kerbrat, A.S., De Fremicourt, I., Darius, H.T., Golubic, S., Chinain, M. & Pauillac, S.: Involvement of cyanobacteria in ciguatera fish poisoning

306

---

Lewis, N., Garnett, C., Leggiadro, C., Rafuse, C. & Quilliam, M.: Production of spirolides in single cells of *Alexandrium ostenfeldii* throughout the diurnal cycle

309

---

Oda, T., Kim, D. & Yamaguchi, K.: Nitric oxide (NO) generation by the harmful red tide phytoplankton *Chattonella marina*

313

---

Pauillac, S., Vernel-Pauillac, F., Kumar-Roine, S., Sauviat, M.-P., Benoit, E., Chinain, M. & Laurent, D.: First evidence for implication of nitric oxide in a mouse model for ciguatera fish poisoning

316

---

Pigozzi, S., Bianchi, L., Boschetti, L., Cangini, M., Ceredi, A., Magnani, F., Milandri, A., Montanari, S., Pompei, M., Riccardi, E. & Rubini, S.: First evidence of spirolide accumulation in north-western Adriatic shellfish.

319

---

Reis, M., Kraberg, A.C., Erler, K., Luckas, B., Amorim, A. & Wiltshire, K.H.: Ecotoxicology of different strains of *Lingulodinium polyedrum* from the Portuguese coast

323

---

Rhodes, L., Munday, R. & Briggs, L.: *Ostreopsis siamensis* and palytoxin-related compounds in New Zealand: a risk to human health?

326

---

Soto, K., Stucken, K., Méndez, M.A., Lagos, N., Cembella, A.D., Krock, B. & Vásquez, M.: The effects of chloramphenicol, arginine and temperature on PST-production by *Cylindrospermopsis raciborskii* strain D9

330

---

Vila, M., Masó, M., Sampedro, N., Illoull, H., Arin, L., Garcés, E., Giacobbe, M.G., Alvarez, J. & Camp, J.: The genus *Ostreopsis* in recreational waters of the Catalan Coast and Balearic islands (NW Mediterranean Sea): is this the origin of human respiratory difficulties?

334

---

## 10. TOXIN ANALYSIS AND SYNTHESIS

Botelho, M.J., Gomes, S.S., Rodrigues, S.M. & Vale, P.: Studies on cryptic PSP toxicity depend on the extraction procedure

338

---

Boyer, G.L., Konopko, E. & Gilbert, H.: Rapid field-based monitoring systems for the detection of toxic cyanobacteria blooms: microcystin ImmunoStrips and fluorescence-based monitoring systems

341

---

Ciminiello, P., Dell'Aversano, E., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C. & Melchiorre, N.: The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method

344

---

de la Iglesia, P., Gago-Martínez, A. & Yasumoto, T.: Application of capillary electrophoresis-mass spectrometry to the determination of lipophilic marine toxins

347

---

Diener, M. & Luckas, B.: LC-MS/MS determination of Paralytic Shellfish Poisoning (PSP) in sea-food by application of a new hydrophilic interaction liquid chromatographic (HILIC) column

350

---

Fuentes, S., Rick, H.J., Scherp, P., Chistoserdov, A. & Noel, J.: Development of Real-Time PCR assays for the detection of *Cylindrospermopsis raciborskii*

353

Hégaret, H., Wikfors, G.H. & Shumway, S.E.: *In vitro* interactions between several species of harmful algae and hemocytes of bivalve mollusks

356

---

Hess, P.: What's new in toxins?

360

---

Hiller, S. Krock, B., Cembella, A. & Luckas., B.: Development of a wide spectrum method for detection of cyanobacterial toxins by mass spectrometry

371

---

Mallat, E., Cañete, E., Caillaud, A., Fernández, M., Bravo, I., Paz, B., Franco, J.M. & Diogène, J.: Evidence of yessotoxins in Alfacs Bay-toxic effect evaluation by cell-based assays and toxin profile determination by liquid chromatography

374

---

Quilliam, M.A., Lewis, N.I., Aasen, J. & Hardstaaf, W.: Analysis of phycotoxins in hand-picked plankton cells by micro-column liquid chromatography-tandem mass spectrometry

377

---

Sekiguchi, R., Suzuki, M., Takahashi, N., Yamamoto, M., Watai, M., Suzuki, T. & Yasumoto, T.: Evaluation of the enzyme inhibition assay for diarrhetic shellfish toxins and the ELISA assay for yessotoxins by LC-MS

381

---

Suzuki, M., Sekiguchi, R., Watai, M. & Yasumoto, T.: Preparation and simultaneous LC-M analysis of fourteen shellfish toxins

384

---

Yoshino, A., Naoki, H. & Yasumoto, T.: Preparation of toxin standards for use in monitoring diarrhetic shellfish toxins by LC/MS

387



# 1. ALLELOPATHY



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Relationship between heterotrophic bacteria and bloom-forming phytoplankton species from the coastal area of Thailand

A. Piumsomboon<sup>1</sup>, P. Soasii<sup>1</sup>, I. Sivaipram<sup>1</sup>, C. Songroop<sup>1</sup>, S. Rungsupa<sup>2</sup> and K. Fukami<sup>3</sup>

<sup>1</sup> Marine Ecology Laboratory, Department of Marine Science, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand, e-mail: Ajcharaporn.P@chula.ac.th

<sup>2</sup> Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand

<sup>3</sup> Laboratory of Aquatic Environmental Science, Kochi University, Nankoku, Kochi 783-8502, Japan

### Abstract

The relationship between the abundance of heterotrophic bacteria and the bloom-forming species of diatoms *Skeletonema costatum* and *Chaetoceros* spp., cyanobacteria of the genus *Oscillatoria* and the dinoflagellates *Noctiluca scintillans* and *Ceratium furca* from the coastal area of Bangpra, in the eastern part of the Upper Gulf of Thailand, was investigated January 2003-June 2004. The increase in abundance of heterotrophic bacteria was observed following peaks of *S. costatum* and *Chaetoceros* spp. in the rainy season of both 2003 and 2004. Three clonal cultures of *Bacillus* sp., *Pseudomonas* sp. and unidentified pale yellow colony-forming bacterium, isolated from seawater in May 2004, inhibited growth of axenic cultures of *C. curvisetus* and *S. costatum* with a threshold abundance exceeding  $10^4$  cells/ml of *Pseudomonas* sp. and  $10^5$  cells/ml of the other bacteria. Growth of a natural population of *Noctiluca scintillans* was affected by all bacterial strains tested, with a threshold abundance above  $10^5$  cells/ml of *Bacillus* sp. and  $10^4$  cells/ml of the other bacteria.

### Introduction

The Upper Gulf of Thailand is a coastal embayment which receives freshwater from four major rivers. Eutrophication resulting in plankton blooms and/or red tides has caused a total of 90 recorded blooms during the past 44 years. In the eastern part of the area there are almost annual blooms of *Noctiluca scintillans* or *Ceratium furca*, particularly in the rainy season, while *Chaetoceros* spp. and *Skeletonema costatum* often bloom during the dry season (Rungsupa *et al.* 2003). Very few studies have been conducted to elucidate the initiation and termination of the blooms in Thai waters. It is well known that bacteria can play an important role in the decline of phytoplankton blooms (Fukami *et al.*, 1996; Doucette *et al.*, 1998; Nagai and Imai 1998; Mayali and Azam 2004), but studies of algicidal bacteria and bloom-forming phytoplankton in the Upper Gulf of Thailand are very limited (Dech-sakulwatana 2006). The objectives of this work are to describe the relationship between bacteria and bloom-forming phytoplankton in the coastal area and to determine the inhibiting effect of bacteria in both natural populations and cultures of phytoplankton.

### Study Site and Study Period

Samples containing phytoplankton and bacteria were collected monthly February 2003-June 2004 at a fixed station (13.22038°N and 100.89058°E) in the coastal area of Bangpra, Chonburi province, east coast of the Upper Gulf of Thailand (Fig. 1). In the rainy season,



**Figure 1.** Sampling site (dot in circle) at Bangpra coast, Chonburi province, eastern part of the Upper Gulf of Thailand.

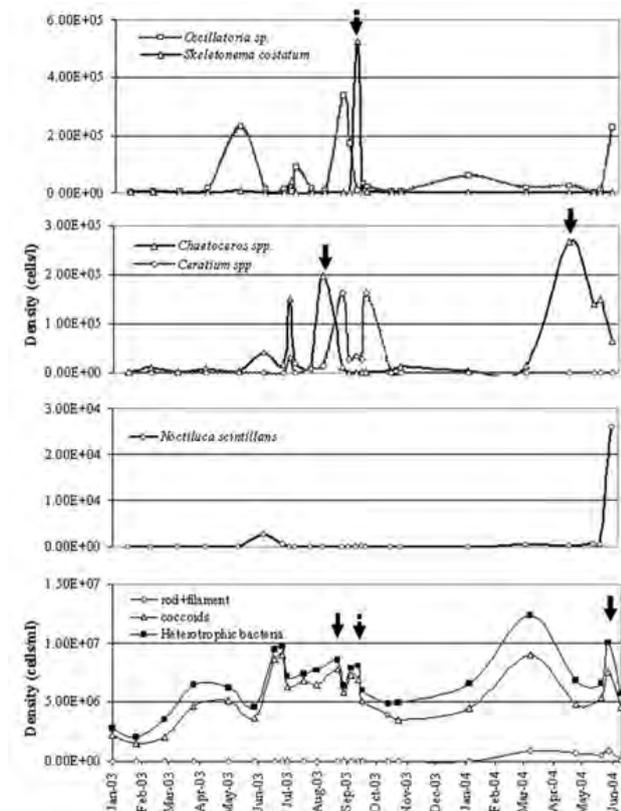
May to September, sampling was repeated weekly when the weather permitted.

### Methods and Materials

Duplicate water samples of at least 10 litres were collected from two depths, 0.5 m and 2.0 m and filtered

onto a 20- $\mu\text{m}$  mesh to retain microphytoplankton cells. The samples were preserved and identified, followed by determination of densities as described by Throndsen (1995). Heterotrophic bacteria from water samples collected in triplicates at each depth were distinguished from autotrophic bacteria by autofluorescence of the chlorophyll a and DAPI staining of the heterotrophic cells (Porter and Feig 1980).

To investigate an adverse effect of bacteria on selected phytoplankton species, three bacterial strains, *Bacillus* sp., *Pseudomonas* sp. and an unidentified pale yellow bacterium, originating from 0.8- $\mu\text{m}$  pre-filtered seawater in May 2004 and cultured in an enriched medium (beef extract and peptone), were tested against axenic cultures of *Skeletonema costatum* and *Chaetoceros curvisetus* as well as a natural population of *Noctiluca scintillans*, using an experimental procedure modified from Fukami *et al.* (1996). A 0.2- $\mu\text{m}$  pre-filtered bacterial culture medium was used as control. The growth rate,  $\mu$ , was calculated from changes in phytoplankton cell numbers during the first 3 days. Two-factor ANOVA with equal replications was used to analyze the difference in growth rate due to bacteria (Zar 1996).



**Figure 2.** Bloom-forming phytoplankton species and heterotrophic bacteria in Bangpra area from January 2003 to June 2004 (arrows indicate concurrent phytoplankton and bacteria abundances).

## Results and Discussion

### Bloom-forming phytoplankton and bacterial abundance in coastal area

Two peaks of the cyanobacterium *Oscillatoria* (*Trichodesmium*) *erythraeum*, density  $>1.69 \times 10^5$  cells/l, occurred in May and September of 2003 followed by a peak of the diatom *Skeletonema costatum*,  $5.25 \times 10^5$  cells/l, in late September. Peaks of *Chaetoceros* spp.,  $>1.40 \times 10^5$  cells/l were found in July and August 2003 and May and June 2004 (Fig. 2). Both *O. erythraeum* and *Chaetoceros* spp. have been responsible for red tide events in the Gulf of Thailand during the past 44 years (Rungsupa *et al.* 2003). High densities of the dinoflagellate *Ceratium furca*, ca.  $1.63 \times 10^5$  cells/l, were noticed during the latter part of the rainy season. Another dinoflagellate, *N. scintillans*, occurred in numbers above  $2 \times 10^3$  cells/l in June 2003 but the density increased ten-fold in June 2004 (Fig. 2). During this period, the density of heterotrophic bacteria varied between  $2.09 \times 10^6$  and  $1.24 \times 10^7$  cells/ml. Bacterial densities above  $10^7$  cells/ml were recorded in July 2003, March and June 2004 (Fig. 2). The bacteria were dominated by coccoid forms, constituting 58-93 % of the density, while rod-shaped and filamentous bacteria contributed less than 10 % of the density (Fig. 2). During the study period, bacteria occurred concurrently with a decrease in the abundance of *S. costatum* and *C. curvisetus* during the latter part of the rainy season 2003 and the early rainy season 2004 (Fig. 2).

### Effect of bacteria on bloom-forming phytoplankton

Specific growth rates of axenic cultures of *S. costatum* and *C. curvisetus* incubated with *Bacillus* sp. (rod-shaped), *Pseudomonas* sp. (coccoid) and unidentified filamentous bacteria at densities  $\geq 1.53 \times 10^5$ ,  $2.89 \times 10^4$  and  $1.16 \times 10^5$  cells/ml, respectively, were significantly different ( $p < 0.001$ ) (Table 1). The decrease in growth rate of a natural assemblage of *N. scintillans* was also noticed when incubated with *Bacillus* sp., *Pseudomonas* sp. and unidentified bacteria at concentrations of  $1.10 \times 10^5$ ,  $8.60 \times 10^4$  and  $8.80 \times 10^4$  cells/ml, respectively (Table 1). These threshold abundances of bacteria were in the same range as bacterial abundance in nature. Both *Bacillus* sp. and *Pseudomonas* sp. have been previously reported to inhibit growths of diatoms and dinoflagellates (Mayali and Azam 2004). A bacterial abundance of ca.  $10^5$  cells/ml was reported to inhibit growth of *S. costatum* in the coastal Chonburi province of Thailand (Dech-sakulwatana 2006).

## Conclusion

Our results showed that the abundance of bacteria increased following peaks of some bloom-forming phytoplankton in the coastal area of Bangpra, Thailand. The laboratory studies also demonstrated an inhibition effect of strains of heterotrophic bacteria on the growth of bloom-forming phytoplankton. However, bacterial strains isolated from this study may contribute only a very small proportion of the bacterial population in natural waters, and a study of the inhibitory effect of bacteria on phytoplankton under natural conditions is necessary.

## Acknowledgements

The authors would like to thank Dr. Sanit Piyapattanakarn for his help in identifying bacteria. This work was supported by the National Research Council of Thailand NRCT-JSPS cooperative research program in coastal oceanography.

## References

- Dechsakulwatana, C., Fukami, K., Pinkaew, K. & Wongsudawan, W. (2006). *Coast. Mar. Sci.* 30: 100-103.
- Doucette, G.J., Kodama, M., Franca, S. & Gallacher, S. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (eds), Springer-Verlag, Berlin Heidelberg, pp. 619-647.
- Fukami K., Sakagushi, K., Kanou, M. & Nishijima, T. (1996). In: *Harmful Algal Blooms*, Yasumoto, T., Oshima, Y. & Fukuyo, Y. (eds), UNESCO, Paris, pp. 335-338.
- Mayali, X. & Azam, F. (2004). *J. Eukaryot. Microbiol.* 51: 139-144.
- Nagai, S. & Imai, I. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, J. & Wyatt, T. (eds), Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 402-405.
- Porter, K.G. & Feig, Y.S. (1980). *Limnol. Oceanogr.* 25: 943-948.
- Rungsupa, S., Songroop, C., Piumsomboon, A., Paphavasit, N., Panichphol, A. & Sophon, A. (2003). In: *Red Tides in Thai Waters*, Paphavasit, N. *et al.* (eds), Aquatic Resources Research Institute, Bangkok, pp. 74-104. (in Thai).
- Thronsen, J. (1995). In: *Manual on Harmful Marine Microalgae IOC Manuals and Guides No.33*, Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. &

Enevoldsen, H.O. (eds), UNESCO, Paris, pp. 63-80.

Zar, J. (1996). *Biostatistical Analysis*. Prentice-Hall, Upper Saddle River, 662 pp..

**Table 1.** Specific growth rate of phytoplankton in the presence of 3 strains of bacteria (mean  $\pm$  SD, n=6; a declining cell density, b no cell)

Phytoplankton species	Bacteria clone	Bacterial concentration (cells/ml)	Phytoplankton Growth rate (day <sup>-1</sup> )
<i>S. costatum</i>	<i>Bacillus</i> sp.	0.00E+00	0.62 $\pm$ 0.02
		1.53E+03	0.48 $\pm$ 0.02
		<b>1.53E+04</b>	0.46 $\pm$ 0.02
		<b>1.53E+05</b>	-0.82 $\pm$ 0.01 <sup>a</sup>
		1.53E+06	-1.42 $\pm$ 0.02 <sup>a</sup>
	<i>Pseudomonas</i> sp.	0.00E+00	0.62 $\pm$ 0.02
		2.89E+02	0.56 $\pm$ 0.01
		2.89E+03	-0.05 $\pm$ 0.01 <sup>a</sup>
		<b>2.89E+04</b>	-0.74 $\pm$ 0.02 <sup>a</sup>
		<b>2.89E+05</b>	-1.33 $\pm$ 0.04 <sup>a</sup>
	Unidentified pale yellow bacteria	0.00E+00	0.62 $\pm$ 0.02
		1.16E+03	0.56 $\pm$ 0.01
		1.16E+04	0.48 $\pm$ 0.01
		<b>1.16E+05</b>	-0.79 $\pm$ 0.01 <sup>a</sup>
		<b>1.16E+06</b>	-1.06 $\pm$ 0.01 <sup>a</sup>
<i>C. curvisetus</i>	<i>Bacillus</i> sp.	0.00E+00	0.20 $\pm$ 0.01
		1.53E+03	0.19 $\pm$ 0.01
		1.53E+04	0.16 $\pm$ 0.01
		<b>1.53E+05</b>	-0.81 $\pm$ 0.02 <sup>a</sup>
		<b>1.53E+06</b>	-1.42 $\pm$ 0.05 <sup>a</sup>
	<i>Pseudomonas</i> sp.	0.00E+00	0.20 $\pm$ 0.01
		2.89E+02	0.17 $\pm$ 0.01
		2.89E+03	0.16 $\pm$ 0.01
		<b>2.89E+04</b>	-0.82 $\pm$ 0.01 <sup>a</sup>
		<b>2.89E+05</b>	-1.58 $\pm$ 0.02 <sup>a</sup>
	Unidentified pale yellow bacteria	0.00E+00	0.20 $\pm$ 0.01
		1.16E+03	0.16 $\pm$ 0.02
		1.16E+04	0.16 $\pm$ 0.02
		<b>1.16E+05</b>	-0.83 $\pm$ 0.04 <sup>a</sup>
		<b>1.16E+06</b>	-1.51 $\pm$ 0.07 <sup>a</sup>
<i>N. scintillans</i>	<i>Bacillus</i> sp.	0.00E+00	0.22 $\pm$ 0.07
		1.22E+02	0.16 $\pm$ 0.06
		3.66E+03	0.16 $\pm$ 0.04
		<b>1.10E+05</b>	- <sup>b</sup>
		0	0.25 $\pm$ 0.08
	<i>Pseudomonas</i> sp.	9.50E+01	0.17 $\pm$ 0.05
		2.86E+03	0.19 $\pm$ 0.08
		<b>8.60E+04</b>	- <sup>b</sup>
		0	0.17 $\pm$ 0.04
		9.76E+01	0.16 $\pm$ 0.06
	bacteria	2.93E+03	0.14 $\pm$ 0.07
		<b>8.80E+04</b>	- <sup>b</sup>

## Responses of bivalve molluscs to the ichthyotoxic dinoflagellate *Karlodinium veneficum*

A.R. Place<sup>1</sup>, E.F. Brownlee<sup>2</sup>, H. Nonogaki<sup>1</sup>, J.E. Adolf<sup>1</sup>, T.R. Bachvaroff<sup>1</sup>, S.G. Sellner<sup>3</sup> and K.G. Sellner<sup>4</sup>

<sup>1</sup>Center of Marine Biotechnology, University of Maryland, 701 E. Pratt Street, Baltimore, MD, USA 21202 and place@umbi.umd.edu, nonogaki@umbi.umd.edu, adolf@umbi.umd.edu, bachvaro@umbi.umd.edu,

<sup>2</sup>Hood College, 401 Rosemont Avenue, Frederick, MD, USA 21701 and efb2@hood.edu and <sup>3</sup>Morgan State University Estuarine Research Laboratory, St. Leonard, MD, USA 20678 and sgsellner@moac.morgan.edu,

<sup>4</sup>Chesapeake Research Consortium, 645 Contees Wharf Road, Edgewater, MD, USA 21037 and sellnerk@si.edu

### Abstract

The Eastern oyster *Crassostrea virginica* and the Suminoe oyster *C. ariakensis* are native and introduced oysters, respectively, in the Chesapeake Bay, and the northern quahog (hard clam) *Mercenaria mercenaria*, common to sandy habitats of the bay and adjacent coastal bays, is a major cultured species in Virginia waters. In the wild, these species are exposed to phytoplankton assemblages, including harmful species, throughout their life cycle. Recent work suggests that at least one prey item, the ichthyotoxic dinoflagellate *Karlodinium veneficum*, occurs frequently throughout the range for the oysters and clam, and produces toxins (karlotoxins, KmTX) which elicit toxicity through formation of sterol-dependent, non-specific membrane pores. Juvenile oysters and clams were exposed to moderately toxic strains [ $18.5 \pm 6.2$  ng KmTX mL<sup>-1</sup>] at environmentally-relevant cell densities. Juvenile oyster growth rates were significantly reduced when feeding on *Karlodinium* as were clearance rates for both oysters and clams relative to a non-toxic prey species. These initial results suggest that the cosmopolitan *K. veneficum* could substantially alter oyster recruitment through growth limitation of 1-2 week old spat as well as temporarily reduce seston clearance, and hence energy intake, in bloom areas.

### Introduction

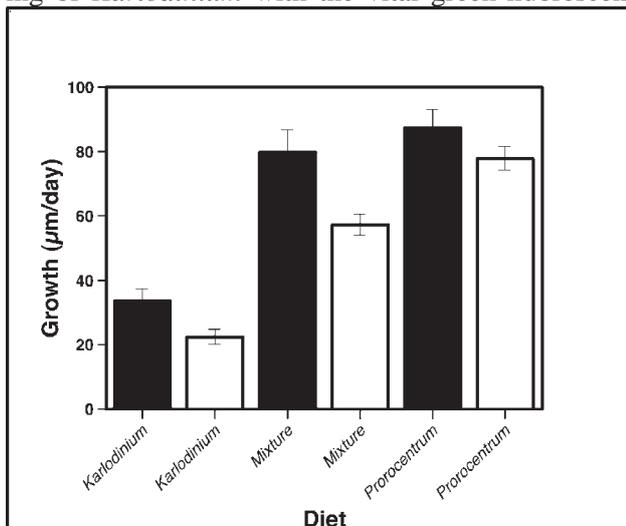
The Chesapeake Bay was once synonymous with the Eastern oyster *Crassostrea virginica*, resulting in the largest US oyster harvest late in the last century. Overfishing and disease have reduced native oyster stocks to ~1 % of historical levels. As a consequence, there is increasing interest in the introduction of the Suminoe oyster *Crassostrea ariakensis*, which initial experiments suggest grows more rapidly and is more disease tolerant than the native oyster. The northern quahog (hard clam), *Mercenaria mercenaria* is a bivalve mollusc found along the eastern and Gulf coasts of North America ranging from the Gulf of St. Lawrence to the Yucatan Peninsula. It has been the focus of important commercial fisheries along the Atlantic coast with the Virginia aquaculture industry evolving into a multi-million-dollar enterprise (Murray and Kirkley 2005). The Chesapeake and its coastal bays experience several recurrent blooms, including the ichthyotoxic dinoflagellate *Karlodinium veneficum* from late spring through fall (Li *et al.* 2000; Goshorn *et al.* 2004). Previous work suggests that this alga might impact suspension feeding bivalves (Abbott and Ballantine 1957; Nielsen & Strømgren 1991) as well as the altered fish morphologies and mortalities reported over the past 50 years (Abbott and Ballantine 1957; Pieterse and van der Post 1967; Nielsen 1993; Deeds

*et al.* 2002; Kempton *et al.* 2002; Deeds *et al.* 2006). Because of the regional interest in restoring bay oyster populations, either *C. virginica* or *C. ariakensis*, and with the potential impact on a growing hard clam industry, the effect of co-occurring blooms of *K. veneficum* on juvenile oyster growth as well as clearance rates for juvenile oysters and clams was assessed in a series of laboratory experiments.

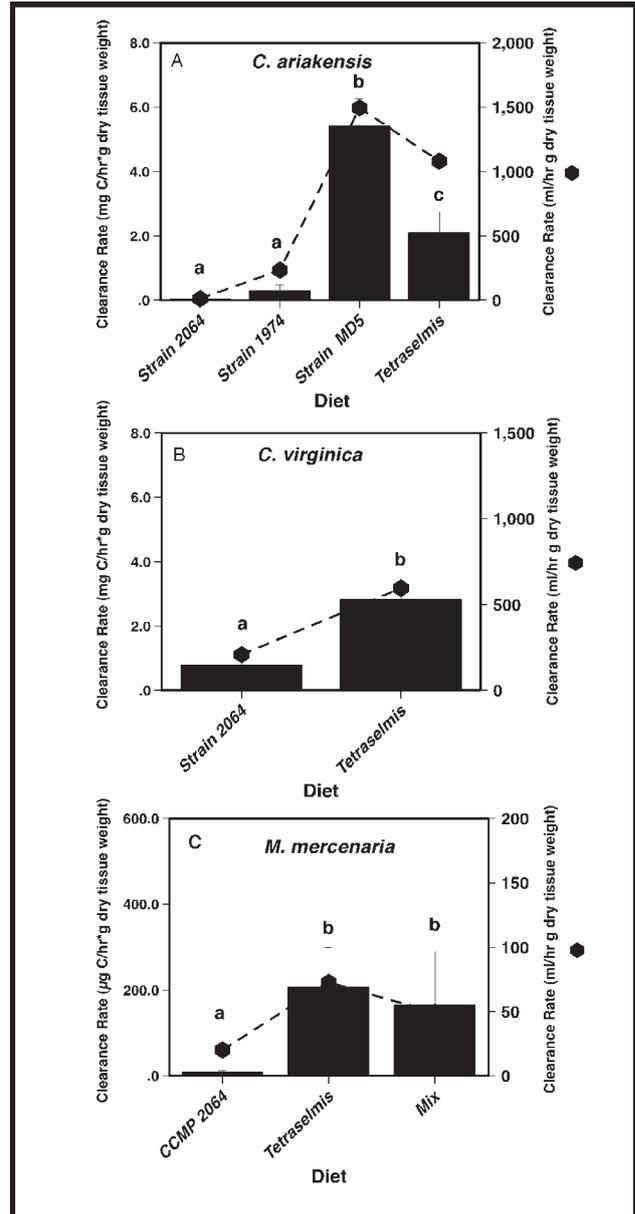
### Methods

Eyed larvae of *C. virginica* and *C. ariakensis* were obtained from S. Allen, VIMS and set on 10 x 10 cm PVC plates in filtered seawater at a salinity of 16 PSU and a temperature of 26 °C. Following 3 d of initial growth, three food mixtures were provided to 5 replicate plates per food type. The food mixtures included a commercial hatchery food (*Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii*), a non-toxic *Prorocentrum minimum* (from D.W. Coats, SERC), and *Karlodinium veneficum* CCMP 1974 (~0.4 pg KmTX1 cell<sup>-1</sup>). Every 4th day, all chambers were emptied and refilled with filtered seawater and similar algal enrichments for each food mixture (hatchery formula 1.4-1.7 µgC mL<sup>-1</sup>; *P. minimum* at 3.1-4.5 µgC mL<sup>-1</sup>; *K. veneficum* at 2.8-4.3 µgC mL<sup>-1</sup>); daily, between each major feeding, smaller food additions (0.47-0.93, 0.25-0.51, and 0.27-0.48 µgC mL<sup>-1</sup>, respectively) were made of the

same food types. Initially and on day 8 and day 13 or 14, dimensions (height, length) of the same 10 individual spat per plate were determined using a dissecting microscope. Growth rates for the two time periods (Period 1: days 3 to 8, Period 2: days 8 to 13-14) were calculated as (length at time 2 – length at time 1), normalized to time interval in days. In separate experiments, juvenile oysters (1-2 cm) and clams (3-4 cm; *M. mercenaria*) were also examined to assess the impact of *K. veneficum* on clearance rates as determined according to Coughlan (1969). Bivalves were placed in 38-L aquaria at 18 °C and a salinity of 15 on a 14L:10D cycle, and live algal food mixtures (2 mgC) of 33 % *Isochrysis*, 33 % *Tetraselmis*, and 33 % *Chaetoceros* were added daily. For feeding trials, individuals were removed and placed in 5-L aquaria containing suspensions of several similar size *Karlodinium* strains, CCMP 2064 (2.2 pg KmTX2 cell<sup>-1</sup>), CCMP 1974 (0.25 pg KmTX1 cell<sup>-1</sup>), and MD5 (0.0 pg KmTX1 cell<sup>-1</sup>), or *Tetraselmis* (0.0 pg karlotoxin) for 6 h at densities of 3.0 x 10<sup>4</sup> cells mL<sup>-1</sup> for oysters and 3.0 x 10<sup>3</sup> cells mL<sup>-1</sup> for clams. Initial and final cell densities were measured with a Coulter® Multisizer II and data analyzed with Coulter® ACCUCOMP software (ver. 2.01). Control aquaria contained the same phytoplankton mixtures without any suspension feeders. Following the 6-h feeding bouts, bivalves were returned to the normal maintenance food mixture for 18 h. This was repeated 5 times over 5 d with no animals used more than once for the feeding trial. Biodeposits (feces, pseudofeces) of oysters and clams were also examined with epifluorescence microscopy (ex. 450 – 490 nm; em. LP 550 nm) following staining of *Karlodinium* with the vital green-fluorescent



**Figure 1.** Food effects on spat growth of *C. ariakensis* (open bars) and *C. virginica* (black bars), respectively, measured for Period 2. Bars represent  $\pm$  se.



**Figure 2.** Clearance rates for bivalves fed on different algal suspensions. Rates with different letters are significantly different at the  $p=0.05$  level within a species. Bars represent means  $\pm$  se.

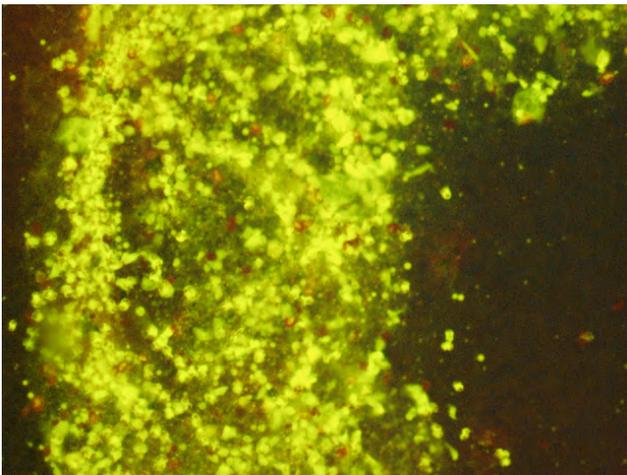
stain, 5-chloromethylfluorescein diacetate (CMFDA) (Adolf *et al.* 2006).

## Results

For both oysters, *K. veneficum* depressed spat growth rates nearly three-fold relative to the rates noted for spat feeding on *P. minimum* or hatchery algal mixtures (Fig. 1, plotted for Period 2). On all food sources, spat growth rates of the non-native oyster *C. ariakensis* were lower (ANOVA,  $p < 0.0001$ ) than those of the native oyster. The reduced growth rates when feeding on *Karlodinium* were also associated with poor soft tissue development in both oyster species. Tis-

sues filled less of the shell cavity when oysters fed on *K. veneficum* than on the other foods, with most dramatic differences noted in gill filament lengths. In a comparison of single oyster species for each food, the ratio of gill filament to total shell length in *Karlodinium*-reared oysters approximated 0.06-0.07 while ratios for the two other diets ranged from 0.10 to 0.14. Additional data are required in the future to validate this as a consistent *K. veneficum* impact.

For older bivalves, *K. veneficum* also altered 'normal' behaviour. *K. veneficum* depressed clearance rates of juveniles of the two oysters and *Mercenaria* to rates measured on non-toxic *K. veneficum* (MD5) or other algae (Fig. 2). Clearance rates were inversely related to toxin content, with lowest rates on the most toxic *K. veneficum* strain 2064, slightly higher rates on less but still toxic strain 1974, and highest clearance rates, equal to those observed on other non-toxic taxa, on strain MD5 (Fig. 2A). Pseudofeces of oysters and clams fed toxic *Karlodinium* were packed with intact cells, as seen in Figure 3 for clam pseudofeces. For example, in a 1:1 mixture of CCMP 2064 and *Tetraselmis*, clams were able to separate *Tetraselmis* (red cells) from CCMP 2064 (green coloured cells).



**Figure 3.** Fluorescent micrograph of clam pseudofeces fed a 1:1 mixture of *Tetraselmis* (red) and CFDMA-stained CCMP 2064 (yellow-green).

### Discussion

As a common mesohaline Chesapeake Bay bloom former, *K. veneficum* is likely to co-occur with all bivalve populations in the bay. As a result, establishing responses of the native Eastern oyster, the non-native Suminoe oyster, and the hard clam to this species is critical to identifying potential population (and harvest) effects in tidal waters of the system. The present results suggest that spat growth rates of both oysters were depressed by bloom levels of *K. veneficum* but

rates were more depressed in the non-native species. This is particularly important as these earliest life stages are most susceptible to Bay predators, including mud and blue crabs and polychaetes. The slower growth was apparently (although only single individuals were examined) associated with slower organ development in *K. veneficum*-exposed individuals, including contraction and stubbing of secondary gill lamellae as seen in mussels (Nielsen & Strøm-gren 1991) and fish larvae (Nielsen 1993; Deeds *et al.* 2006) exposed to karlotoxin. Juveniles of both oysters and clams also experience marked reductions in clearance rates on exposure to toxic *K. veneficum*, with depression proportional to toxin content of the *K. veneficum* strain. The impact in nature still needs to be assessed but the laboratory studies clearly establish that *K. veneficum* can influence several aspects of bivalve physiology.

### Acknowledgements

The authors appreciate use of Morgan State University's Estuarine Research Center facilities and thank G. Abbe and M. Luckenbach for bivalves, their insight, and recommendations, S. Allen for eyed larvae and suggestions, and A.M. Hartsig for microscopy assistance. This research is partially funded by the National Oceanic and Atmospheric Administration Coastal Ocean Program under award #NA04NOS4780276 to the University of Maryland Biotechnology Institute and Grant #U50/CCU 323376, Centers for Disease Control and Prevention and the MD Department of Health and Mental Hygiene. This is contribution #07-162 of the Center of Marine Biotechnology and #209 from the ECOHAB program.

### References

- Abbott, B.C. & Ballantine, D. (1957). *J. Mar. Biol. Ass. U.K.* 36: 169-189.
- Adolf, J.E., Bachvaroff, T.R., Krupatkina, D.N., Nonogaki, H., Brown, P.J.P. Lewitus, A.J., Harvey, H.R. & Place, A.R. (2006). *African J. Mar. Sci.* 28: 415-421.
- Coughlan, J. (1969) *Mar. Biol.* 2: 356-358.
- Deeds, J.R., Terlizzi, D.E., Adolf, J.E., Stoecker, D.K. & Place, A.R., (2002). *Harmful Algae* 1: 169-189.
- Deeds, J.R. & Place, A.R. (2006). *African J. Mar. Sci.* 28: 421-427.
- Deeds, J.R., Reimschuessel, R. & Place, A.R. (2006). *J. Aq. Animal Hlth.* 18: 136-148.
- Goshorn, D., Deeds, J., Tango, P., Poukish, C., Place, A.R., McGinty, M., Butler, W., Lucket, C.

- & Magnien, R. (2004). In: Harmful Algae 2002, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), Florida Institute of Oceanography and IOC UNESCO, St. Petersburg, FL., pp. 361–363.
- Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M. & Place, A.R., (2002). Harmful Algae 1: 233-241.
- Li, A., Stoecker, D.K. & Coats, D.W. (2000). J. Plankton Res. 22: 2105-2124.
- Murray, T. I.. & Kirkley, J. E. (2005) Economic Activity Associated with Clam Aquaculture in Virginia – 2004 VIMS Marine Resource Report No. 2005-5, pp. 21.
- Nielsen, M.V. (1993). Mar. Ecol. Prog. Ser. 95: 273-277.
- Nielsen, M.V. & Strømgren, T. (1991). Mar. Biol. 108: 263-267.
- Pieterse, F. & van der Post, D.C. (1967). The Pilchard of South West Africa. Admin. SW Africa, Mar. Res. Lab., Investig. Rpt. 14. pp. 27.

## Diatom effect on dinoflagellate growth

K. Spilling

Finnish Environment Institute, P.O. Box 140, 00251 Helsinki, Finland, kristian.spilling@ymparisto.fi

### Abstract

Diatoms release organic compounds, but little is known about the effect of the exudates on co-occurring phytoplankton. In this study, the growth rate of cold-water dinoflagellates from the Baltic Sea: *Peridiniella catenata*, *Scrippsiella hangoei* and *Woloszynskia halophila* were determined in filtrates (0.2 µm and 2.0 µm) of co-occurring diatoms, established in monocultures (*Melosira arctica*, *Chaetoceros wighamii*, *Diatoma tenuis*, *Thalassiosira baltica*, *Skeletonema costatum*). Aged, autoclaved seawater was used as control. The growth of *P. catenata* and *W. halophila* was reduced in filtrates of diatom cultures compared with the control; *S. hangoei*, on the other hand, had generally increased growth rate in diatom filtrates. Indications are that diatom exudates affect the growth of the studied dinoflagellates and the effect is species specific. The effect on growth rate was stable over the duration of the experiment (2 weeks), suggesting that the effect is caused by compounds that are not easily degradable.

### Introduction

Diatoms are known to produce and release organic compounds, mainly extracellular polymeric substances (EPS) and non-polymeric extracellular carbohydrates (NPEC) (Hoagland *et al.* 1993; Underwood *et al.* 2004). Excretion of EPS and NPEC by diatoms can be affected by several abiotic factors such as nutrients or light (Smith and Underwood 1998; Straats *et al.* 2000). Generally the release of EPS is low during active growth, but increases once growth slows down (Myklestand and Haug 1972). EPS might have several functions for diatoms such as propulsion or adhesion and may be important for bacterial growth and sediment stability (e.g. Paterson 1989; Decho 1990; Hoagland *et al.* 1993), but little is known about what effect diatom exudates have on other phytoplankton groups.

In the Baltic Sea there is an annual spring bloom of phytoplankton. During the bloom a succession from diatom to dinoflagellate dominance is often observed, and this has been attributed to the stratification pattern (Högländer *et al.* 2004). During the decline of diatom growth there is potentially large production and excretion of organic compounds, which in turn might precondition the water at the time when dinoflagellates start to grow.

The objective of this study was to investigate potential effects of diatom exudates on dinoflagellate growth. This was studied by growing cold-water dinoflagellates from the Baltic Sea in filtrates of co-occurring diatoms.

### Materials and Methods

Cultures of the diatoms *Melosira arctica*, *Chaetoceros wighamii*, *Diatoma tenuis*, *Thalassiosira baltica*, *Skeletonema costatum* and the dinoflagellates *Peridiniella catenata*, *Woloszynskia halophila* and *Scrippsiella hangoei* were established from single cells.

Filtrations of the diatom cultures were done after they had reached the stationary growth phase. Membrane filters (Poretics) with pore sizes of 2.0 and 0.2 µm were used. The filtrate was bubbled with air (~1 min) in order to saturate the CO<sub>2</sub> concentration and bring the pH down to ~7.5. Subsequently, inorganic nutrients were added in F/2 concentrations (Guillard 1975). Aged, autoclaved, bubbled water with the same nutrient additions were used as control. All treatments were done with 4 replicates.

The dinoflagellates were grown in 400-µl well-plate chambers. The initial number of dinoflagellate cells in each well was approximately 20. Well plates were placed in a temperature-controlled room (4 °C), at ~50 µmol photons m<sup>-2</sup> s<sup>-1</sup> and 12:12 L:D cycle. Growth was monitored by fluorescence using a fluorometer (Varian) with a well-plate reader. Fluorescence is not a direct measure, but a good proxy of biomass for monocultures grown in a constant light environment (Raven and Beardall 2006). Measurements of fluorescence were conducted daily for two weeks. Growth rate was calculated according to the equation:

$$\mu = (\ln N_t - \ln N_0)/t$$

where  $\mu$  is the specific growth rate d<sup>-1</sup>,  $N_0$  is the initial fluorescence,  $N_t$  is the fluorescence at time  $t$  and  $t$  is

the time in days. Statistical tests were conducted with Student's two tailed t-test assuming unequal variance using Microsoft Excel software.

After ~12 d the fluorescence signal was out of range for the best growing treatments, using the original measurement protocol. The measurement protocol of the fluorometer was changed in order to determine the higher fluorescence intensities, but to avoid any uncertainty in the conversion between measurement protocols, the growth rate presented here was calculated from the first week of exponential growth (not including the lag phase).

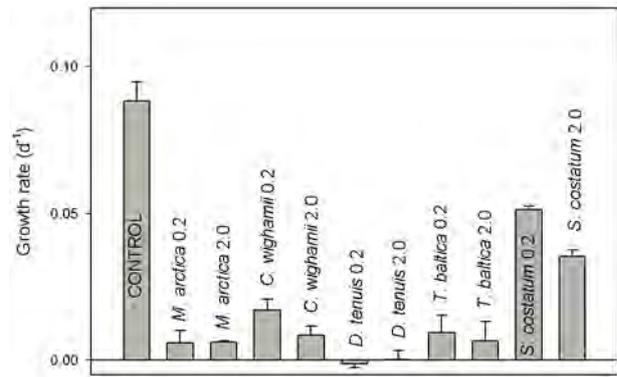
## Results

There was a lag phase of 4 d in all treatments before exponential growth started. The average growth rates of the controls during the first week of exponential growth was for *P. catenata*, *S. hangoei* and *W. halophila* 0.09 d<sup>-1</sup>, 0.02 d<sup>-1</sup> and 0.03 d<sup>-1</sup> respectively (Figs 1-3).

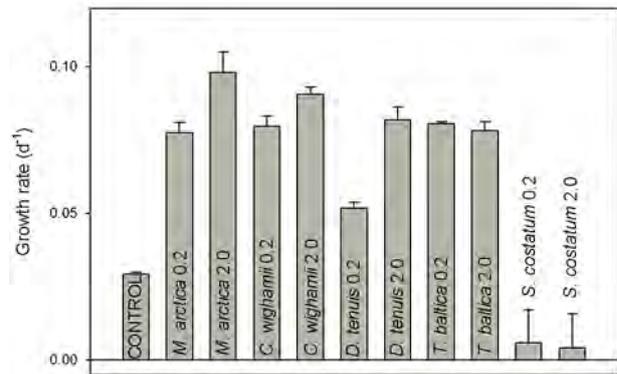
The diatom filtrate did affect the growth of the dinoflagellates. *P. catenata* had a lower growth rate ( $p < 0.002$ ) in all diatom filtrates compared with the control (Fig. 1), while *S. hangoei* had clearly higher growth rates in diatom filtrates ( $p < 0.001$ ) with the exception of the *S. costatum* filtrate, where the growth rate was lower compared to the control (Fig. 2). For *W. halophila* the growth was generally lower than for the other species and the variation between replicates higher. However, generally, the average growth rate in filtrates was lower compared to the control (Fig. 3), and the statistical analysis revealed that *W. halophila* growth was significantly lower in the filtrates of *D. tenuis* and *S. costatum* ( $p < 0.05$ ) compared to the control.

## Discussion

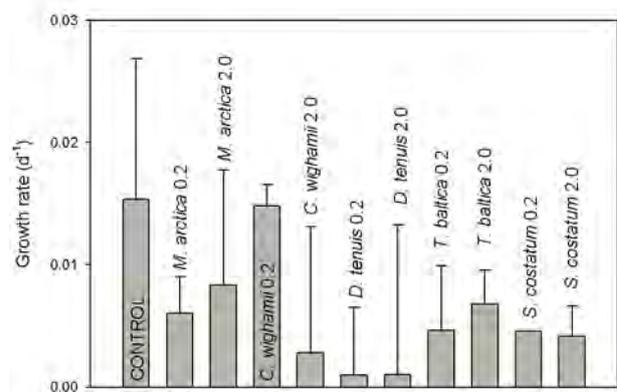
The results indicated that exudates produced and released from diatoms affect the growth of co-occurring dinoflagellates in the Baltic Sea. The effect of diatom filtrates compared to the control was species specific: generally negative for *P. catenata* and *W. halophila*, while *S. hangoei* had increased growth in diatom filtrates compared to the control. Additionally, there was some indication of different effect between filtrates from different diatom species. Interestingly, the filtrate of *S. costatum* was the only filtrate giving lower growth compared with the control for *S. hangoei*, while for *P. catenata*, this filtrate reduced growth less, compared with the other filtrates (Figs 1, 2). This could be the result of less exudates produced by *S. costatum*, or



**Figure 1.** Average specific growth rate of *Peridiniella catenata* in control and filtrates of *Melosira arctica*, *Chaetoceros wighamii*, *Diatoma tenuis*, *Thalassiosira baltica* and *Skeletonema costatum* measured over the first week of exponential growth. The filtrates were made with filters of 0.2 and 2.0  $\mu\text{m}$  pore size, indicated by 0.2 and 2.0 on the graph. Error bars represent SD,  $n = 4$ .



**Figure 2.** Average specific growth rate of *Scrippsiella hangoei*, measured over the first week of exponential growth. See Fig. 1 for legend details.



**Figure 3.** Average specific growth rate of *Woloszynskia halophila* measured over the first week of exponential growth. See Fig. 1 for legend details.

alternately exudates with different chemical composition compared with the other diatoms. Furthermore, there was some difference between the different filter sizes used for the same diatom species. For *S. hangoei*, the 2.0- $\mu\text{m}$  diatom filtrate generally produced better growth than the 0.2- $\mu\text{m}$  filtrate of the same species. For *P. catenata* the difference between filtrates of the same diatom species was less, but the 2.0- $\mu\text{m}$  filtrate from *C. wighamii* and *S. costatum* resulted in lower growth rates compared with the 0.2- $\mu\text{m}$  filtrate. The combined result suggest that the 0.2- $\mu\text{m}$  filter remove some of the filtrate effect, i.e. there are particles with size  $>0.2 \mu\text{m}$  enhancing the filter effect.

In dense cultures, high fixation rate of  $\text{CO}_2$  may increase the pH to growth-limiting levels (Goldman *et al.* 1982; Hansen 2002). In this study the treatments were not bubbled after the initial set-up, and pH was not monitored. However, the observed effect of diatom filtrates on growth was present from the beginning of the experiment when the pH was  $\sim 7.5$ . Additionally, in the well-plate chambers there were relatively short maximal distances (1 cm) to the surface allowing relatively high exchange rates of  $\text{CO}_2$  with the air. Thus, the pH did most likely not rise to growth limiting levels, at least not in the initial part of the experiment.

The chemical composition of the diatom exudates are not well known, but can be separated into different fractions (Smith and Underwood 2000; de Brouwer and Stal 2002). Some of these compounds are degradable by bacteria and will increase bacterial production (Decho 1990). However, the result shows that the preconditioning of diatoms had a long-lasting effect as there was no sign of changes in dinoflagellate growth in the filtrates during the whole experiment, i.e. the growth rates shown in Figs 1-3 persisted for the duration of the experiment. This indicates that the compounds released by diatoms affecting dinoflagellate growth are not easily degradable, and thus presumably of high molecular weight.

Preliminary results of carbon assimilation in dinoflagellates (results not shown) revealed no difference between control and filtrates in short-term incubations. This suggests that the effect of diatom filtrates on dinoflagellates growth is not an immediate effect, reducing photosynthetic production (1-2 h incubations), but rather affects growth on a longer time scale.

The results give no grounds for conclusions about the mechanisms behind the observed effect of diatom filtrates on dinoflagellate growth. One might speculate that compounds in the filtrate have nutritional val-

ue for *S. hangoei*, stimulating growth, but this needs further studies. However, the fact that filtrates may limit or enhance growth depending on the dinoflagellate species, indicate that the effect is not due to toxic compounds, nor an active allelopathic strategy of the diatoms.

In conclusion, filtrates of diatoms did affect the growth rate of the studied dinoflagellates, and the effect was species specific. What effect these exudates have in a natural environment remains an open question, but this could potentially be a factor that influences phytoplankton growth during spring bloom in the Baltic Sea.

### Acknowledgements

This study was funded by Finnish Academy and Walter and Andr e de Nottbeck Foundation. I would like to thank the staff at Tv rminne Zoological Station, Univ. Helsinki, in particular M. Sjöblom and E. Salminen, for assistance in the laboratory.

### References

- de Brouwer, J.F.C. & Stal, L.J. (2002). *J. Phycol.* 38: 1-10.
- Decho, A.W. (1990). *Oceanogr. Mar. Biol. Ann. Rev.* 28: 73-153.
- Goldman, J.C., Azov, Y., Riley, C.B. & Dennett, M.R. (1982). *J. Exp. Mar. Biol. Ecol.* 57: 1-13.
- Guillard, R.R.L. (1975). In: *Culture of Marine Invertebrate Animals*, Smith, W.L. & Chanley, M.H. (eds), Plenum Press, New York, pp. 26-60.
- Hansen, P.J. (2002). *Aquat. Microb. Ecol.* 28: 279-288.
- Hoagland, K.D., Rosowski, J.R., Gretz, M.R. & Roemer, S.C. (1993). *J. Phycol.* 29: 537-566.
- Högländer, H., Larsson, U. & Hajdu, S. (2004). *Mar. Ecol. Prog. Ser.* 283: 15-27.
- Myklestad, S. & Haug, A. (1972). *J. Exp. Mar. Biol. Ecol.* 9: 125-136.
- Paterson, D.M. (1989). *Limnol. Oceanogr.* 34: 223-234.
- Raven, J.A. & Beardall, J. (2006). *New Phytol.* 169: 449-450.
- Smith, D.J. & Underwood, G.J.C. (1998). *Limnol. Oceanogr.* 43: 1578-1591.
- Smith, D.J. & Underwood, G.J.C. (2000). *J. Phycol.* 36: 321-333.
- Straats, N., Stal, L.J., de Winder, B. & Mur, L.R. (2000). *Mar. Ecol. Prog. Ser.* 193: 261-269.
- Underwood, G.J.C., Boulcott, M., Raines, C.A. & Waldron, K. (2004). *J. Phycol.* 40: 293-304.

## Allelopathic effects of bioactive compounds produced by harmful algae

Urban Tillmann, Uwe John, Bernd Krock and Allan Cembella  
 Alfred Wegener Institute, Am Handelshafen 12, D-27570 Bremerhaven, Germany,  
 utillmann@awi-bremerhaven.de

### Abstract

Chemical interactions between species have been widely studied in terrestrial ecology but are less well known in marine ecosystems, especially among protists. In the marine plankton, temporal and spatial changes of biomass and species composition have traditionally been thought to be mainly regulated by resource availability and abiotic factors. However, there is increasing evidence that interspecific interactions in the plankton play a major role in succession, food web structure and bloom development. Many HAB species are regarded as rather poor exploitation competitors in terms of growth rate and/or resource uptake capabilities. There is some evidence that a number of HAB species may gain dominance by the production of bioactive compounds, particularly secondary metabolites that affect growth or elicit other physiological responses in other organisms. Such allelochemicals may be targeted to exclude competitors from exploiting limited resources (interference competition), as well as to avoid/reduce predation. HAB species with allelopathic potency include cyanobacteria, raphidophytes, haptophytes and dinoflagellates. Although the causative substances are poorly described, such allelochemicals seem to be distinct from the commonly known phycotoxins, many of which are neurotoxins.

### Definition and Scope

The chemical ecology of microalgae has received much less attention than such inter-specific interactions in terrestrial ecosystems. Nevertheless, in recent years there has been an emerging awareness of the importance of chemically mediated processes in the plankton. This is reflected by a number of recent reviews on allelopathy and chemical ecology of microalgae in aquatic systems (Cembella 2003; Gross 2003; Legrand *et al.* 2003; Granéli and Hansen 2006).

The term allelopathy was traditionally used exclusively for plant-plant interactions, and originally applied only to negative interactions. Allelopathy has since been redefined as “any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment” (Rice 1984). Within the unicellular planktonic organisms (protists), however, it is notoriously difficult to differentiate “plants” from “animals”, because motility via flagella, mixotrophy and kleptochloroplasty are rather widespread. We will therefore use allelopathy to refer to chemically mediated interactions between unicellular organisms in general, including both competitors and grazers. There is much more known on inter-specific effects of bioactive compounds produced by cyanobacteria than for protistan flagellates. Indeed, a recent review of Babica *et al.* (2006) on allelopathy of microcystins, for example, presents a 5-page table on

studies on that topic. Although we recognize the importance of cyanobacteria as legitimate HAB species and many of the allelochemical mechanisms involving cyanobacterial blooms and toxins in food web dynamics are undoubtedly analogous to those associated with eukaryotic microalgae, we will focus this review on planktonic protists.

### Introduction

In the marine plankton, temporal and spatial changes of biomass and species composition have traditionally been thought to be mainly regulated by resource availability and abiotic factors, the so-called “bottom up” processes. In order to explain HAB blooms, which are often dominated by a single species, conceptual and experimental approaches thus tended to focus on species-specific physiological and ecological traits of individual HAB species. This “autecological approach” has been refined with the recognition of cryptic genetic variability and the requirement for using multiple strains in physiological experiments to determine rate constants. Nevertheless, the interpretation of such results to explain bloom dynamics remains largely unsatisfactory.

There is increasing evidence that inter-specific interactions in the plankton play a major role in succession, food web structure and bloom development. Competition and grazing are undoubtedly among the most important interactions between planktonic spe-

cies. Resource competition may occur in two forms – exploitation and interference. Exploitation competition is the direct use of a resource, thereby reducing the availability of this resource to competing species. On the other hand, interference competition occurs when access to a resource is denied to competitors. The ability of species to be successful exploitation competitors may be evaluated by their  $K_s$  values, the half-saturation constant for nutrient uptake. Smayda (1997) compiled  $K_s$  values available in the literature and pointed out a paradox in that HAB species generally lack the expected high affinity for nutrient uptake thought to be essential for their frequent bloom occurrence in nutrient-depleted waters. So he suggested four major adaptations of HAB flagellates to have been evolved to offset the ecological disadvantages of their low nutrient affinity: (1) nutrient retrieval migrations; (2) mixotrophic tendencies; (3) allelochemically enhanced inter-specific competition; and (4) allelochemical, anti-predation defence mechanisms (Smayda 1997).

With respect to grazing, there is increasing evidence that protistan grazing is an important loss factor, even for larger bloom-forming phytoplankton (Tillmann 2004). A recent data compilation (Strom *et al.* 2001) showed that the ratio of microplankton grazing rate and phytoplankton growth rate for coastal area, where most phytoplankton blooms occur, is on average as high as 0.71, meaning that a very large proportion of primary production is immediately removed by these micrograzers. This tight coupling has two major implications: first, we expect an intense selective pressure for the evolution of defences against micrograzers; second, because blooms do occur, there must be mechanisms promoting uncoupling of phytoplankton growth and microzooplankton grazing. Chemically mediated reduction of grazing losses is believed to one of these processes.

### Allelopathic HAB Species

A number of protistan HAB species for which allelopathic effects have been described, are listed in Table 1. This list is not meant to be exclusive. For example, there are a number of species, for which haemolytic effects of cell extracts have been described (e.g., Fu *et al.* 2004). Although it is tempting to assume allelopathic effects for these species *in vivo* as well, more research and evidence is needed for such conclusions.

For many of the species listed in Table 1, negative effects towards both competitors and protistan graz-

**Table 1.** Eukaryotic HAB species for which allelopathic effects towards competitors and protistan grazers are described. (1) Arzul *et al.* 1999 (2) Tillmann *et al.* (submitted a, b) (3) Fistarol *et al.* 2004 (4) Hansen 1989 (5) Tillmann & John 2002 (6) Kubanek *et al.* 2005 (7) Sugg & VanDolah 1999 (8) Uchida 1995 (9) Kamiyama & Arima 1997 (10) Parrish *et al.* 1998 (11) Hansen 1995 (12) Adolf *et al.* 2006 (13) Adolf *et al.* (in press) (14) Schmidt & Hansen 2001 (15) John *et al.* 2002 (16) Fistarol *et al.* 2003 (17) Granéli & Johannsson 2003 (18) Tillmann 2003 (19) Pratt 1966 (20) Verity & Stoecker 1982 (21) Clough & Strom 2005.

Species	Compounds	competitors	Protistan grazers
<i>Alexandrium</i> spp.	?	(1), (2), (3)	(3), (4), (5)
<i>Karenia brevis</i>	?	(6)	-
<i>Prorocentrum lima</i>	?	(7)	-
<i>Heterocapsa circularisquama</i>	?	(8)	(9)
	Porphyrin deriv. ?		
<i>Karenia mikimotoi</i>	Glycoglycerolipids; Fatty acids	(10)	(11)
<i>Karlodinium veneficum</i>	Karlotoxins	(12)	(13)
<i>Chrysochromulina polylepis</i>	?	(14)	(15)
<i>Prymnesium parvum</i>	?	(16), (17)	(18)
	Prymnesin?		
<i>Heterosigma akashiwo</i>	?	(19)	(20), (21)

ers have been described. However, with a few exceptions, compounds responsible for allelopathic effects are poorly characterised. *Karenia mikimotoi* has been shown to produce some compounds which are detrimental to the growth of other algae (Gentien and Arzul 1990) and large amounts of haemolytic glycoglycerolipids have been determined for this species (Parrish *et al.* 1998). Haemolytic and ichthyotoxic glycoglycerolipids have also been identified in extracts of the haptophyte *Chrysochromulina polylepis* (Yasumoto *et al.* 1990). However, it is not clear whether or not toxicity is a function of such lipid components, as analysis of lipid classes and fatty acids derived from toxic and non-toxic clones of *C. polylepis* exhibited no identifiable difference (John *et al.* 2002). Very potent haemolytic and ichthyotoxic polyethers known as prymnesins have been identified (Igarashi *et al.* 1996) from the closely related haptophyte *Prymnesium parvum*. However, because standard material is lacking it is not clear if prymnesins are indeed the major extracellular compounds responsible for the observed allelopathic effects of this species. The dinoflagellate *Karlodinium veneficum* has been shown to produce cytotoxic compounds (karlotoxins) that render cell membranes permeable to a range of small ions and molecules, resulting in cell death through osmotic lysis (Place *et*

al. 2006). Addition of purified karlotoxin resulted in both growth inhibition of competitors (Adolf *et al.* 2006) and reduced grazing rates of protistan grazers (Adolf *et al.*, in press). This is one rare example where allelopathic effects, and not just haemolysis, have been shown to be due to a known compound. For the dinoflagellate *Heterocapsa circularisquama*, direct cell contact is obviously involved in negative effects on both competitors (Uchida *et al.* 1995) and grazers (Kamiyama and Arima 1997). Miyazaki *et al.* (2005) recently characterised haemolytic porphyrine derivatives in cell extracts of *H. circularisquama*, but it has to be shown that these compounds are involved in the allelopathic effects.

Within the dinoflagellate genus *Alexandrium*, lytic activity of extracellular metabolites and other negative effects upon other planktonic protists is rather widely expressed. Extracellular compounds produced by *Alexandrium* heavily affect cells of various protistan species, including both competitors (Blanco and Campos 1988; Arzul *et al.* 1999; Fistarol *et al.* 2004; Tillmann *et al.* submitted a, b) and protistan grazers (Hansen 1989; Hansen *et al.* 1992; Matsuoka *et al.* 2000; Tillmann and John 2002). Observed negative effects of allelochemicals from *Alexandrium* include immobilisation, and morphological and/or behavioural changes, mainly as preliminary stages of cell lysis (Tillmann *et al.* submitted a).

Allelochemical effects, such as target cell lysis, can be expressed very rapidly. For example, when the cryptophyte *Rhodomonas baltica* is incubated with a cell-free filtrate of *A. ostensfeldii*, complete lysis, after a short pre-lytic phase, was observed after a couple of minutes (Fig. 1). By using cell-free filtrate in this experiment, it was clear that the responsible lytic compounds were extracellular. Nevertheless, quantitative comparison showed that % activity in filtrates decreased with decreasing pore size with various polycarbonate filters (Table 2).

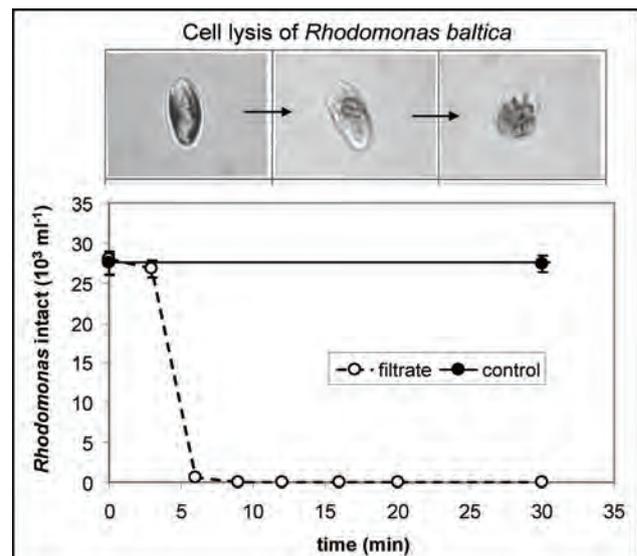
With pore sizes  $\leq 0.2 \mu\text{m}$ , no measurable lytic activity was found in the filtrate. With glass-fibre filters (Whatman GF/C) of a nominal pore size of  $1 \mu\text{m}$ , activity in the filtrate was reduced by an order of magnitude, indicating that both pore size and adsorption of the bioactive components to the filter material may be responsible for activity reduction.

### Examples of Allelopathy

Allelopathic effects of bioactive compounds produced by HAB species that might play an important role for species succession and bloom development

**Table 2.** Loss of lytic activity of allelopathic compounds after filtration. Fifty ml each of a culture of *A. tamarense* were filtered through polycarbonate filters of various pore sizes and one glass-fibre filter (Whatman GF/C, nominal pore size  $1 \mu\text{m}$ ). For each sample, a dilution/response (mortality of *Rhodomonas baltica*) curve was recorded over a 3 h incubation period. EC50 concentration (% dilution of the sample causing 50 % mortality) was calculated from a sigmoidal fit of data to log-normalised x-values.

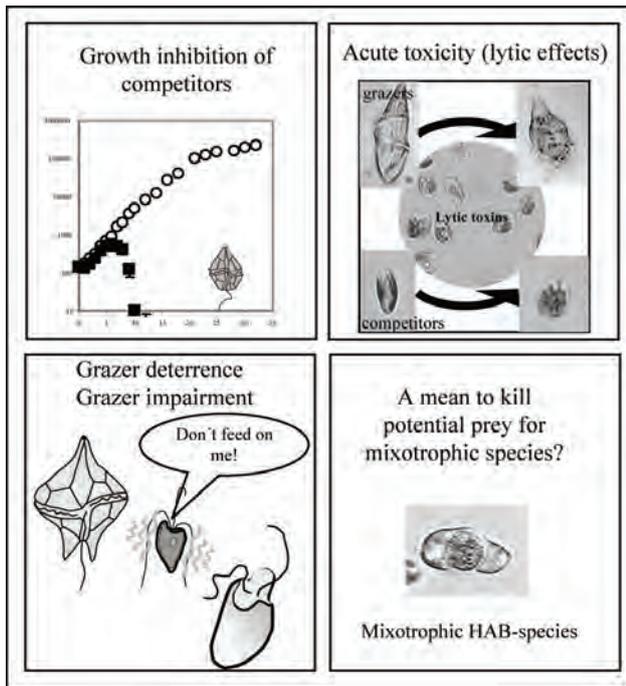
typ	EC <sub>50</sub>	% activity in filtrate
Algae	1.64	–
8.0 $\mu\text{m}$	1.68	97.6
5.0 $\mu\text{m}$	1.73	94.5
3.0 $\mu\text{m}$	2.13	77.0
1.2 $\mu\text{m}$	2.41	68.9
0.4 $\mu\text{m}$	2.49	65.7
0.2 $\mu\text{m}$	-	0
0.1 $\mu\text{m}$	-	0
GF/C	16.99	9.7



**Figure 1.** Cell lysis of *Rhodomonas baltica* after incubation with cell-free filtrate of *Alexandrium ostensfeldii* (2000 cells  $\text{ml}^{-1}$ ).

include growth inhibition of competitors or acute toxic effect (mainly via cell lysis) towards both competitors and protistan grazers. With respect to protistan grazers, compounds may act as grazer deterrents or may cause all kind of grazer impairment. Finally, as a number of HAB species are in fact mixotrophic, bioactive compounds may be involved in immobilizing or killing potential prey (Fig. 2).

Growth experiments involving species mixtures or addition of cell-free filtrate of the putative allelo-



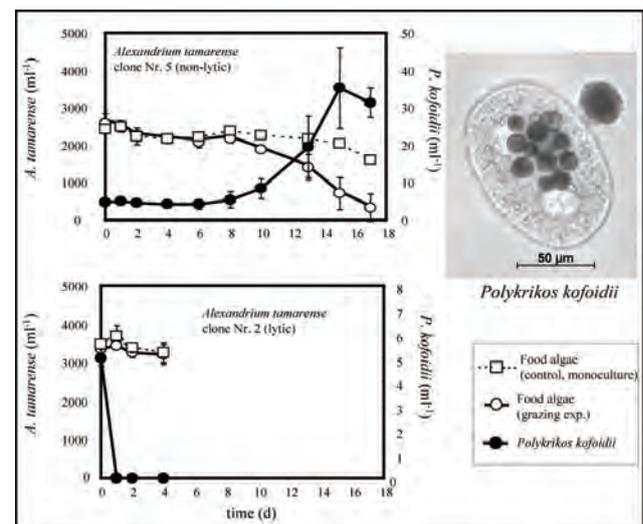
**Figure 2.** Potential effects of bioactive compounds on marine protists.

pathic species to cultures of target species can be used to study growth inhibition and/or induced mortality of competitors. Mixed growth experiments by Schmidt & Hansen (2001) showed that *Chrysochromulina polylepis* outcompeted most other algae in mixed cultures. Similarly, exposure to *Alexandrium tamarens* killed all nine target phytoplankton species tested in mixed growth experiments (Tillmann & Hansen, in prep.). In these types of experiments, it is important to distinguish allelopathic effects from those of physico-chemical factors because a number of autotrophic (Schmidt and Hansen 2001) and heterotrophic (Pedersen and Hansen 2003) eukaryotes are negatively affected simply by the high pH often generated in dense cultures.

Whole-cell cultures or filtrates can also be used in short-term incubation experiments to quantify mortality of target species (Tillmann *et al.* submitted a, b). By adding cell-free filtrate to target species (Granéli and Johansson 2003; Fistarol *et al.* 2005), potential competition for nutrients or other inter-specific interactions can be avoided. Addition of filtrate has also been used to study allelopathic effects of *Alexandrium tamarens* (Fistarol *et al.* 2004) and *Prymnesium parvum* (Fistarol *et al.* 2003) on natural communities.

Allelopathically enhanced grazer defence can be expressed as acute mortality of micrograzers or by sublethal effects such as reduced feeding rates. In comparative grazing studies comparing toxic and non-toxic clones of *Chrysochromulina polylepis* (screened

by *Artemia* bioassay), John *et al.* (2002) confirmed profound differences in ingestion, clearance and growth rates of the protistan predator *Oxyrrhis marina*. Even at algal concentrations of  $400 \times 10^3$  cells  $\text{ml}^{-1}$ , *O. marina* was not killed by the presence or ingestion of toxic *C. polylepis*, indicating that involved compounds act as grazer deterrence. An example of acute toxicity towards protistan grazers is shown in Fig. 3. In this comparative approach, a lytic and a non-lytic clone of *A. tamarens*, isolated from the same geographical location on the Scottish east coast (Alpermann *et al.* 2004), were offered as food to the large heterotrophic dinoflagellate *Polykrikos kofoidii*. Whereas the non-lytic strain was grazed and allowed *Polykrikos* cell numbers to increase, the lytic clone caused *Polykrikos* to die out after only one day of incubation. This acute mortality is not due to starvation because starved *Polykrikos* are able to survive for at least a couple of days. Similarly, some strains of both *A. tamarens* and *A. ostenfeldii* have been shown to be



**Figure 3.** Grazing experiments with *Polykrikos kofoidii* offered a non-lytic (upper panel) and a lytic (lower panel) clone of *Alexandrium tamarens*.

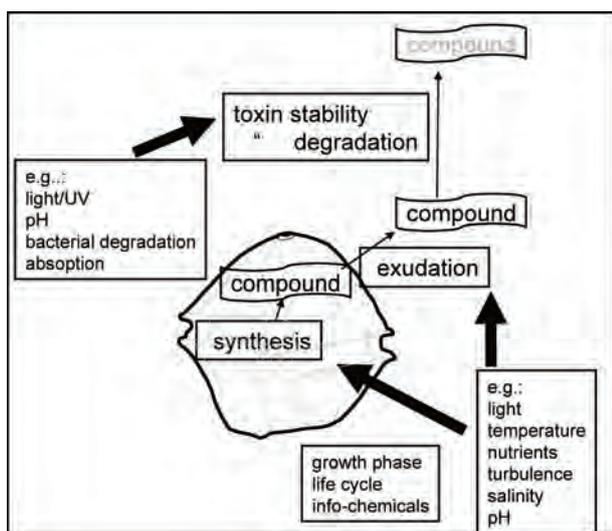
lethal to the large tintinnid species *Favella ehrenbergii* (Hansen 1989, 1995).

Although many toxic microalgae that produce potent known phycotoxins (e.g. tetrahydropurines, domoic acid, linear-, cyclic- and ladder-frame polyethers) are primarily photoautotrophic (Cembella 2003), species that express allelochemical activity and in which the bioactive substances are poorly defined are often phagotrophic or closely related to known phagotrophs (Stoecker *et al.* 2006). For *Prymnesium parvum* there is good evidence that toxins are involved in prey capture. The feeding frequency

of *P. parvum* on motile prey is positively correlated with toxicity (Skovgaard and Hansen 2003). Toxicity has also been shown to be a key factor in determining the interaction of *P. parvum* with protistan grazers (Tillmann 2003). Whereas low toxicity *P. parvum* is a suitable prey for the heterotrophic dinoflagellate *Oxryrrhis marina*, the dinoflagellate is rapidly killed by *P. parvum* at high toxicity levels. Moreover, under these latter conditions, the former predator (*O. marina*) is ingested by *P. parvum*, thus reversing the normal direction of grazing interactions. Similarly, toxic strains of *Karlodinium veneficum*, a mixotrophic dinoflagellate, are grazed less by *O. marina* than are non-toxic strains (Adolf *et al.*, in press). Furthermore, grazing of *O. marina* on non-toxic prey was inhibited by a sub-lethal dose of purified karlotoxin. There is evidence that karlotoxins are also involved in prey capture (Adolf *et al.* 2006). Immobilization of flagellates is probably the common mechanism behind anti-herbivory and prey immobilization.

### Variability

Factors affecting allelopathic activity are, in most cases, poorly known. As is typical for most secondary metabolites, high inter-specific (Tillmann *et al.*, in prep. b), as well as intra-specific variability (John *et al.* 2002; Alpermann *et al.* 2004; Tillmann *et al.* submitted a) is often expressed. Presumably, much of this variation is a function of intrinsic genetic factors determined at the genomic level with modulation of expression at the transcriptional and translational stages. In addition, there is the whole suite of exogenous biotic and abiotic factors that are involved in allelopathy (Fig. 4).



**Figure 4.** Factors potentially affecting allelochemical potency.

For extracellular compounds, two processes, biosynthesis and exudation, are affected by numerous intrinsic and extrinsic factors. In addition, excreted compounds in the aqueous, colloidal or particulate phases are subject to biological and physico-chemical factors affecting toxin stability and degradation. Moreover, a single factor might affect different processes in different ways. For example, light has been claimed to be essential for toxin production in *Prymnesium parvum* (Shilo 1967), with higher light conditions augmenting toxin production (Shilo and Aschner 1953). On the other hand, the extracellular toxins are known to be rapidly inactivated by exposure to both visible and UV light (Parnas *et al.* 1962). Other factors shown to affect allelopathy include pH (Ulitzur and Shilo 1964; Schmidt and Hansen 2001) and nutrient concentration. Under nutrient-limiting conditions, both intracellular concentrations of haemolytic compounds and allelopathic effects on other microalgae increased for *P. parvum* (Granéli and Johansson 2003).

### Compounds

Although allelopathic compounds of HAB species are, in most cases, poorly characterized, the limited evidence based on solubility, polarity, and molecular weight estimates of “toxic” fractions tend to indicate that they are not consistent with the known phyco-toxins. With respect to *Alexandrium* spp., PSP toxins (saxitoxin and derivatives) are clearly not responsible for observed allelopathic effects against other protists (Tillmann and John 2002). Furthermore, Tillmann *et al.* (submitted a) recently showed that the allelochemical potency of certain *A. ostentfeldii* strains is not related to spiroside cell quota. Sugg and VanDolah (1999) showed that allelopathy of the DSP toxin-producing dinoflagellate *Prorocentrum lima* was not obviously related to cell content of the toxin okadaic acid. Although this DSP toxin exhibited growth inhibitory potential against other microalgae, it did not represent the major growth-inhibitory activity found in the growth medium of *P. lima* cultures. The brevetoxin-producing dinoflagellate *Karenia brevis* was shown to negatively affect a number of co-occurring phytoplankton species in mixed growth experiments (Kubanek *et al.* 2005). However, by adding pure brevetoxins, these workers showed that unidentified compounds (other than brevetoxins) must be involved in allelopathy in this species. The domoic acid-producing diatom *Pseudo-nitzschia multiseries* apparently had no allelopathic effects on other microalgae, and bioassays testing the effect of purified domoic acid

confirmed a lack of allelopathic effects of this toxin (Lundholm *et al.* 2005). In summary, there is little conclusive evidence that the algal-derived “shellfish toxins”, which are toxic to higher trophic levels including humans, play a major role in allelopathic interactions, at least among protists. On the other hand, almost all allelopathic HAB species seem to fit within the group of ichthyotoxic species. Even *Alexandrium* spp. may be grouped here, since blooms of *Alexandrium* have been involved in massive fish kills when these dinoflagellates are at high cell concentrations (Mortensen 1985; Cembella *et al.* 2002). Pathology studies have tended to support the hypothesis that fish mortalities are associated with damage to the gills and/or asphyxiation. Such ichthyotoxic effects make sense because allelochemical activity in protists appears to be related to membrane-disruption of target cells - immobilization and/or cell lysis are the most commonly observed effects.

### Conclusions

Chemically mediated interference with other protistan species has been demonstrated and growth reduction or mortality has been quantified for a number of HAB species. However, this represents only the first step in a “to do” list proposed by Putnam & Tang (1986) for a proof of allelopathy. According to these authors the next steps would be to: (2) isolate, characterise, and assay the chemicals against species that were previously affected. Identification of chemicals that are not artefacts is a key step in proof of allelopathy; (3) obtain toxicity with similar symptomology when chemical(s) are added back to the system and (4) monitor release of chemicals from the donor (plant) and detect them in the environment. Thus, despite increasing evidence for a potentially high allelochemical capacity among HAB species, its relevance to *in situ* dynamics and bloom formation is still uncertain. We therefore remain in agreement with the elegant conclusion of Smayda (1997) from almost a decade ago that: “until studies are carried out with pure phycotoxins and allelochemical metabolites, or there is a definitive chemical characterization of the active metabolites, and these are unequivocally linked to a particular regulatory process, allelochemical involvement in HAB events remains speculative.” Although much progress has been made in the few years – the remarkable work on karlotoxins (Place *et al.* 2006) stands as a noteworthy model for such allelochemical studies – the need remains to identify causative compounds and to develop methods to measure their

*in situ* concentration and bioactivity. Only in this way, under environmentally realistic conditions, will we be able to demonstrate that allelopathic effects shown in the laboratory are indeed important for *in situ* bloom dynamics.

### Acknowledgements

Thanks to Per Juel Hansen, Catherine Legrand and Jason Adolf for fruitful discussions.

### References

- Adolf, J. *et al.* (in press). Harmful Algae  
 Adolf, J.E., Bachvaroff, T.R., Krupatkina, D.N., Nogaki, H., Brown, P.J.P., Lewitus, A.J., Harvey, H.R. & Place, A.R. (2006). African J. Mar. Sci. 28: 415-419.  
 Adolf, J.E., Krupatkina, D., Bachvaroff, T.R. & Place, A.R. (2006). 12th International Conference on Harmful Algae. Abstract Book, p. 61, Copenhagen, Denmark.  
 Alpermann, T., John, U., Tillmann, U., Evans, K. & Cembella, A. (2004). XI International Conference on Harmful Algal Blooms. Abstract Book, page 57 Cape Town, South Africa.  
 Arzul, G., Seguel, M., Guzman, L. & Erard-LeDenn, E. (1999). J. Exp. Mar. Biol. Ecol. 232: 285-295.  
 Babica, P., Bláha, L. & Marsalek, B. (2006). J. Phycol. 42: 9-20.  
 Blanco, J. & Campos, M.J. (1988). Aquaculture 68: 289-298.  
 Cembella, A.D. (2003). Phycologia 42: 420-447.  
 Cembella, A.D., Quilliam, M.A., Lewis, N.I., Bauder, A.G., Dell Aversano, C., Thomas, K., Jellet, J. & Cusack, C.R. (2002). Harmful Algae 1: 313-325.  
 Clough, J. & Strom, S. (2005). Aquat. Microb. Ecol. 39: 121-134.  
 Fistarol, G.O., Legrand C. & Granéli, E. (2003). Mar. Ecol. Prog. Ser. 255: 115-125.  
 Fistarol, G.O., Legrand, C. & Granéli, E. (2005). Aquat. Microb. Ecol. 41: 153-161.  
 Fistarol, G.O., Legrand, C., Selander, E., Hummert, C., Stolte, W. & Granéli, E. (2004). Aquat. Microb. Ecol. 35: 45-56.  
 Fu, M., Koulman, A., van Rijssel, M., Lützen, A., de Boer, K., Tyl, M.R. & Liebezeit, G. (2004). Toxicol. 43: 355-363.  
 Gentien, P. & Arzul, G. (1990). J. Mar. Biol. Ass. U.K. 70: 571-581.  
 Granéli, E. & Hansen, P.J. (2006). In: Ecology of Harmful Algae, Granéli, E. & Turner, J.T., (eds), Springer, Berlin, pp. 189-201.

- Granéli, E. & Johansson, N. (2003). *Harmful Algae* 2: 135-145.
- Gross, E.M. (2003). *Crit. Rev. Plant Sci.* 22: 313-339.
- Hansen, P.J. (1989). *Mar. Ecol. Prog. Ser.* 53: 105-116.
- Hansen, P.J. (1995). *Mar. Ecol. Prog. Ser.* 121: 65-72.
- Hansen, P.J., Cembella, A.D. & Moestrup, Ø. (1992). *J. Phycol.* 28: 597-603.
- Igarashi, T., Satake, M. & Yasumoto, T. (1996). *J. Am. Chem. Soc.* 118: 479-480.
- John, U., Tillmann, U. & Medlin, L. (2002). *Harmful Algae* 1: 45-57.
- Kamiyama, T. & Arima, S. (1997). *Mar. Ecol. Prog. Ser.* 160: 27-33.
- Kubanek, J., Hicks, M.K., Naar, J. & Villareal, T.A. (2005). *Limnol. Oceanogr.* 50: 883-895.
- Legrand, C., Rengefors, K., Fistarol, G.O. & Granéli, E. (2003). *Phycologia* 42: 406-419.
- Lundholm, N., Hansen, P.J. & Kotaki, Y. (2005). *Mar. Ecol. Prog. Ser.* 288: 21-33.
- Matsuoka, K., Cho, H.J. & Jacobson, D.M. (2000). *Phycologia* 39: 82-86.
- Miyazaki, Y., Nakashima, T., Iwashita, T., Fujita, T., Yamaguchi, K. & Oda, T. (2005). *Aquat. Toxicol.* 73: 382-393.
- Mortensen, A.M. (1985). In: *Toxic Dinoflagellates*, Anderson, D.M., White, A.W. and Baden, D.G. (eds), Elsevier, Amsterdam, pp. 165-170.
- Parnas, I., Reich, K. & Bergmann, F. (1962). *Appl. Microbiol.* 10: 237-239.
- Parrish, C.C., Bodennec, G. & Gentien, P. (1998). *Phytochemistry* 47: 783-787.
- Pedersen, M.F. & Hansen, P.J. (2003). *Mar. Ecol. Prog. Ser.* 33: 33-41.
- Place, A.R., Harvey, H.R., Bai, X. & Coats, D.W. (2006). *Afr. J. Mar. Sci.* 28: 347-351.
- Pratt, D.M. (1966). *Limnol. Oceanogr.* 11: 447-455.
- Putnam, A.R. & Tang, C.S. (1986). *The Science of Allelopathy*. Wiley, New York.
- Rice, E.L. (1984) *Allelopathy*. Academic Press, New York.
- Schmidt, L.E. & Hansen, P.J. (2001). *Mar. Ecol. Prog. Ser.* 216: 67-81.
- Shilo, M. (1967). *Bact. Rev.* 31: 180-193.
- Shilo, M. & Aschner, M. (1953). *J. Gen. Microbiol.* 8: 333-343.
- Skovgaard, A. & Hansen, P.J. (2003). *Limnol. Oceanogr.* 48: 1161-1166.
- Smayda, T.J. (1997). *Limnol. Oceanogr.* 42: 1137-1153.
- Stoecker, D.K., Tillmann, U. & Granéli, E. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer, Berlin, pp. 177-187.
- Strom, S.L., Brainard, M.A., Holmes, J.L. & Olson, M.B. (2001). *Mar. Biol.* 138: 355-368.
- Sugg, L.M. & VanDolah, F.M. (1999). *J. Phycol.* 35: 93-103.
- Tillmann, U. (2003). *Aquat. Microb. Ecol.* 32: 73-84.
- Tillmann, U. (2004). *J. Eukaryot. Microbiol.* 51: 156-168.
- Tillmann, U. & John, U. (2002). *Mar. Ecol. Prog. Ser.* 230: 47-58.
- Tillmann, U., John, U. & Cembella, A.D. (submitted a). *J. Plankton Res.*
- Tillmann, U., Alpermann, T., John, U. & Cembella A.D. (submitted b). *Harmful Algae*
- Uchida, T., Yamaguchi, M., Matsuyama, Y. & Honjo, T. (1995). *Mar. Ecol. Prog. Ser.* 118: 301-303.
- Ulitzur, S. & Shilo, M. (1964). *J. Gen. Microbiol.* 36: 161-169.
- Verity, P.G. & Stoecker, D. (1982). *Mar. Biol.* 72: 79-87.
- Yasumoto, T., Underdahl, B., Aune, T., Hormazabal, V. & Skulberg, O.M. (1990). In: *Toxic Marine Phytoplankton*, Granéli, E., Sundström, B., Edler, L. & Anderson, D.M. (eds), Elsevier, New York, pp. 436-440.

## Promotion of oxidative stress in *Synechocystis* sp. strain PCC 6803 via cyanobacterial toxins

M. Vassilakaki and S. Pflugmacher

Leibnitz Institute of Freshwater Ecology and Inland Fisheries, RG Biochemical Regulation, Müggelseedamm 301, 12587 Berlin, Germany, Forschungsverbund e.V., vassilakaki@igb-berlin.de

### Abstract

Cyanobacteria produce a variety of different secondary metabolites including microcystins. Until now the ecological role of microcystins for cyanobacteria themselves and the aquatic ecosystem has not been thoroughly understood. The aim of this study is to evaluate if microcystins might be used as a communication tool for interspecies cyanobacterial communication via the promotion of oxidative stress. Reactive oxygen species are known to be used as plant signals. Do cyanobacterial toxins promote oxidative stress in *Synechocystis* sp. strain PCC 6803 and what are the physiological effects? This study shows an increase of intra cellular hydrogen peroxide and catalase, both markers for oxidative stress, upon exposure to microcystin.

### Introduction

It is well known that cyanobacteria can produce different types of secondary metabolites with fungicidal and/or antibacterial effects. Some of them are so-called cyanotoxins, e.g. the microcystins, which are the most prominent group of cyanobacterial secondary metabolites. Unfortunately, their ecological function is all but unknown (Jüttner 1999). Microcystins are mainly set free from producer cells during cell lysis or bloom breakdown, but in field samples without cyanobacterial cell lysis a release of toxin from intact cells in the range of 0.001 – 16  $\mu\text{g L}^{-1}$  has been shown (Fastner *et al.* 2001).

Many bacteria produce signalling molecules and release them into the environment to communicate with other bacteria (Cámara *et al.* 2002). ‘Quorum Sensing’ (QS) is the most common terminology used to describe this phenomenon. The goal of this study was to examine if cyanobacterial secondary metabolites, in this case the microcystins, execute this function in the QS system. As the microcystins are also released from intact cells in small quantities, there is the possibility that they work as signal molecules. Pure microcystin-LR (MC-LR) was tested to examine the possible function as a signal molecule and to trace down the effects on this toxin. Additionally, cell-free cyanobacterial crude extracts containing the same amount of MC-LR were tested to simulate an environmentally more relevant situation. The following hypothesis was tested: Are microcystins able to generate oxidative stress via the enhanced formation of reactive oxygen species in *Synechocystis* sp. cells? Can microcystins act as a communication tool in the cells? In higher plants reactive oxygen species (ROS)

are known to be part of a cell internal signalling pathway (Ryals *et al.* 1996; Dat *et al.* 2000; Gechev *et al.* 2006).

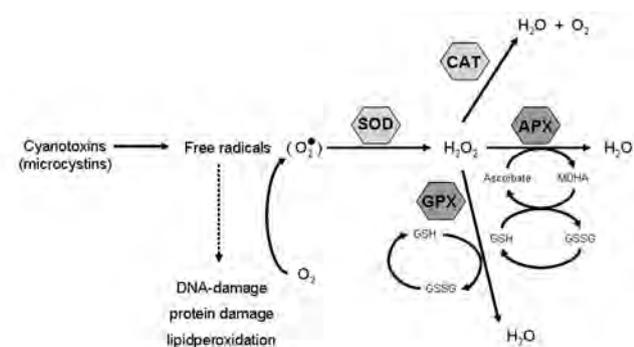
Under normal physiological conditions ROS do not pose a threat for organisms because of their efficient protection systems. Under stress conditions such as exposure to cyanobacterial toxins, the balance between the formation of ROS and the antioxidative defence system controlling them is disturbed. Oxidative stress is imposed on cells as a result of one of three factors: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species. Under normal conditions, ROS (e.g. hydroxyl radical, superoxide and hydrogen peroxide) are cleared from the cell by the action of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), or ascorbate peroxidase (APX) (Fig. 1).

The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Additionally, oxidative stress and ROS might have implications for growth and reproduction as well and, therefore, are able to influence ecosystems (Pflugmacher *et al.* 2006).

### Materials and Methods

#### Culture conditions

*Synechocystis* sp. strain PCC 6803 was cultured in a diaphanous column with a capacity of 10 liters. The culture was grown with a L:D cycle of 14:10 h at a temperature of 20 °C and the light intensity was about 116  $\mu\text{mol s}^{-1} \text{m}^{-2}$ . The medium used was described by Nicklisch *et al.* (1999).



**Figure 1.** Generation of oxidative stress due to exposure to cyanobacterial toxins (microcystins). One of the reactive oxygen species (ROS) generated is the superoxide anion radical, which is detoxified by the activity of superoxide dismutase (SOD) resulting in the formation of hydrogen peroxide ( $H_2O_2$ ).  $H_2O_2$  is toxic to the cell and will be transformed to water by catalase (CAT), glutathione peroxidase (GPX) or ascorbat peroxidase (APX). The activation of this antioxidative defence system prevents DNA-damage, protein inhibition or lipid peroxidation. (Figure redrawn after Pflugmacher *et al.* 2006).

#### Crude extract and Microcystin-LR

For the crude extract, cyanobacterial cells were collected from the lake Müggelsee (Berlin, Germany) in August 2001. The cyanobacterial bloom material contained mainly *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*. The material was lyophilized and stored at  $-80\text{ }^\circ\text{C}$ . The lyophilized cells were suspended in dd $H_2O$ , ultrasonicated on ice to break the cells and centrifuged for 15 min at 10,000 g. In the resulting supernatant a concentration of MC-LR of  $28\text{ }\mu\text{g mL}^{-1}$  was measured by HPLC-PDA according to Pflugmacher *et al.* (1998). The crude extract was diluted to result in MC-LR concentrations of 0.5 and  $1.0\text{ }\mu\text{g L}^{-1}$ . The purified MC-LR was kindly donated by Dr. Linda Lawton (RGU, Aberdeen Scotland, UK). It was dissolved in water.

#### Exposures

*Synechocystis* sp. cells (cell density:  $1.39 \times 10^9$  cells  $\text{mL}^{-1}$  using a Neubauer chamber) were exposed in 200-mL beakers to two different concentrations ( $0.5$  and  $1\text{ }\mu\text{g L}^{-1}$ ) of the pure toxin MC-LR and cell-free cyanobacterial crude extract containing the same amount of MC-LR for 24 h. The same light conditions and temperature were used as described above.

#### Enzyme preparation

Enzyme preparation follows Pflugmacher & Steinberg (1997) with minor modification as listed below. 25 mL of the culture were centrifuged at 10,000 g for 10 min to obtain a pure cyanobacterial suspension. Cyanobacteria were homogenised using a glass

homogenizer with 3 mL of 0.1 M sodium phosphate buffer (pH 6.5) containing 1.4 mM dithioerythrol (DTE), 1 mM EDTA and 20 % glycerol. To break the cells, glass pearls on a vortex were used for 3 min. The suspension was centrifuged at 2000 g for 5 min to remove cell debris. The supernatant was fractionated by two solid ammonium sulphate precipitations from 0 to 35 % and from 35 to 80 %. Each was centrifuged for 20 min at 35,000 g and 30 min at 80,000 g. The pellet of the last centrifugation step was collected and suspended in 20 mM sodium phosphate buffer. To remove the ammonium sulphate the extract was applied on a NAP-5 column according to the manufacturer's protocol. The protein samples were frozen in liquid nitrogen and stored at  $-80\text{ }^\circ\text{C}$  until use.

#### Protein determination and enzyme measurement

Due to the low protein concentration in the samples, the "Advanced Protein Assay (ADV01)" was used. The assay principle follows Bradford (1976), but the reagent was five times more concentrated. Serum bovine albumin (initial fraction 98 %, Sigma) was used as protein standard for calibration.

Catalase activity was measured according to Baudhuin *et al.* (1964) and is given in  $\mu\text{kat mg}^{-1}$  protein (kat = rate of reaction in mol substrate per second).

#### Measurement of intracellular hydrogen peroxide

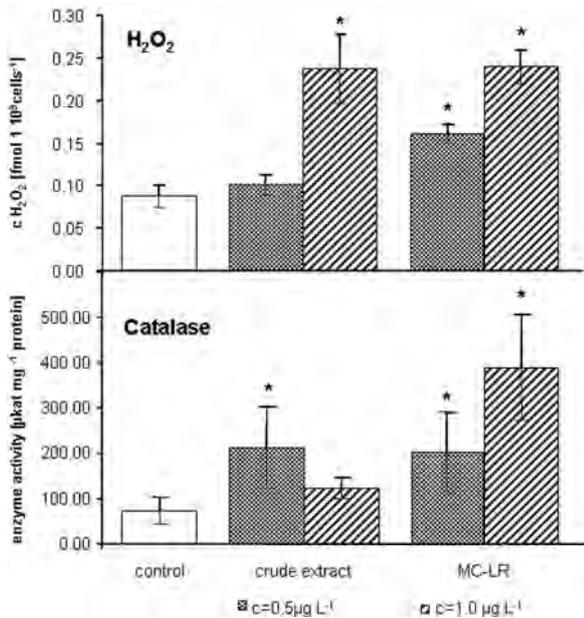
$H_2O_2$  levels were determined according to Jana *et al.* (1981). 25 mL of *Synechocystis* sp. was homogenized in 1.5 mL of sodium phosphate buffer 50 mM pH 6.5. The homogenate was centrifuged at 20,000 g for 90 s.  $H_2O_2$  was determined by mixing 250  $\mu\text{L}$  of the supernatant with 750  $\mu\text{L}$  of 0.1 % titanium chloride in 20 % (v/v)  $H_2SO_4$ . After 1 min, the intensity of the developed yellow colour was measured at 410 nm. Levels of  $H_2O_2$  were calculated using the extinction coefficient of  $0.28\text{ }\mu\text{mol cm}^{-1}$ .

#### Analysis of Data

To test the significance a statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey test ( $p < 0.05$ ) (SPSS 9.0 for Windows).

#### Results

Exposure to  $0.5\text{ }\mu\text{g L}^{-1}$  crude extract did not significantly increase the concentration of intracellular  $H_2O_2$ , whereas the CAT activity was significant increased compared to the control (Fig. 2). A significant enhancement of intracellular  $H_2O_2$  was determined after exposure to  $1.0\text{ }\mu\text{g L}^{-1}$  crude extract, whereas the CAT activity was not significantly increased.



**Figure 2.** Intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration and enzyme activity of catalase (CAT) after exposure the *Synechocystis* sp. cells to 0.5 µg L<sup>-1</sup> and 1 µg L<sup>-1</sup> crude extract or MC-LR. Bars show mean and standard deviation. Asterisks indicate significance (p = 0.05).

Exposure to 0.5 and 1.0 µg L<sup>-1</sup> MC-LR significant increased the intracellular concentration of H<sub>2</sub>O<sub>2</sub> as well as CAT activity (Fig. 2).

## Discussion

Intracellular H<sub>2</sub>O<sub>2</sub> levels and the CAT activity are both markers for oxidative stress. Enhancement of CAT activity but not of the intracellular concentration of H<sub>2</sub>O<sub>2</sub> after exposure to 0.5 µL<sup>-1</sup> crude extract could mean that after 24 hours H<sub>2</sub>O<sub>2</sub> was already cleared by CAT while the enzyme was still active. Exposure to higher concentration of crude extract showed the opposite result: H<sub>2</sub>O<sub>2</sub> increased but CAT activity was similar to the control. Maybe other components within the crude extract such as lipopolysaccharides are able to influence CAT activity, or other enzymes such as GPX or APX are able to detoxify H<sub>2</sub>O<sub>2</sub>. On the other hand, exposure to both concentrations of pure toxin MC-LR led to an elevation of intracellular H<sub>2</sub>O<sub>2</sub> and CAT activity in a dose-dependent manner. These results support the hypothesis described above.

In this study we evaluated if microcystins might be used as a communication tool for interspecies cyanobacterial communication via the promotion of oxidative stress. The results indicate that crude extract as well as MC-LR are able to promote oxidative stress in *Synechocystis* sp. Therefore the possibility arises that oxidative stress in another cyanobacterium, *Synechocystis* sp., can be promoted by cyanotoxins. Be-

cause ROS are known to be involved in communication processes in cells and between cells, it is possible that ROS, generated by exposure to cyanotoxins, is part of a communication pathway between two different cyanobacteria.

The results from this study indicate that cyanotoxins may play a role in facilitating communication between cyanobacteria involving the promotion of oxidative stress as an enhancement signal in the cells. Additional studies are necessary to elucidate the natural function of cyanobacterial secondary metabolites.

## Acknowledgements

We would like to thank Jorge Nimtsch (IGB Berlin) for his helpful comments and suggestions.

## References

- Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O., Wattiaux, R., Jacques, P. & DeDuve, C. (1964). *Biochem. J.* 92: 179-184.
- Bradford, M.M. (1976). *Anal. Biochem.* 72: 248-254.
- Cámara, M., Hardman, A., Williams, P. & Milton, D. (2002). *Nat. Genet.* 32: 217-218.
- Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D. & Van Breusegem, F. (2000). *Cell. Mol. Life Sci* 57: 779-795.
- Fastner, F., Wirsing, B., Wiedner, C., Heinze, R., Neumann, U. & Chorus, I. (2001). In: *Cyanotoxins – Occurrence, Causes, Consequences*, Chorus, I. (ed.), Springer Verlag, Berlin Heidelberg, pp. 22-37.
- Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I. & Laloi, C. (2006). *Bioassays* 28: 1091-1101.
- Jana, S. & Choudhuri, M.A. (1981). *Aquat. Bot.* 12: 345-354.
- Jüttner, F. (1999). In: *Biofilms in the Aquatic Environment*, Keevil, C.W., Godfree, A., Holt, D. & Dow, C. (eds), Cambridge, UK, pp. 43-50.
- Nicklisch, A. (1999). *Int. Rev. Hydrobiol.* 84: 233-241.
- Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A. & Steinberg, C.E.W. (1998). *BBA-Gen. Subjects* 1425: 527-533.
- Pflugmacher, S., Jung, K., Lundvall, L. & Peuthert, A. (2006). *Environ. Toxicol. Chem.* 25: 2381-2387.
- Pflugmacher, S. & Steinberg, C. (1997). *J. Appl. Bot.–Angew. Bot.* 71: 144-146.
- Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y. & Hunt, M.D. (1996). *Plant Cell* 8: 1809-1819.

## Physiological stress responses of *Daphnia magna* exposed to cyanobacterial compounds

C. Wiegand<sup>1</sup>, A. Jarosch<sup>1</sup>, A. Behrend<sup>1</sup> and E. Krause<sup>2</sup>

<sup>1</sup>Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 301, 12587 Berlin, Germany, cwiegand@igb-berlin.de, <sup>2</sup>Leibniz-Institute of Molecular Pharmacology, Robert Rössele Str. 10, 13125 Berlin, Germany, ekrause@fmp-berlin.de

### Abstract

Cyanobacteria frequently flourish in eutrophic aquatic systems, and several species are capable of producing toxic secondary metabolites, including microcystins that specifically inhibit serine/threonine protein phosphatases, cyanopeptolines and micropeptins which lead to inhibition of trypsin and chymotrypsin. The grazing zooplankter *Daphnia magna* can detoxify microcystins by conjugation to glutathione via the glutathione-S transferases. Kinetics of the antioxidative reactions showed that antioxidative enzymes (superoxide dismutase, catalase, and glutathione peroxidase) reduce oxidative stress, caused by microcystin-LR, but to much lower extent if the daphnids were exposed to a cyanobacterial crude extract of same microcystin-LR concentration. A proteomic approach revealed enhanced induction of proteins involved in oxygen transport and oxidative metabolism following exposure to cyanobacterial crude extract, whereas the pure toxin caused a down regulation of protein expression.

### Introduction

In many eutrophic freshwater lakes the diversity of the phytoplankton community is drastically changed towards dominance of cyanobacteria. Cyanobacteria of the genus *Microcystis*, *Planktothrix* and *Anabaena* are frequently bloom forming and may produce bio-active or toxic metabolites, such as the microcystins, inhibitors of serin/threonin protein phosphatases (Sivonen & Jones 1999). Other bio-active peptides are cyanopeptolines, micropeptins and microviridin J, inhibitors of the digestive enzymes trypsin and chymotrypsin. Besides the specific molecular mechanisms of the individual compounds, generation of oxidative stress is a further mechanism adding to cyanobacterial toxicity (e.g. Ding *et al.* 1998). Population sustainability of phytoplanktivorous zooplankton is negatively impacted by grazing inhibition (Lampert 1981; Ferrao-Filho *et al.* 2002). A further disadvantage for the grazers is the low sterol content and the total lack of polyunsaturated fatty acids (Mueller-Navarra *et al.* 2000; von Elert *et al.* 2003).

The grazing zooplankter *Daphnia magna* also suffers from lowered food uptake efficiency, the toxicity correlating with the amount of microcystin absorbed via the gut epithelia, which undergoes disruption (Rohrlack *et al.* 1999, 2005; Ghandouani *et al.* 2004). On the other hand, *D. magna* and *D. pulicaria* develop tolerance against cyanobacteria (Gustavsson *et al.* 2005) and pre-exposure enables them to better tolerate toxic *Microcystis* in the food (Sarnelle &

Wilson 2005). Biotransformation and antioxidative mechanisms may aid to this tolerance. As biotransformation of microcystin-LR in *D. magna* was investigated previously (Pflugmacher *et al.* 1998; Wiegand *et al.* 2002), the focus of this study was the kinetics of antioxidative enzymes. In addition, additional proteins following exposure to cyanobacterial compounds were identified.

### Materials and Methods

*Daphnia magna* were reared and exposed at 20 +/- 1 °C and a L:D regime of 14:10 in Elendt M7 medium (Elendt 1990). They were fed with Microcell algal powder, and 50 % of the water was exchanged every 3–5 days.

#### Exposure

Pure MC-LR was applied at 5.0 µg/L. To resemble the environmentally relevant situation a cyanobacterial crude extract was prepared from a natural bloom in Lake Müggelsee in 2000, consisting of 80 % *Microcystis aeruginosa* and 20 % *Aphanizomenon flos-aquae*, by repeated freeze thaw cycles and ultrasonication. Cell debris was removed by centrifugation. Determination by HPLC-PDA revealed MC-LR as the main compound in the sample. The crude extract was applied at a concentration of 5.0 µg/L MC-LR/L. After exposure, daphnia were rinsed, frozen in liquid nitrogen and stored at -80 °C until enzyme extraction.

### Enzyme assays

Triplicate batches of 100 daphnia (six to nine days of age) were exposed to test and control solutions for 0.5, 1, 2, 4, 9 and 24 h. The enzyme preparation followed Wiegand *et al.* (2000). Enzyme activities were determined colourimetrically: superoxide dismutase according to Elstner *et al.* (1995); catalase (Cat) and glutathione peroxidase (GPx) according to Chang and Kao (1998) and Drotar *et al.* (1985). Enzymatic activities were calculated in terms of the protein content of the sample (Bradford 1976).

### Hydrogen peroxide determination

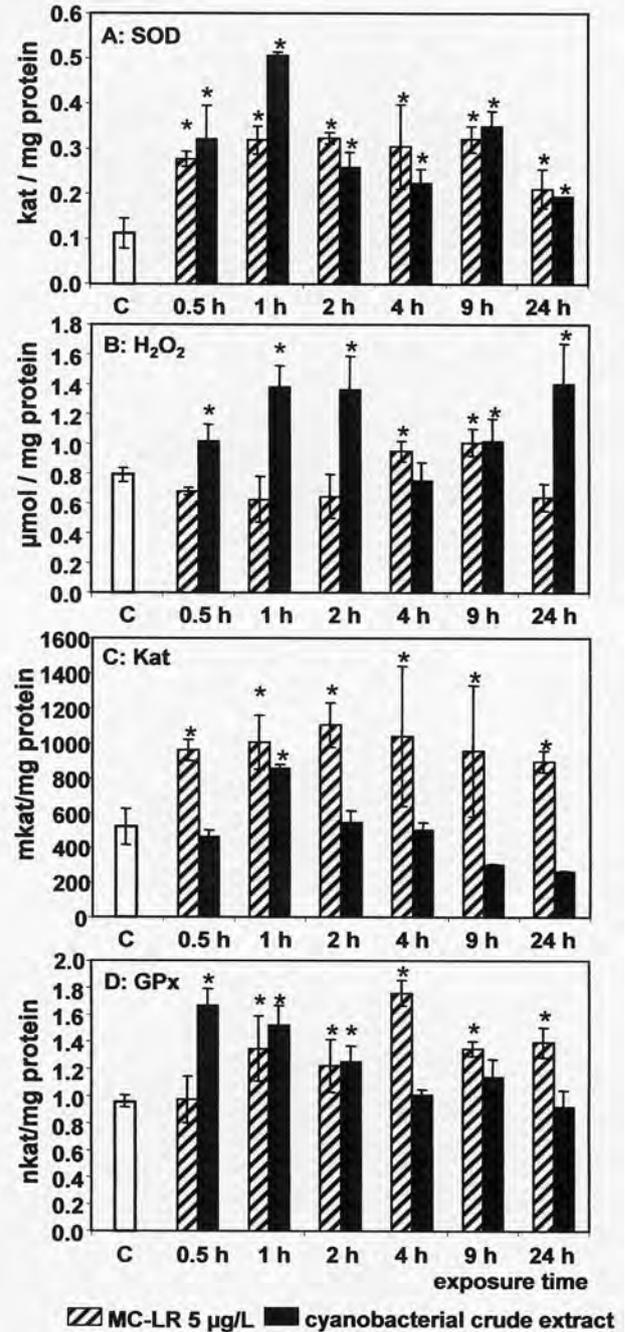
Triplicates of 10 daphnia were exposed as for the enzymatic studies. The H<sub>2</sub>O<sub>2</sub> content was assayed after homogenization in sodium phosphate buffer (50 mM, pH 6.5), and centrifugation (12.000g) using titanium sulphate according to Jana and Choudhuri (1982). Significance of differences from control was tested by one-way analysis of variance followed by student Newman-Keuls test,  $P < 0.05$  (SPSS 9.0).

### Proteome study

Daphnia were exposed for 24 h to either MC-LR or cyanobacterial crude extract, both at 5.0 µg/L. After homogenization and separation of membrane bound proteins, cytosolic proteins were focused to their isoelectrical point (IP) between pH 4 and 7, and separated to their molecular weight (MW) on 10 % SDS PAGE. The most prominent changed proteins were tryptic digested and analyzed by MALDI-TOF/TOF mass spectrometry and determined via peptide mass fingerprint and additional MS/MS measurements (Bente *et al.* 2003). NCBI database (*Daphnia* sp, when possible) and a Mascot-score probability (P) of at least 70 were used for identification of the proteins.

## Results and Discussion

An increased SOD-activity in *D. magna* throughout both exposure durations (Fig. 1A) proves the formation of superoxide. Whereas MC-LR provoked a uniform elevation, the cyanobacterial crude extract resulted in a peak activity at 1 h followed by a decrease between 2 and 4 h and a second increase peaking at 9 h. The kinetic of H<sub>2</sub>O<sub>2</sub> content in *D. magna* did not change much during MC-LR exposure but exposure to crude extract led to an increase during the first 2 h, which was reduced after 4 h but increased a second time starting at 9 h (Fig. 1B). H<sub>2</sub>O<sub>2</sub> is reduced to water by catalase and glutathione peroxidase. Activity of catalase was increased during the whole exposure to MC-LR but only for 1 h after exposure to crude extract (Fig. 1C). Similarly, the activity of GPx was elevated



**Figure 1.** Activity of antioxidative enzymes (A: superoxide dismutase, C: catalase, D: glutathione peroxidase) in *Daphnia magna* during exposure to MC-LR or a cyanobacterial crude extract of the same MC-LR concentration. B: development of hydrogen peroxide.

from 1 h onwards due to exposure to MC-LR, but after exposure to crude extract only for the first 2 h (Fig. 1D). The inactivity of both enzymes explains the increased hydrogen peroxide content in *D. magna* after exposure to crude extract. It furthermore corresponds to the pattern of the second increase of hydrogen peroxide, as both enzymes were at or below control values. Currently, this inactivity can not be explained.

Our results suggest that daphnia suffer from oxidative stress caused by other cyanobacterial products than the toxin MC-LR, due to short term activities of anti-oxidative enzymes. Previous results demonstrated a concentration-dependent increase of the biotransformation enzyme glutathione S-transferase in *D. magna* between 0.5 and 5 µg/L MC-LR, decreasing again at 50 µg/L (Wiegand *et al.* 2002). In conclusion, biotransformation and antioxidative enzymes enable *D. magna* to withstand exposure to cyanobacterial compounds for short time or at low concentration, but the mechanism for long-time adaptation processes needs to be investigated further. It further needs to be considered whether the natural uptake route, ingestion of whole cyanobacteria with the food, may include even more harmful compounds.

The proteomic analysis revealed more proteins involved in the response of *Daphnia magna* to cyanobacterial compounds. Exposure to pure toxin enhanced the production of hemoglobin (MW 38048, pI 6.55, P: 370), which was not detected in the crude extract treatment. In the latter treatment, enhanced production of a precursor of the hemoglobin di-domain (MW 38491, pI 6.61, P: 150) was identified, and furthermore, triose-phosphate-isomerase (MW 16159, pI 5.42, P: 204). Enhanced induction of hemoglobin or the precursor of one hemoglobin domain can be interpreted as a general stress signal, and may support the oxygen supply required for all additional physiological reactions in the course of the treatment, such as biotransformation and antioxidative reactions, to prevent damages or reactions to mediate their reparation. Additionally, we may see that the life cycle of daphnia is affected, as both the synthesis of hemoglobin and the induction of sexual reproduction is genetically controlled by the same factor, the hormone methy-farnesoat (Rider *et al.* 2005).

The triose phosphate isomerase is involved in the glycogene cycle and the observed enhancement of this enzyme matches an increased energy demand, in parallel with the oxygen demand. Internal energy allocation might also be caused by other cyanopeptides included in the crude extract, such as cyanopeptolins or microviridins. These compounds inhibit serin/threonin proteases, preventing the ingested food from being digested, and the required energy must be derived from internal storages. To prove these assumptions, further proteins involved in metabolic pathways of *D. magna* to cyanobacterial compounds need to be identified, in particular their kinetics during chronic or lifetime exposure.

## Acknowledgements

S. Lamer is greatly acknowledged for help in protein identification.

## References

- Bente, M., Harder, S., Wiesgigl, M., Heukeshoven, J., Gelhaus, C., Krause, E., Clos, J. & Bruchhaus, I. (2003). *Proteomics*: 1811–1829.
- Bradford, M. (1976). *Anal. Biochem.*, 72: 248-254.
- Chang, C.J. & Kao, C.H. (1998). *Plant Growth Regul.* 25: 11–15.
- Ding, W.X., Shen, H.M., Zhu, H.G. & Ohng, C.N. (1998). *Environ. Res.* 78: 12-18.
- Drotar, A., Phelps, P. & Fall, R. (1985). *Plant Sci.* 42: 35–40.
- Elendt, B. P. (1990). *Protoplasma* 154: 25-33.
- Elstner, E.F., Youngman, R.J. & Oßwald, W. (1995). In: *Methods of Enzymatic Analysis*, Bergmayer, H.U. (ed.), VCH Weinheim, pp. 293-302.
- Ferrao-Filho, A.S., Domingos, P. & Azevedo, S.M.F.O. (2002). *Limnologia* 32: 295-308.
- Ghadouani, A., Pinel-Alloul, B., Plath, K., Codd, G.A. & Lambert, W. (2004). *Limnol. Oceanogr.* 49: 666-679.
- Gustafsson, S., Rengefors, K. & Hansson, L.A. (2005). *Ecology* 86: 2561-2567.
- Jana, S. & Choudhuri, M.A. (1982). *Aquat. Bot.* 12: 345–354.
- Lampert, W. (1981). *Int. Rev. Ges. Hydrobiol.* 66: 285-298.
- Mueller-Navarra, D., Brett, M.T., Liston, A.M. & Goldmann, C.R. (2000). *Nature* 403: 74-77.
- Pflugmacher, S., Wiegand, C., Oberemm, A., Beatie, K.A., Krause, E., Codd, G.A. & Steinberg, C. (1998). *Biochim. Biophys. Acta* 1425: 527-533.
- Rider C.V., Olmstead A.W., Wasilak B.A., LeBlanc G. & Gorr T.A. (2005). *J. Exp. Biol.* 208: 15-23.
- Rohrlack, T., Christoffersen, K., Dittmann, E., Nogueira, I., Vasconcelos, V. & Borner, T. (2005). *Limnol. Oceanogr.* 50: 440-448.
- Rohrlack, T., Henning, M. & Kohl, J.G. (1999). *Arch. Hydrobiol.* 146: 385-395.
- Sarnelle, O. & Wilson, A.E. (2005). *Limnol. Oceanogr.* 50: 1565-1570
- Sivonen, K. & Jones, G., (1999). In: *Toxic Cyanobacteria in Water*, Chorus, I. & Bartram, J. (eds), Spon, London, pp. 41-111.
- Von Elert, E., Martin-Creuzburg, D. & LeCoz, J.R. (2003). *Proc. Royal Soc. London, Series B, Biol. Sci.* 270: 1209-1214.
- Wiegand, C., Peuthert, A., Pflugmacher, S. & Carmeli, S. (2002). *Environ. Toxicol.* 17: 400-406.
- Wiegand, C., Pflugmacher, S., Oberemm, A. & Steinberg, C. (2000). *Internat. Rev. Hydrobiol.* 85: 413-422.

## 2. ECOPHYSIOLOGY AND AUTECOLOGY



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Metal concentration in freshwater sediments seasonally subjected to toxin-producing cyanobacterial blooms

Mafalda S. Baptista and M. Teresa S.D. Vasconcelos

CIIMAR, Rua dos Bragas, 289, 4050-123 Porto, Portugal and Chemistry Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 687, 4169-071 Porto, Portugal  
 abaptista@fc.up.pt and mtvascon@fc.up.pt

### Abstract

Cyanobacteria represent a sink for metals in the aquatic environment, as they are effective metal sorbents, affecting metal speciation and bioavailability. In the ambit of a project aimed at investigating causes and consequences of cyanobacterial blooms in freshwater, trace metal (Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn) concentrations in sediments were monitored monthly, throughout one year, at two sites of Tâmega river (North Portugal): (1) the Marco de Canaveses reservoir, which has been dominated by toxin-producing cyanobacteria from June to September; and (2) the city of Amarante (ca. 20 km upstream) where no such blooms have been recorded. The trace metal concentrations were similar at the two sites, in spite of Marco de Canaveses being a lentic system and Amarante a lotic habitat. However, extreme drought characterized the sampling year; its influence on the results remains to be ascertained. Atypically, the bloom event registered annually at Marco de Canaveses only took place during October and November. For most of the analysed metals, regulation of metal content in the sediment was independent of the bloom event.

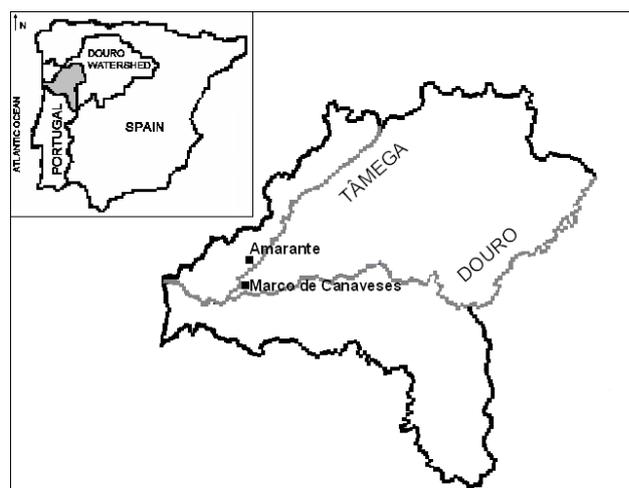
### Introduction

Toxic cyanobacterial populations have been reported all over the world and, naturally, most recent research has focused on toxin identification and quantification (Codd *et al.* 2005). It is known, however, that cyanobacteria are a sink for metals in the aquatic environment, affecting metal speciation and bioavailability (Yee *et al.* 2004). Therefore, environmental assessments considering potential impacts of toxic agents should take into account the metal status of the aquatic environment. Cyanobacterial interactions with metals have been reported, but seldom related to bloom-formation. Cu, Cr, Co, Fe, Mn, Ni and Zn are known to be essential (micro)-nutrients for cyanobacteria. Cd or Pb, however, are toxic at very low levels, and no cellular functions are known (Baptista and Vasconcelos 2006). This work aimed at studying variations in the concentration of trace metals in freshwater sediments affected by the seasonal occurrence of toxin-producing cyanobacterial blooms.

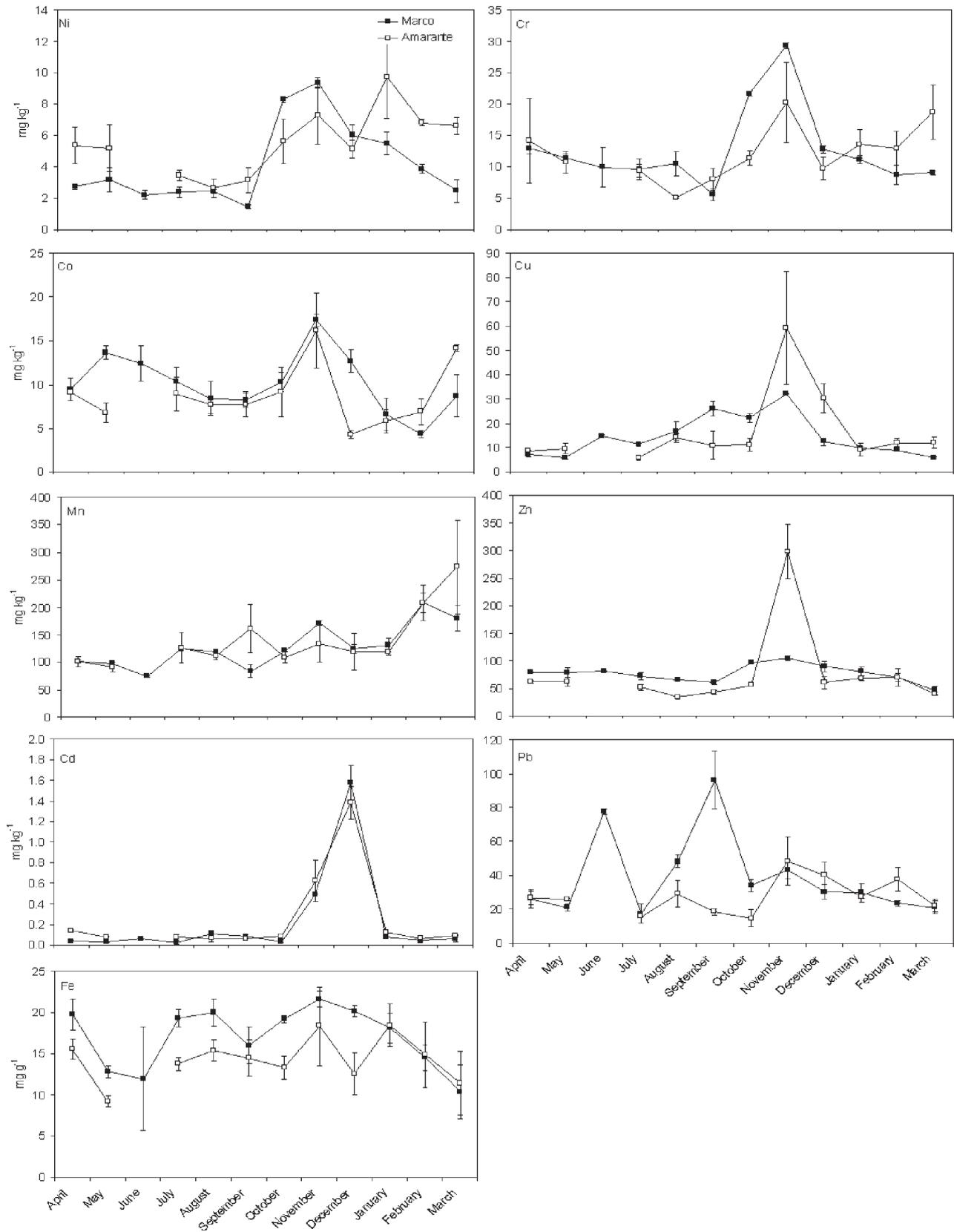
### Material and Methods

Sediments were collected from April 2005 to March 2006, at Tâmega river, Portugal, at Amarante and Marco de Canaveses. Tâmega is located within the river Douro watershed (Fig. 1). Since the building of a dam in 1989, near the city of Marco de Canaveses, seasonal blooms dominated by *Microcystis aeruginosa*

Kütz. have occurred, mainly from June to September, with confirmed toxin production (Saker *et al.* 2005). Upstream (ca. 20 km), at the city of Amarante, no such blooms have been recorded. Sediments were collected into individual plastic bags with a plastic shovel, and oven-dried at 30 °C. For each analysis, ca. 0.25 g of the fraction < 2 mm was microwave digested using supra-pure HNO<sub>3</sub> (methodological details in Mucha *et al.* 2004). HNO<sub>3</sub> digestion of the sediments only permits determination of total recoverable metal contents; regardless it dissolves all elements that could become “environmentally available” (Almeida *et al.*



**Figure 1.** Map of mainland Portugal showing the Portuguese portion of the Douro watershed and the location of the sampling cities within it.



**Figure 2.** Metal concentrations in sediments collected in Marco de Canaveses and Amarante. Symbols are means and bars are standard deviations of three replicates.

2004). Metal content in sediments was determined by atomic absorption spectrophotometry. Flame atomization (PU 9200X, Philips) was used for Cu, Cr, Co, Fe, Mn, Pb and Zn, while electrothermal atomization provided with a Zeeman background correction (4100 ZL, Perkin-Elmer coupled to an AS 70 autosampler), was used for Cd and Ni. Aqueous matched standards were used for external calibrations. Reference standard sediment (BCR 277 estuarine sediment No. 068) was used to check the accuracy of the digestion and analysis procedure. For Cd, Cr, Cu, Ni, Pb, and Zn statistically identical results were obtained. For Fe and Mn only indicative values were available, the recovery percentages being 70 % and 80 %, respectively; for Co no values were available. The chemicals used were of pro analysis grade or equivalent. Standard solutions for metal analysis were prepared from 1000 mg L<sup>-1</sup> stock solutions (BDH (Spectrosol) and Panreac) by weighing with deionised water. All glassware and plastic containers were soaked in 20 % (v/v) HNO<sub>3</sub> for 24 h, and rinsed several times with deionised water (conductivity < 0.1 µS cm<sup>-1</sup>) before use.

## Results and Discussion

We hypothesised that an increase in cell numbers would increase sediment metal concentration, given the fact that at the death, the cells would liberate their metal content into the environment. Other workers have reported increases in sediment metal content during the occurrence of a harmful algal bloom (García-Hernández *et al.* 2005). The analysed metals are known to be essential (micro)-nutrients to cyanobacteria (Cu, Fe, Mn, Zn) or essential co-factors (Co, Cr, Ni). Only Cd and Pb do not have a known cellular function and, given their toxicity, allow for an account of sediment contamination.

Trace metal concentration was similar at the two sites, with Cd < Ni < Co < Cr < Cu < Pb < Zn < Mn < Fe (Fig. 2). Given this fact, the lack of blooms at Amarante is not due to a difference in sediment metal content. Atypically, the Marco de Canaveses *M. aeruginosa* bloom only occurred in October and November, while in previous years it usually extended from June to September. In Marco de Canaveses, the

highest metal concentration was registered in October and November for all metals except Cd, Mn and Pb. In Amarante, an increase in metal concentration was also registered in October and November, although for most elements this was not the highest concentration. For Cd, an increase in concentration took place in December at both sites. We hypothesize that this is due to anthropogenic contamination.

The results do not suggest that harmful algal blooms contribute to higher metal concentrations in the sediment. If anything, the increase in metal content promoted the bloom occurrence, although a combination of other factors such as temperature, N:P ratio and water flow may have also contributed. However, the sampling took place in a year of extreme drought; it is therefore difficult to ascertain whether the results reflect the usual status of sediment metal concentration or whether the drought contributed to a shift.

## Acknowledgements

This work was partially funded by Fundação para a Ciência e a Tecnologia (FCT), Portugal, through a fellowship awarded to Mafalda Baptista (SFRH/BD/16292/2004).

## References

- Almeida, C.M.R., Mucha, A.P. & Vasconcelos M.T.S.D. (2004). *Env. Sci. Technol.* 38: 3112-3118.
- Baptista, M.S. & Vasconcelos, M.T. (2006). *Crit. Rev. Microbiol.* 32: 127-137.
- Codd, G.A., Morrison, L.F. & Metcalf, J.S. (2005). *Toxicol. Appl. Pharmacol.* 203: 264-272.
- García-Hernández, J., García-Rico, L., Jara-Marini, M.E., Barraza-Guardado, R. & Weaver, A.M. (2005). *Mar. Poll. Bull.* 50: 733-739.
- Mucha, A.P., Vasconcelos, M.T.S.D. & Bordalo A.A. (2004). *Estuar. Coast. Shelf Sci.* 59: 663-673.
- Saker, M. L., Fastner, J., Dittmann, E., Christiansen, G. & Vasconcelos, V.M. (2005). *J. Appl. Microbiol.* 99: 749-757.
- Yee, N., Benning Liane, G., Phoenix, V.R. & Ferris, F.G. (2004). *Env. Sci. Technol.* 38: 775-782.

# *Pseudo-nitzschia* in south central coastal waters of Vietnam: growth and occurrence related to temperature and salinity

H. Doan-Nhu, M.A. Nguyen Thi and T.G. Nguyen-Ngoc

Department of Marine Plankton, Institute of Oceanography, Nha Trang, Vietnam, habsea@dng.vnn.vn

## Abstract

*Pseudo-nitzschia* species are widely distributed along the coast of Vietnam with abundance varying in time and space. Along the southern central coast, they tend to occur in high numbers during the rainy season when the nutrient loading from land/rivers is high. The seasonal variation shows local differences, depending on the hydro-chemical characteristics of each embayment along the coast. *Pseudo-nitzschia* concentration was observed to be as high as 480,000 cells L<sup>-1</sup>. Temperature and salinity compared with cell abundance indicated that *Pseudo-nitzschia* can adapt to a wide range of temperatures and salinities. In Nha Phu Lagoon, *Pseudo-nitzschia* spp. were distributed in a wider range of both salinity and temperature. However, they were stenohaline but eurythermal in Nha Trang Bay and the northern coastal waters of Binh Thuan Province. In laboratory experiments, *P. cuspidata* had the highest growth rate at 35 psu and 30 °C (1.8 divisions d<sup>-1</sup>), but grew well at 20-30 °C and 25-35 psu. Maximal *P. pungens* growth rate was 1.54 div d<sup>-1</sup>, at 25 psu and 26 °C.

## Introduction

The diatom genus *Pseudo-nitzschia* comprises ca. 30 species (Hasle 1994; Lundholm *et al.* 2002) of which 10 species have been confirmed to produce toxin (Moestrup & Lundholm 2004). There is little research on how temperature and salinity affect the growth and/or distribution of *Pseudo-nitzschia* species, although records of some species in nature and in culture experiments indicate that they are euryhaline (Jackson *et al.* 1992; Lundholm *et al.* 1997; Thessen *et al.* 2005).

*Pseudo-nitzschia* species are commonly recorded along the coast of Vietnam (Chu 1998; Nguyen-Ngoc *et al.* 1999; Nguyen & Ho 2001; Skov *et al.* 2004). Surveys during 1998-1999 identified 11 species and tested 14 strains of four species with molecular probes (Skov *et al.* 2004). The seasonal variation of cell density in different waters along the coast of Vietnam has been investigated, revealing peaks of potential risks in time and space (Nguyen *et al.* 2004).

The present study focuses on long-term data of cell density versus temperature and salinity in embayments along the coast of the southern central Vietnam. In addition, *P. pungens* and *P. cuspidata* were isolated into culture to examine the growth rate at different salinities and temperatures.

## Materials and Methods

The present study combined data from various projects (Table 1). Locations and stations are shown in Fig. 1. Enumeration of *Pseudo-nitzschia* species was carried out using a Sedgewick-Rafter chamber.

**Table 1.** Sources of data used in this study

Location	Project	Year	No of St.	Sampling Frequency
Dam Mon, Van Phong Bay	Pearl Oyster Co.	1998-2000	12	Weekly
Xuan Tu, Van Phong Bay	HABViet I	1999	2	2nd week
Nha Phu Lagoon	National project	2004-2005	5	Monthly
Baodai, Nha Trang Bay	National monitoring programme	1996-2006	1	3rd month
Nha Trang Bay	HABViet II/DMP-ION	2003	7	Monthly
Cam Ranh Bay	HABViet I	1999	3	2nd week
North Binh Thuan waters	HABViet II/DMP-ION	2003-2004	9	Monthly

*Pseudo-nitzschia pungens* and *P. cuspidata* were isolated from Nha Trang Bay and maintained in a culture room at 26 °C, with L:D cycle of 12:12 at 28 µmol photos m<sup>-2</sup> s<sup>-1</sup>. *P. cuspidata* experiments were set up at 20, 25 and 30 °C, at salinities of 15, 20, 25, 30 and 35 psu. *P. pungens* experiments were done at 26 °C and salinities of 15, 20, 25, 30 and 35 psu. The experiments were done in an incubator (Gallenkamp, SANYO, UK) at a L:D of 12:12 and 39 µmol photos m<sup>-2</sup> s<sup>-1</sup>. Each experiment was performed in triplicate. The cells were counted daily, starting from the second day. Three subsamples were counted for each repli-

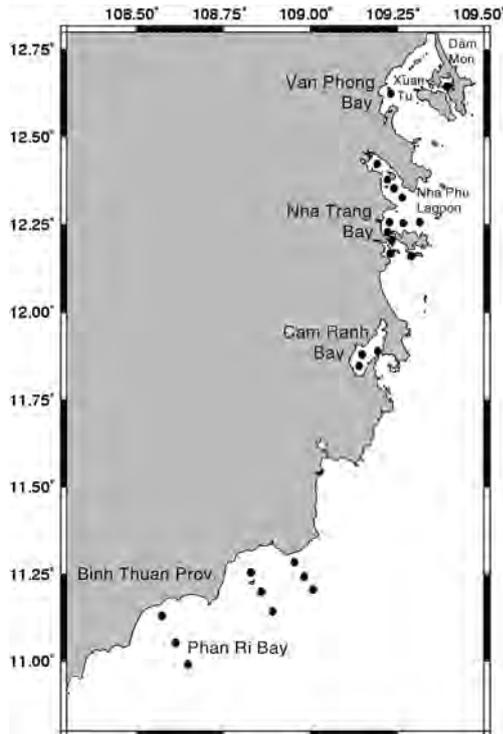


Figure 1. Map showing sampling sites.

cate. Growth rate (division  $d^{-1}$ ) was calculated following Wood *et al.* (2005).

## Results and Discussion

*Geographical and temporal variation in cell density*  
*Pseudo-nitzschia* species were widely distributed along the coast of Vietnam and showed high degree of variation in abundance in time and space. In the southern central coastal waters, from Khanh Hoa to Binh Thuan Provinces, *Pseudo-nitzschia* spp. tended to occur with the highest abundance during the rainy season when the nutrient loading from land/rivers is high. Six species have previously been recorded along the southern central coast and *P. pungens*, *P. calliantha*, *P. cuspidata* and *P. cf. sinica* were the most common. The last three species were recorded in Northern Binh Thuan Province waters (Skov *et al.* 2004). *P. pungens* was present all year round along the southern central coast while *P. cuspidata* in Khanh Hoa Province only occurred from February–September (Skov *et al.* 2004).

In Van Phong Bay, peaks in *Pseudo-nitzschia* were observed after rainy periods, e.g. in Dam Mon in November 1998–January 1999 and February–April 2000, and in Xuan Tu in March–April 1999. The peaks were recorded during periods of low solar radiation (ca. < 100 hours/month). It was obvious during the investigation in Nha Trang Bay in 2003 that the higher

number of cells was found at stations near the estuaries at the south side of the bay, and during the rainy season. This accorded with previous observations that N and P inputs from rivers or sediments can cause decreases in the Si/N ratio and favour *Pseudo-nitzschia* blooms (Bates *et al.* 1998). In the northern coastal waters of the Binh Thuan province, the *Pseudo-nitzschia* assemblages reached 480,000 cells  $L^{-1}$  in September 2003, which is close to the critical limit used in the management of shellfish harvesting in Europe (Anderson *et al.* 2001).

The seasonal variation in abundance was site-specific, depending on hydro-chemical characteristics of each embayment. Because the rainy season starts 1–2 months earlier in the south (Binh Thuan province) than in the northern Khanh Hoa province, the peaks

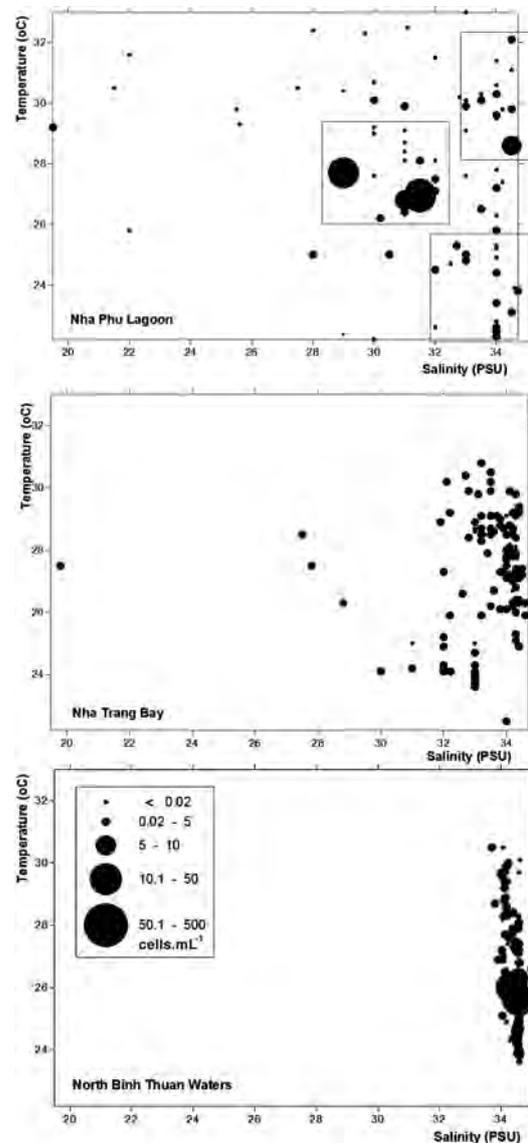


Figure 2. Cell abundance of *Pseudo-nitzschia* plotted against salinity and temperature in different embayments.

of *Pseudo-nitzschia* were skewed accordingly. Generally, *Pseudo-nitzschia* abundance at all investigated sites peaked during periods of lower water temperature.

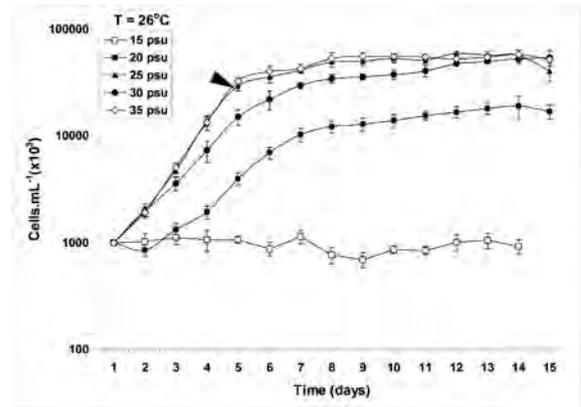
#### Temperature and salinity versus abundance of *Pseudo-nitzschia*

Cell abundance of *Pseudo-nitzschia* indicate that *Pseudo-nitzschia* can adapt to a wide range of temperature and salinity. In Nha Phu Lagoon, *Pseudo-nitzschia* spp. were distributed at a wide range of both salinity and temperature (Fig. 2a). Fig. 2a may include different *Pseudo-nitzschia* assemblages characterized according to different combinations of salinity and temperature, and additional observations on species composition are needed to examine this. In contrast, *Pseudo-nitzschia* spp. were stenohaline and eurythermal in Nha Trang Bay (Fig. 2b) and in the northern waters of the Binh Thuan Province (Fig. 2c).

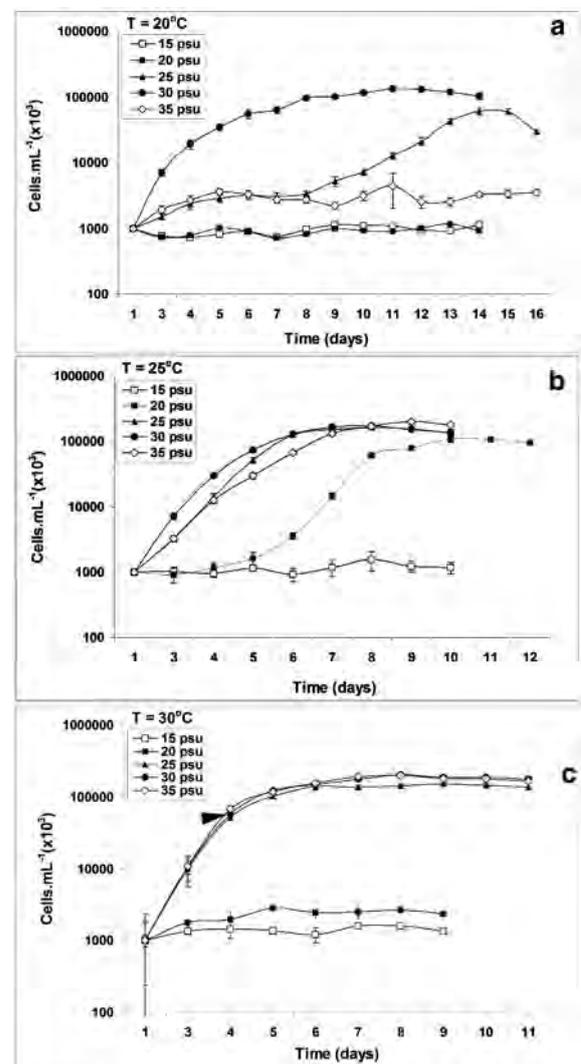
#### Growth rates of *Pseudo-nitzschia* in culture

*P. pungens* grew best at salinities of 25-35 psu, with a maximum growth rate of 1.54 divisions.d<sup>-1</sup> at 25 psu and 26 °C (Fig. 3). There was a significant effect of salinity on growth at 26 °C (ANOVA-one-way,  $p < 0.01$ ). The Tukey test showed no significant difference between growth at salinities of 25, 30 and 35 psu ( $q_{cal} < q_{critical} = 3.95$ ). Growth rate was considerably lower at 20 psu, and *P. pungens* did not grow at 15 psu.

*P. cuspidata* showed the highest growth rate at 35 psu and 30 °C (1.8 divisions d<sup>-1</sup>), but grew well within a range of 20-30 °C and 25-35 psu (Fig. 4). It seems that *P. cuspidata* can tolerate a wider range of salinities when grown at higher temperatures (Fig. 4). Growth was significantly affected by temperature (ANOVA- one-way,  $p < 0.01$ ), except when grown at a salinity of 30 psu. This indicates that *P. cuspidata* has the ability to grow at even wider ranges of temperature at salinities around 30 psu. A Tukey test (after an ANOVA-one-way analysis) for growth at 20 °C and at different salinities showed significant differences between cells grown at 30 psu compared to those grown at other salinities ( $q_{cal} > q_{critical} = 3.73$ ). At 25 °C, the cells grew very slowly at 15 psu, significantly slower than at 25-35 psu ( $q_{cal} > q_{critical} = 3.79$ ) but not than those grown at 20 psu ( $q_{cal} = 2.02$ ) (Fig. 4b). At 30 °C, cells did not grow at 15 and 20 psu (Fig. 4c). This species may be a representative of the high temperature and salinity assemblages observed in Nha Trang Bay and Nha Phu lagoon.



**Figure 3.** Growth rate (divisions.d<sup>-1</sup>) of *P. pungens* at different salinities.



**Figure 4.** Growth rate (divisions.d<sup>-1</sup>) of *P. cuspidata* at different salinities and temperatures

### Acknowledgements

This work used data from NT Pearl Oyster Co., HAB-Viet project phases I-II, National project KC-09-19, National environmental monitoring programme, and the DMP-ION project 2003-2005. Thanks to HAB-Viet phase III for funding the authors to present this work at the XII HAB conference. Thanks to the two reviewers for their valuable comments and language improvement.

### References

- Anderson D.M., Andersen P., Bricelj V.M. & Resel J.E.J. (2001). IOC Technical Series No 59, Paris.
- Bates, S.S., Garrison D.L. & Horner R.A. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson, D.M., Cembella A.D. & Hallegraeff G.M. (eds), Springer-Verlag, pp. 267-292.
- Chu, V.T. (1998). *J. Mar. Env. Res.* 5: 155-166. (in Vietnamese)
- Doan-Nhu, H., Nguyen-Ngoc, L., Nguyen, T.M.A. & Ho, V.T. (2004). *Coll. Mar. Res. W.* 14: 89-98.
- Hasle, G.R. (1994). *J. Phycol.* 30: 1036 -1039.
- Jackson, A.E., Ayer, S.W. & Laycock, M.V. (1992). *Can. J. Bot.* 70: 2198-2201.
- Lundholm, N., Hasle, G.R., Fryxell, G.A. & Hargraves, P.E. (2002). *Phycologia* 41: 480-497.
- Lundholm, N., Skov, J., Pocklington, R. & Moestrup, Ø., (1997). *Phycologia* 36: 381-388.
- Moestrup, Ø. & Lundholm, N. (2004). In: *IOC Taxonomic Reference List of Toxic Plankton Algae*, [www.bi.ku.dk/ioc/group1.asp](http://www.bi.ku.dk/ioc/group1.asp)
- Nguyen, T.M.A. & Ho, V.T. (2001). *Coll. Mar. Res. W.* 11: 135 - 144. (in Vietnamese).
- Nguyen-Ngoc, L. & Doan-Nhu, H. (1996). In: *Harmful and Toxic Algal Blooms*, Yasumoto, T. & Osahima, Y. (eds), UNESCO, Paris, pp. 45 - 48.
- Nguyen-Ngoc, L., Doan-Nhu, H. & Ho, V.T. (1999). *Coll. Mar. Res. W.* 9: 179-195. (in Vietnamese).
- Nguyen-Ngoc, L., Doan-Nhu, H., Andersen, P., Ho, V.T., Skov, J., Chu, V.T. & Do, T.B.L. (2004). *Opera Botanica* 140: 159-180.
- Skov, J., Ton, T.P. & Do, T.B.L. (2004). *Opera Botanica* 140: 23-52.
- Thessen, A.E., Dortch, Q., Parsons, M.L. & Morrison, W. (2005). *J. Phycol.* 41: 21-29.
- Wood, A.M., Everroad, R.C. & Wingard, L.M. (2005). In: *Algal Culturing Techniques*, Andersen R.A. (ed.), Elsevier, pp. 269-285.

## Going beyond nutrients: the role of environmental factors in shaping harmful algal blooms in estuarine waters

E. Fensin<sup>1</sup> and B.W. Touchette<sup>2</sup>

<sup>1</sup>NC Division of Water Quality, Raleigh, NC, USA 27607, elizabeth.fensin@ncmail.net,

<sup>2</sup>Center for Environmental Studies, Elon University, Elon, NC, USA 27244, btouchette@elon.edu

### Abstract

Nutrient availability is often considered a major contributory factor in algal productivity. However, other environmental components can selectively displace individual species even under eutrophic conditions. Therefore, it is necessary to identify other contributory factors that promote HABs if we are to develop better predictive models for bloom occurrence and duration. This study focused on four potentially harmful algae (dinoflagellates *Gyrodinium instriatum*, *Karlodinium veneficum*, and *Prorocentrum minimum*, and the cyanobacterium *Cylindrospermopsis raciborskii*) that occur in the estuaries of North Carolina, USA. Multivariate analyses were conducted on environmental data collected 2000-2005 in three tributaries (Neuse, New and Pamlico Rivers) of the Albemarle-Pamlico estuarine system. In general, *K. veneficum* and *C. raciborskii* were intolerant to relatively high flow conditions, as abundances greater than 1,000 units mL<sup>-1</sup> only occurred at flows less than 50 m<sup>3</sup> s<sup>-1</sup>. Whereas, *P. minimum* abundance was as high as 21,000 units mL<sup>-1</sup> at flows greater than 230 m<sup>3</sup> s<sup>-1</sup>. *P. minimum* populations were also more pronounced during cooler temperatures, with greater total Kjeldahl nitrogen levels and higher turbidity. Both *C. raciborskii* and *G. instriatum* were more prevalent during periods of lower salinities, and *K. veneficum* was most abundant during higher salinities and higher total phytoplankton abundances.

### Introduction

A critical mechanism for the development and maintenance of harmful algal blooms (HABs) is nutrient supply (e.g., Smayda 1989; Sierra-Beltrán *et al.* 2005), although low algal abundances under high nutrient conditions have been observed (Christian *et al.* 1986). Comparatively few studies have considered secondary environmental factors that may work in concert with nutrients in promoting HABs. The Albemarle-Pamlico estuarine system is characterized as having comparatively high spatiotemporal variability in both biotic and abiotic components (Springer *et al.* 2005). This variability likely influences the development, duration, magnitude and type of HAB, thus allowing the Albemarle-Pamlico estuary to serve as a suitable system to consider secondary environmental factors on algal productivity. In this study we focused on four potentially harmful algal species (dinoflagellates *Gyrodinium instriatum* Freudenthal & Lee, *Karlodinium veneficum* (Ballantine) J. Larsen and *Prorocentrum minimum* (Pavillard) Schiller, and the cyanobacterium *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya & Subba Raju observed over a 6-yr period (2000-2005) within the three tributaries Neuse, New, and Pamlico Rivers of the Albemarle-Pamlico estuarine system. The objective of this study was to use an expansive biological and environmental data set as a frame-work to ascertain secondary factors that may promote HAB development.

### Material and Methods

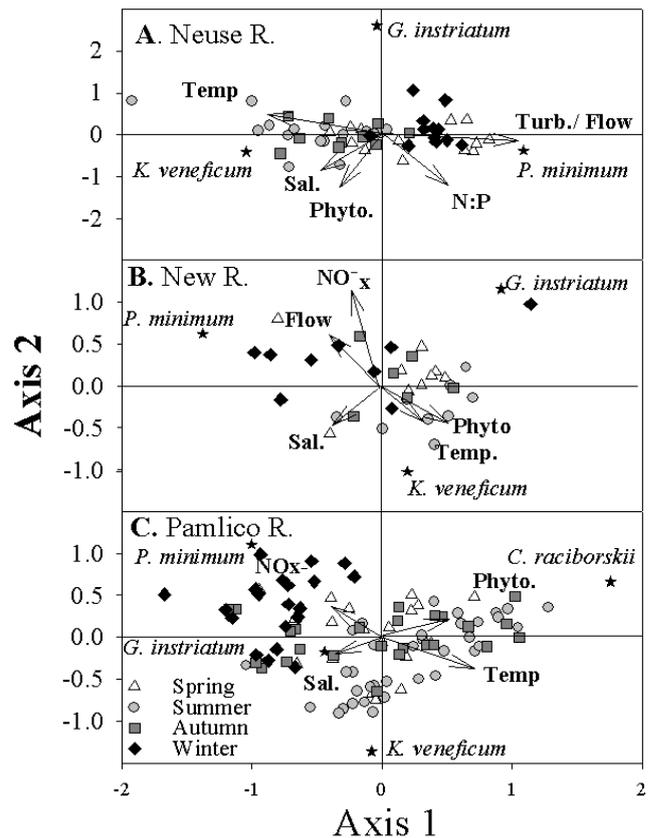
Environmental and biological data were collected (Jan 2000-Dec 2005) as part of a routine monitoring programme conducted by the North Carolina Division of Water Quality (NCDWQ). Samples were collected monthly, with increased frequency during high biomass periods. Nutrients collected from each site included NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, total Kjeldahl nitrogen (TKN), and TP (as described in Fensin 2006). Subsurface physiochemical measurements of DO, pH, temperature and salinity were recorded using a Hydrolab Surveyer-4 (Hydrolab Corp., Austin, Texas, USA). Phytoplankton samples were sampled within the photic zone (defined by NCDWQ as twice the Secchi depth), preserved in the field with acidic Lugol's solution, and stored on ice (~4 °C) during transit to the laboratory. Total microplankton greater than 5 µm were enumerated using an Utermöhl settling chamber (Phycotech Inc., St. Joseph, MI, USA) under 300x magnification on an inverted Leitz Diavert microscope. Phytoplankton populations were reported as units, and defined as one cell (e.g., *G. instriatum*, *K. veneficum*, and *P. minimum*), one filament (e.g., *C. raciborskii*), or single colony (few taxa within the total microplankton). Water flow measurements were provided by the United States Geological Survey, and were based on flow rates observed from gauges proximate to the sampling locations.

A multivariate canonical correspondence analysis (CAA) was performed for each estuarine river system on data collected over the 6-year period using PC-ORD software (version 4; MJM Software Design, Gleneden Beach, Oregon, USA). Square-root transformations of individual algal abundances were compared against environmental variables, and the resultant factors after 100 randomizations were tested for significance at  $p < 0.05$  using a Monte Carlo permutation test. Data were also grouped between high and low algal abundances (high  $> 500$  units  $\text{mL}^{-1}$  for *G. instriatum* and  $> 1,000$  units  $\text{mL}^{-1}$  for *C. raciborskii*, *K. veneficum* and *P. minimum*), and statistical comparisons between environmental parameters at high and low (including absence of designated species) algal abundances were conducted using a nonparametric Mann-Whitney rank sum test.

## Results and Discussion

CAA revealed significant patterns between harmful algal abundance and environmental physiochemical parameters for the first three components ( $p < 0.03$ , Monte Carlo test results), with the first two factors explaining between 38 (Pamlico River) and 42 percent of the total variance (New and Neuse Rivers). In general, eigenvalues were higher for the first Axis, and therefore interpretation of the figures should largely be based upon horizontal tendencies. Overall, the strongest correlations between environmental parameters and the first two components were temperature (Axis-1 for Pamlico, New and Neuse Rivers, respectively), salinity (Axis-2 for all three rivers), river flow (Axis-1 for New and Neuse rivers), total algal abundance (Axis-1 in the New River, and Axis-2 in the Neuse River), and nutrients ( $\text{NO}_x^-$  with Axis-1 in the Pamlico River,  $\text{NO}_x^-$  with Axis-2 in the New River, P and N:P ratios, respectively, in the Neuse River).

The only parameter strongly correlated with Axis-1 for all three study areas was temperature (Fig. 1). Its prominent control over HAB development in temperate waters is not surprising when considering seasonal dynamics in algal productivity (Mallin 1994; Pickney *et al.* 1998). In this study, *C. raciborskii* and *P. minimum* were significantly influenced by environmental temperature (Table 1), *C. raciborskii* preferring warmer temperatures typical of late summer and early autumn ( $28.1 \pm 0.6$  °C) and *P. minimum* favouring cooler winter/early spring temperatures ( $11.4 \pm 1.3$  °C). While this preference towards cooler temperatures by *P. minimum* is inconsistent with some studies in tropical and subtropical locations (temper-



**Figure 1.** CCA defined ordination of seasonally grouped samples within environmental space. The strongest environmental variables ( $r^2 > 0.40$  with any axis) are presented as vectors in the biplot overlay. The first (Axis 1) and second (Axis 2) components account for 42 (Neuse [A], and New [B]) and 38 % (Pamlico [C]) of the total variance.

ature range 22.5–33 °C; Sierra-Beltrán *et al.* 2005), it agrees remarkably well with studies conducted in northern Europe (greatest abundance at 14 °C; Hajdu *et al.* 2005).

Flow rates have been shown to influence algal assemblages in the Albemarle-Pamlico Estuarine system (Christian *et al.* 1986; Fensin 2006). In this study both *C. raciborskii* and *K. veneficum* were unable to maintain significant populations when flow rates exceeded  $50 \text{ m}^3 \text{ s}^{-1}$  (mean flow rates were  $3.3 \pm 0.4$ ,  $9.9 \pm 1.5$ , and  $90.6 \pm 7.8 \text{ m}^3 \text{ s}^{-1}$  for the New, Neuse and Pamlico Rivers, respectively). In contrast, both *G. instriatum* and *P. minimum* were present ( $> 500$  units  $\text{mL}^{-1}$ ) at flows greater than  $200 \text{ m}^3 \text{ s}^{-1}$  and did not appear to have a strong flow preference (Table 1). While the Pamlico River had the greatest flow of all three tributaries investigated, it was also the only river that supported appreciable *C. raciborskii* populations, but only during periods of comparatively low flow ( $11.9 \pm 4.0 \text{ m}^3 \text{ s}^{-1}$ ; Table 1). Similarly, *K. veneficum*, which occurred in all three rivers, was most prevalent at flows approx-

**Table 1.** Biotic and abiotic parameters collected from three major tributaries (Neuse, New and Pamlico Rivers) of the Albemarle-Pamlico estuarine system.

Parameter	<i>C. raciborskii</i>		<i>G. instriatum</i>		<i>K. veneficum</i>		<i>P. minimum</i>	
	Low (n=298)	High (n=16)	Low (n=291)	High (n=30)	Low (n=278)	High (n=37)	Low (n=287)	High (n=27)
Phyto. (units x 1000 mL <sup>-1</sup> )	<b>44.7 ± 2.3</b>	<b>76.8 ± 12.0</b>	45.7 ± 2.3	52.8 ± 6.3	<b>44.0 ± 2.3</b>	<b>63.5 ± 5.5</b>	47.0 ± 2.3	35.5 ± 5.9
Flow (m <sup>3</sup> s <sup>-1</sup> )	<b>35.1 ± 4.5</b>	<b>11.9 ± 4.0</b>	39.2 ± 5.5	29.5 ± 7.9	<b>37.7 ± 4.9</b>	<b>10.6 ± 3.4</b>	34.3 ± 4.8	37.1 ± 9.9
Chlorophyll <i>a</i> (µg L <sup>-1</sup> )	21.8 ± 1.1	17.9 ± 5.9	20.3 ± 0.9	32.8 ± 5.2	<b>20.8 ± 1.1</b>	<b>26.5 ± 2.7</b>	<b>20.5 ± 1.0</b>	<b>30.9 ± 4.6</b>
Turbidity (NTU)	6.19 ± 0.22	ND	<b>5.8 ± 0.19</b>	<b>10.2 ± 1.3</b>	6.33 ± 0.2	5.22 ± 0.3	<b>5.65 ± 1.03</b>	<b>10.4 ± 1.48</b>
pH	<b>9.04 ± 0.27</b>	<b>13.4 ± 1.87</b>	9.4 ± 0.31	7.8 ± 0.32	9.5 ± 0.31	7.9 ± 0.07	9.44 ± 0.31	7.95 ± 0.10
Salinity (PSU)	<b>6.38 ± 0.29</b>	<b>2.15 ± 0.25</b>	<b>6.3 ± 0.31</b>	<b>5.9 ± 0.62</b>	<b>5.78 ± 0.29</b>	<b>9.19 ± 0.96</b>	6.13 ± 0.31	6.95 ± 0.85
Temperature (°C)	<b>19.0 ± 0.46</b>	<b>28.1 ± 0.66</b>	19.6 ± 0.47	17.1 ± 1.4	19.1 ± 0.49	21.1 ± 1.1	<b>20.3 ± 0.45</b>	<b>11.4 ± 1.3</b>
Dissolved oxygen (mg L <sup>-1</sup> )	<b>9.51 ± 0.32</b>	<b>7.23 ± 0.34</b>	9.4 ± 0.34	9.3 ± 0.54	9.5 ± 0.34	8.91 ± 0.48	<b>9.15 ± 0.34</b>	<b>11.5 ± 0.46</b>
TKN (µg L <sup>-1</sup> )	<b>0.74 ± 0.02</b>	<b>0.84 ± 0.03</b>	<b>0.72 ± 0.02</b>	<b>0.94 ± 0.13</b>	0.72 ± 0.02	0.85 ± 0.11	<b>0.71 ± 0.02</b>	<b>1.02 ± 0.12</b>
NO <sub>x</sub> <sup>-</sup> (µg L <sup>-1</sup> )	<b>0.11 ± 0.01</b>	<b>0.02 ± 0.01</b>	0.10 ± 0.01	0.11 ± 0.03	0.11 ± 0.01	0.07 ± 0.03	0.10 ± 0.01	0.10 ± 0.02
TP (µg L <sup>-1</sup> )	<b>0.10 ± 0.01</b>	<b>0.13 ± 0.01</b>	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.003	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
N:P	<b>9.57 ± 0.34</b>	<b>6.72 ± 0.34</b>	9.4 ± 0.34	9.45 ± 0.97	9.38 ± 0.35	10.1 ± 0.86	<b>9.11 ± 0.32</b>	<b>12.5 ± 1.3</b>
Bloom Density (units mL <sup>-1</sup> )		5,700 ± 620		1,700 ± 400		2,900 ± 60		4,200 ± 170

Data is grouped between high and low algal abundances, where high abundance was arbitrarily selected as greater than 500 units mL<sup>-1</sup> (*G. instriatum*) or greater than 1,000 units mL<sup>-1</sup> (*C. raciborskii*, *K. veneficum*, and *P. minimum*). Number of observations for high and low abundances are in parentheses below each algal species. Total microplankton (Phyto) represents phytoplankton densities (harmful and non-harmful algal species collectively) greater than 5 µm in diameter. Statistical differences where  $p < 0.05$  are in bold and  $p < 0.001$  are bold and italicized. Typical bloom densities for each species are presented in the last line. Data are presented as mean ± 1 SE.

imating  $10.6 \pm 3.4$  m<sup>3</sup> s<sup>-1</sup>. High flow rates tended to be sporadic with little to no seasonality; nevertheless, the lowest baseline flows were typically observed in summer. Therefore the warm temperatures and low river flow of summer may contribute, in part, to the development of some algal blooms.

In general, salinities at these sites were low (mean 6.2 psu, range 0-23.0 psu). Nevertheless, three (*C. raciborskii*, *G. instriatum* and *K. veneficum*) of the four species studied showed significant salinity preferences. Both *C. raciborskii* and *G. instriatum* favoured salinities that were lower than average ( $2.2 \pm 0.3$  and  $5.9 \pm 0.6$  psu, respectively), whereas *K. veneficum* tended to accumulate at elevated salinities ( $9.2 \pm 1.0$  psu).

While secondary environmental factors can influence HAB development and maintenance, it must be emphasized that these factors often work in concert with nutrient supply (Christian *et al.* 1986; Mallin 1994). In this study, significant nutrient responses were observed in three (*C. raciborskii*, *G. instriatum* and *P. minimum*) of the four species, with *C. raciborskii* favouring higher P levels, and the dinoflagellates favouring higher N.

## Conclusions

The results illustrate the importance of non-nutrient environmental parameters in shaping HABs in estuarine systems. In general river flow, temperature,

turbidity, and salinity were important secondary components in influencing algal abundances. River discharge, particularly, was important in ameliorating favourable nutrient and temperature conditions for *C. raciborskii* and *K. veneficum* as algal populations declined with increasing river flow.

## Acknowledgements

We are indebted to the NCDWQ's field personnel. D. Vandermast kindly provided statistical counsel, and K. Sellner along with two anonymous reviewers provided helpful comments that strengthened this manuscript.

## References

- Christian, R.R., Bryant, W.L. & Stanley, D.W. (1986). Report No. 223, UNC WRRI, Raleigh, NC.
- Hajdu, S., Pertola, S. & Kuosa, H. (2005). Harmful Algae 4: 471-480.
- Fensin, E.E. (2006). Afr. J. Mar. Sci. 28: 277-281.
- Mallin, M.A. (1994). Estuaries 17: 561-574.
- Pinckney, J.L., Paerl, H., Harrington, M. & Howe, K. (1998). Mar. Biol. 131: 371-381.
- Sierra-Beltrán, A.P., Cortés-Altamirano, R. & Cortés-Lara, M.C. (2005). Harmful Algae 4: 507-517.
- Smayda, T.J. (1989). In: Novel Phytoplankton Blooms, E. Cosper *et al.* (eds), Springer, pp. 449-483.
- Springer, J.J., Burkholder, J.M., Glibert, P.M. & Reed, R.E. (2005). Harmful Algae 4: 533-551.

## Effects of varying salinity and N:P ratios on the growth and toxicity of *Karenia brevis*

D.K. Lekan<sup>1</sup> and C.R. Tomas<sup>2</sup>

UNCW Center for Marine Science, 5600 Marvin K. Moss Lane, Wilmington, NC, USA 28409,

<sup>1</sup>dkl5447@uncw.edu <sup>2</sup>tomasc@uncw.edu

### Abstract

The toxic dinoflagellate *Karenia brevis* forms extensive blooms in the Gulf of Mexico releasing brevetoxins with implications to human health, mortalities of marine mammals and fishes. Historically, *K. brevis* was considered to form blooms above a salinity barrier of 24 PSU. Recently, blooms in low salinity waters were recorded in the Florida Panhandle and near the Mississippi River outflow. In this study, *K. brevis* was cultured in salinities of 15-40 at 5-PSU intervals and N:P ratios of 16:1, 4:1 and 80:1, to measure the influence of salinity and nutrients on growth and toxicity. No growth of *K. brevis* occurred at 20 PSU or below, but good growth occurred at 25-40 PSU. Growth varied from 0.36 to 0.64 div. day<sup>-1</sup>. The highest mean growth rate was at 35 and the lowest at 25 PSU. Salinity was a primary factor regulating growth and nutrients as secondary factors. Using an ELISA assay the highest per cell concentration occurred in the 25- PSU N-limited treatment with 100 and 40 Balanced with 88 pg toxin•cell<sup>-1</sup>. Similarly, salinity was a primary factor regulating toxin production particularly at 25 and 40 PSU. Nutrient stress appears to stimulate toxin production, prompting further investigation on the relationship between nutrients and toxins.

### Introduction

The toxic dinoflagellate *Karenia brevis* forms extensive red tide blooms in the Gulf of Mexico. The blooms produce potent brevetoxins that cause human Neurotoxic Shellfish Poisoning (NSP) and mass mortalities of fishes and marine mammals (Rounsefell & Nelson 1966). The blooms are a concern in coastal areas, where the unarmoured dinoflagellates easily lyse from wave action, releasing a brevetoxin aerosol (Kirkpatrick *et al.* 2004).

A multitude of physical, biological, environmental and chemical factors interact to determine bloom development but nutrient supply and salinity are of primary importance for *K. brevis* (Liu *et al.* 2001). Nitrogen and phosphorus are often limiting factors for phytoplankton growth in the open ocean, where the relative amounts may limit or encourage growth and toxin production in *K. brevis*.

Aldrich and Wilson (1960) first examined the salinity tolerance of *K. brevis* and found good growth at 27-37 PSU, with less than optimum growth below 24 or above 44 PSU, suggesting a "salinity barrier" of 24. However, within the past fifty years *K. brevis* blooms have occurred more frequently at low salinities (Maier Brown *et al.* 2006), such as the Florida Panhandle and mouth of the Mississippi River where salinity is significantly lower due to freshwater outflow (Dortch *et al.* 1998).

With increasing evidence of blooms in low salinity waters, is salinity truly a barrier for bloom de-

velopment of *Karenia* spp.? It is hypothesized that changing environmental conditions such as differing nutrient ratios and salinities may regulate *K. brevis*' ability to initiate blooms, induce toxin production and affect growth rate. In this study, *K. brevis* was cultured in N:P balanced, P-limited, and N-limited conditions, for each salinity of 15, 20, 25, 30, 35 and 40 PSU, in order to observe growth and toxin production.

### Materials and Methods

A clonal culture of *K. brevis* SP3 S-tox clone from Texas was originally cultured in 33 PSU with modified L1 media at 20°C (Guillard and Hargraves 1993). For this study, *K. brevis* was cultured in 15, 20, 25, 30, 35 and 40 PSU. For each salinity three different N:P ratios were created; balanced (N:P 16:1), P-limited (80:1), and N-limited (4:1). Three 30-mL aliquots of each specific salinity-nutrient treatment were dispensed into sterile 25x160 mm glass test tubes. A 1-mL inoculum of the original culture was added to each tube, and initial fluorometric readings were taken (Day 0) to measure *in vivo* chlorophyll fluorescence. The culture tubes were placed in test tube racks in a constant temperature water bath of 20 °C, a 16:8 h L:D photoperiod cycle and fluence rate of 60 μmol m<sup>-2</sup> s<sup>-1</sup>.

Growth as relative fluorometric readings were taken at the same time daily for 21 days. Growth curves were plotted as day vs. (log<sub>10</sub> + 1) of the average fluorometric readings. The standard deviation of each average was also plotted.

The relationship between increased fluorescence and increased cell number was established with a linear regression analysis yielding the equation  $y = 0.4217x + 0.073$  and a correlation coefficient ( $r^2$ ) of 0.965.

Growth rates were calculated as divisions per day from the steepest slope of the log growth curve. One 5-mL aliquot of each treatment was harvested on day 21 and frozen overnight in a  $-20\text{ }^\circ\text{C}$  freezer to analyze pg-toxin cell<sup>-1</sup> using a very sensitive ELISA (Naar *et al.* 2002).

## Results

At 15 and 20 PSU, cells did not grow in any of the nutrient conditions (Fig. 1). At 25, 30, 35 and 40 PSU, *K. brevis* exhibited growth, but growth curves were modified by nutrient variations (Figs 2,3). Growth under N-limited conditions at all salinities reached their maxima of log growth, and relative fluorescence immediately declined. Balanced and P-limited treat-

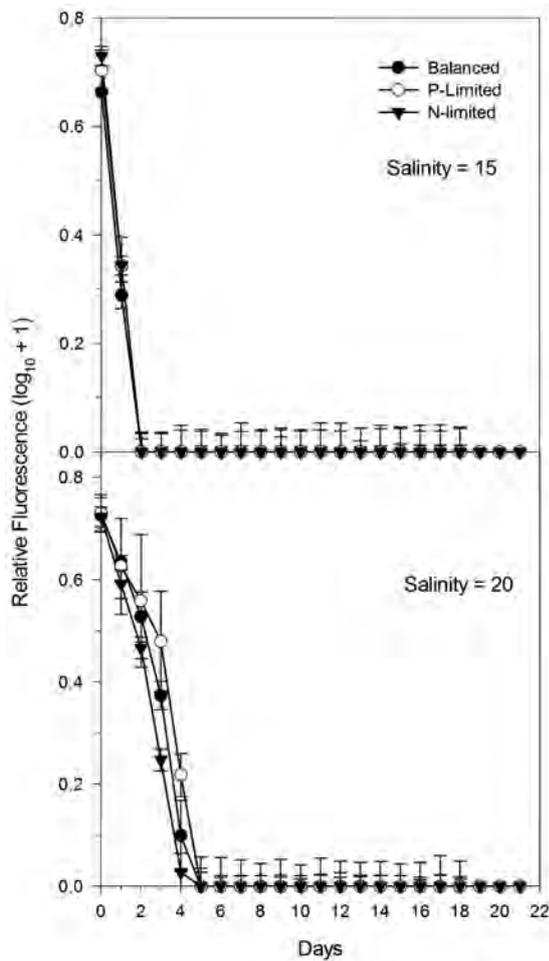
ments showed normal growth curves after the log growth phase.

Highest mean growth rate according to salinity occurred in 35 PSU with 0.61 div. day<sup>-1</sup> and the lowest in 25 PSU with 0.38 div. day<sup>-1</sup> (Table 1). A positive correlation exists between salinity increase and growth rate increase, except at 40 PSU in the N- and P-limited treatments where growth rate dropped (Table 1).

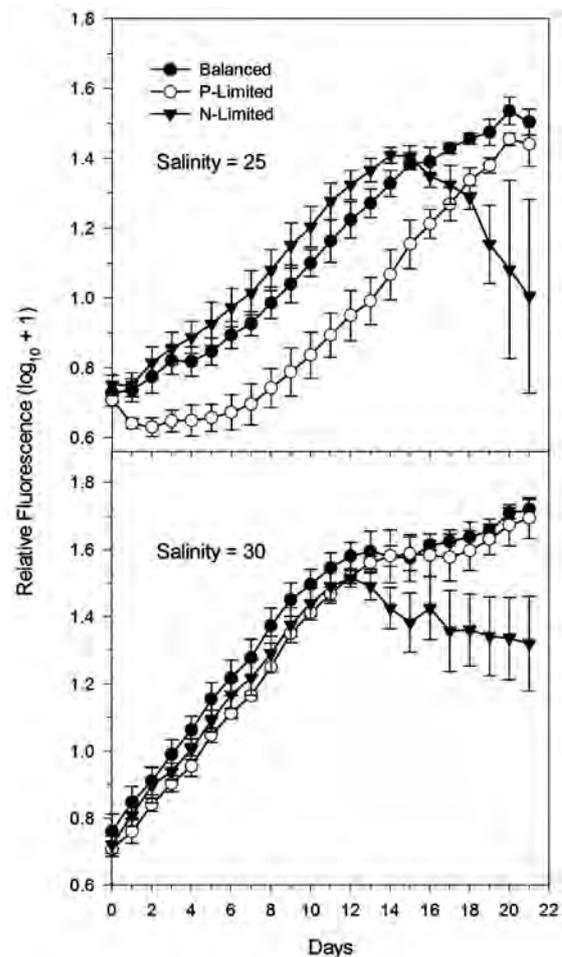
Total toxin levels for each treatment were determined by a single-point ELISA. The highest toxin level, 100 pg-toxin cell<sup>-1</sup>, occurred at 25 PSU N-limited (Table 1). At 35 PSU, there was a drop in toxin concentration for each N:P ratio relative to the other salinity treatments (Table 1).

## Discussion

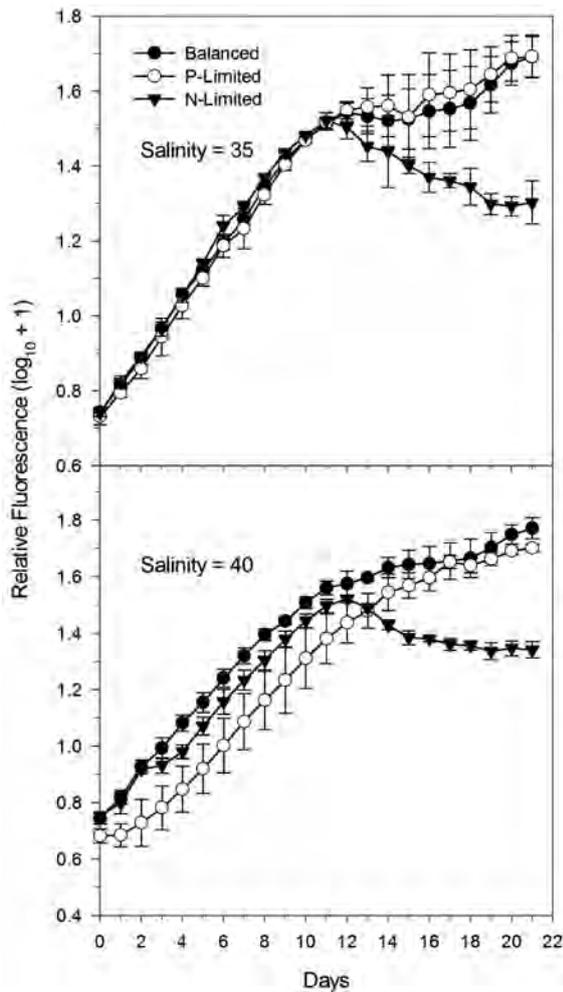
Using the Texas Sp3 S-tox clone, this study supports the historical view that *K. brevis* blooms prefer high salinity waters, and that *K. brevis* cannot survive at



**Figure 1.** Growth curves of *K. brevis* grown at salinities of 15 to 20 and Balanced, P-limited and N-limited treatment over a 21-day growth period



**Figure 2.** Growth curves of *K. brevis* grown at salinities of 25 to 30 and Balanced, P-limited and N-limited treatment over a 21-day growth period.



**Figure 3.** Growth curves of *K. brevis* grown at salinities of 35 to 40 and Balanced, P-limited and N-limited treatment over a 21-day growth period.

or below 20 PSU, and at 25 PSU growth is reduced compared to higher salinities.

Blooms at lower salinities were most likely transported to these areas, where mixing water masses of high and low salinities allowed *K. brevis* to acclimate. Initial salinity studies (Aldrich and Wilson 1960), that did not acclimate *K. brevis*, suggested a narrow salinity tolerance. Maier Brown *et al.* (2006) acclimated several clones of *K. brevis* gradually at 5-PSU intervals allowing the clones to grow below 24 PSU. Clonal variation may also explain the different abilities to acclimate to lower salinities, providing a possible explanation for the *K. brevis* bloom cited by Dortch *et al.* (1998) in the mouth of the Mississippi River.

There was no significant lag phase for any treatment at 30, 35 and 40 PSU, suggesting that *K. brevis* was able to adjust to the salinity well within that range. At 25 PSU there was a short, but noticeable lag phase,

**Table 1.** Growth rates (div. day<sup>-1</sup>) and cellular toxin (pg toxin cell<sup>-1</sup>) of *K. brevis* cultures grown at different salinities (15-40 PSU) and different N:P ratios.

Treatment	div day <sup>-1</sup>	pg toxin cell <sup>-1</sup>
15 Bal	0	0
15 P-Lim	0	0
15 N-Lim	0	0
20 Bal	0	0
20 P-Lim	0	0
20 N-Lim	0	0
25 Bal	0.39	39
25 P-Lim	0.36	37
25 N-Lim	0.40	100
30 Bal	0.51	59
30 P-Lim	0.55	85
30 N-Lim	0.63	73
35 Bal	0.62	35
35 P-Lim	0.61	74
35 N-Lim	0.60	54
40 Bal	0.64	88
40 P-Lim	0.51	87
40 N-Lim	0.48	87

\* Bal N:P=16:1; P-Lim N:P=80:1; N-Lim 4:1

where *K. brevis* required time to acclimate (Figs 2,3), especially in the P-limited culture.

At 35 PSU, there was no significant difference in log phase values for the balanced, N- and P-limited treatments, suggesting that salinity is the principal factor regulating growth.

Toxin production also appears to be a function of salinity. Cellular toxin was greatest at 25 PSU N-limited and 40 PSU balanced conditions. This supports previous findings by Kim and Martin (1974), where toxin concentration in *K. brevis* was highest at low and high salinities, rather than at intermediate salinities. The higher level of toxin in the N-limited treatments may be due to the decline in cell numbers immediately after the log phase. P-limited nutrient stress has been shown to stimulate toxin production in other harmful algae, such as *Prorocentrum lima* and *Chrysochromulina polylepsis* (Granéli *et al.* 1993 ; Tomas & Baden 1993), suggesting that nutrient stress plays a large role in toxin production.

Baden and Tomas (1988) found differences in brevetoxin fractions among different clones. They also found that the toxin profile was directly related to the potency of the clone analyzed. In the present study we examined SP3 Super toxic clone, which is suggested to be highly toxic. To verify and account for any variances, a more detailed approach in which cellular toxin is examined by multiple techniques must be performed.

## Conclusions

At a drastic drop in salinity, cells cannot grow and the cells apparently lyse, releasing a great amount of brevetoxins. Future studies should examine if *K. brevis* has the ability to migrate in the water column during a heavy rainstorm, allowing them to avoid a drastic salinity change, or if cells lyse releasing brevetoxin, which may be important in terms of brevetoxin exposure to fishes or marine mammals.

It is suggested that cellular toxin varies with growth and environmental conditions. Various clones should be studied under the same conditions as they may respond differently to environmental factors.

## Acknowledgements

This work was supported by the Center for Disease Control and Prevention grant #01-504-4, awarded to Carmelo R. Tomas through the North Carolina Department of Health and Human Services.

## References

- Aldrich, D.V. & Wilson, W.B. (1960). *Biol. Bull.* 119: 57-64.
- Baden, D.G. & Tomas, C.R. (1988). *Toxicon* 26: 961-963.
- Dortch, Q., Moncreiff, C.A., Mendenhall, W., Parsons, M.L., Franks, J.S. & Hemphill, K. W. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M. L. & Wyatt T. (eds), Xunta de Galicia and IOC of UNESCO, Spain, pp. 143-144.
- Granéli, E., Paasche, E. & Maestrini, S.Y. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J., Shimizu, Y. (eds), Elsevier, New York, pp. 23-32.
- Guillard, R.R.L. & Hargraves, P.E. (1993). *Phycologia* 32: 234-236.
- Kim, Y. S. & Martin, D. F. (1974). *Phytochem.* 13: 533-538.
- Kirkpatrick, B., Fleming, L.E., Squicciarini, D., Backer, L. C., Clark, R., Abraham, W., Benson, J., Cheng, Y.S., Johnson, D. & Pierce, R. (2004). *Harmful Algae* 3: 99-115.
- Liu, G., Janowitz, G.S. & Kamykowski, D. (2001). *Mar. Ecol. Progr. Ser.* 213: 13-37.
- Maier Brown, A.F., Dortch, Q. Van Dolah, F.M., Leighfield, T.A., Morrison, W., Thessen, A.E., Steidinger, K., Richardson, B., Moncreiff, C.A. & Pennock, J. R. (2006). *Harmful Algae* 5: 199-212.
- Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P.L., Flewelling, L., Steidinger, K., Lancaster, J. & Baden, D. G. (2002). *Env. Health Perspect.* 110: 179-185.
- Rounsefell, G.A. & Nelson, W.R. (1966). U.S. Fish and Wildlife Services Special Scientific Report 535: 85 pp.
- Tomas, C. & Baden, D.G. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J., Shimizu, Y. (eds), Elsevier, New York, pp. 565-570.

## Nitrogen uptake rates during a dinoflagellate bloom in the East China Sea, 2005: variation with N:P ratio

J. Li<sup>1</sup>, P. M. Glibert<sup>1</sup>, S. Lu<sup>2</sup>, D. Lu<sup>3</sup>, X. Shi<sup>4</sup> and C. Zhang<sup>4</sup>

<sup>1</sup>University of Maryland Center for Environmental Science, Horn Point Laboratory, PO Box 775, Cambridge MD 21613 USA, jili@hpl.umces.edu, glibert@hpl.umces.edu, <sup>2</sup>Institute of Hydrobiology, Jinan University, Guangzhou, 510632, China, lusonghui1963@163.com, <sup>3</sup>Second Institute of Oceanography, State Oceanographic Administration, Hangzhou, 310012, China, ludouding@sio.zj.edu.cn, <sup>4</sup>College of Chemistry & Chemical Engineering, Ocean University of China, Qingdao, 266001, China, shixy@mail.ouc.edu.cn, zcsong@mail.ouc.edu.cn

### Abstract

During late spring and early summer of 2005, large scale (>15,000 km<sup>2</sup>) mixed dinoflagellate blooms developed in the coastal East China Sea. *Karenia mikimotoi* was the dominant HAB species in the first stage of the bloom and was succeeded by *Prorocentrum donghaiense*. Samples were collected from different stations along both north-south and west-east transects during 3 cruises of the Chinese Ecology and Oceanography of Harmful Algal Blooms (CEOHAB) Programme, before and during the bloom progression. Nitrogen isotope methods were used to measure rates of NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, and glycine uptake during the blooms. The progression of the blooms was related to a change in available nitrogen (N) and phosphorus (P) composition. As the ambient N:P decreased from > 50 to ~16, bloom strength increased. Reduced nitrogen, especially NH<sub>4</sub><sup>+</sup> and urea were preferentially taken up during the blooms, and supplemental phosphorus enrichment increased the rates of nitrogen uptake, suggesting phosphorus limitation in the bloom area.

### Introduction

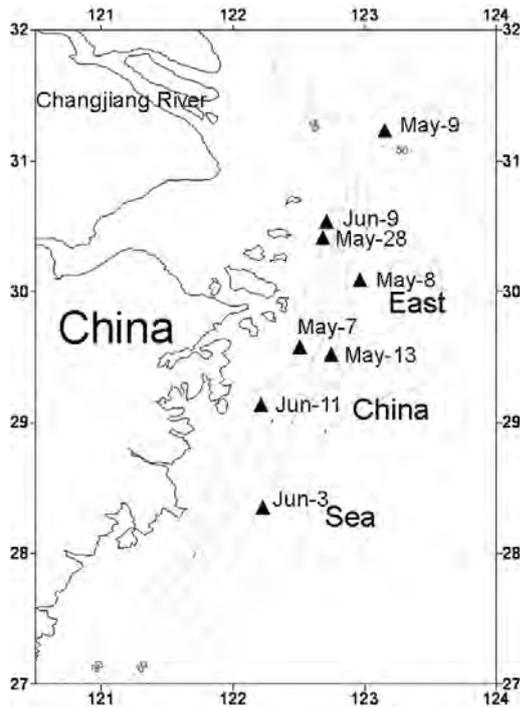
Large-scale dinoflagellate blooms in the coastal area of East China Sea in late spring and early summer have been recorded in the past decade (Zhou *et al.* 2003). Research cruises in this area supported by the Chinese Ecology and Oceanography of Harmful Algal Blooms (CEOHAB) programme over the past five years have focused on the dominant bloom species, *Prorocentrum donghaiense*. However, in 2005, the typical *P. donghaiense* bloom did not develop in the spring. Instead, a massive (>15,000 km<sup>2</sup>) *Karenia mikimotoi* bloom was observed in late May and was gradually replaced by *P. donghaiense* as the summer progressed (Lu *et al.* 2006). The *K. mikimotoi* bloom was associated with a massive fish kill in coastal aquaculture areas. Aquaculture fish worth about \$2.5 million were lost due to this bloom (Zhou 2005). The *Karenia* and *Prorocentrum* blooms decayed in late June and were succeeded by a bloom of *Noctiluca scintillans* (Lu *et al.* 2006).

The bloom area is adjacent to the Changjiang (Yangtze) River estuary. Changjiang River is the third longest river of the world. It has a population of over 400 million in its watershed, including the most developed area of China along the east coast (Chen *et al.* 2001), and exports large amounts of nutrients to the coastal sea. The objectives of this study were to assess nitrogen uptake rates, and the relationships be-

tween ambient N:P ratio, phytoplankton community composition, and the effect of phosphorus limitation on these rates. Three cruises were conducted as part of the CEOHAB project in April-June 2005. The sampling area included the plume of the periphery of Changjiang River estuary, Zhoushan archipelago and most of the coast of Zhejiang province.

### Materials and Methods

The data reported here are from experiments conducted on May 7, 8, 9, 13, 28 and June 3, 9, 11, 2005, representing the period before and during the blooms (Fig. 1). One experiment was conducted per day. Samples were collected around noon, using a bucket for surface sample and a 30-L Niskin sampler for a middle layer sample. Sample water was transferred into a clear 20-L carboy and experiments were started immediately. Water samples were dispensed into 250-ml bottles and inoculated with <sup>15</sup>N-labelled substrates (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, urea, and glycine) at concentrations representing ~ 10 % of ambient. Incubations were 0.5 h on-deck in a flowing-seawater incubator. Samples were then collected onto pre-combusted (2 h at 400 °C) GF/F filters. The potential effect of P limitation of N uptake was tested in separate experiments which involved pre-incubation with added PO<sub>4</sub><sup>3-</sup> at 3 µg atom-P L<sup>-1</sup> and incubated for 2 h before the start of the isotope <sup>15</sup>N enrichment experiments. Due to

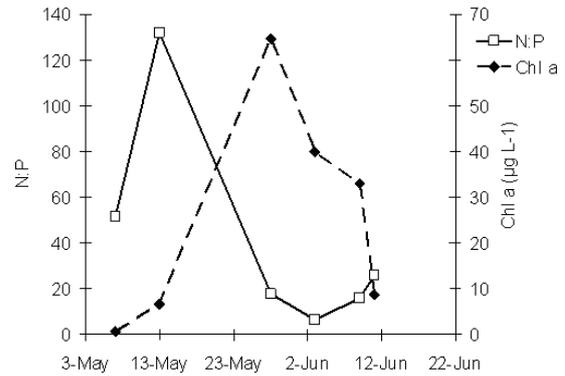


**Figure 1.** Location and magnitude of sampling stations (▲) in the East China Sea.

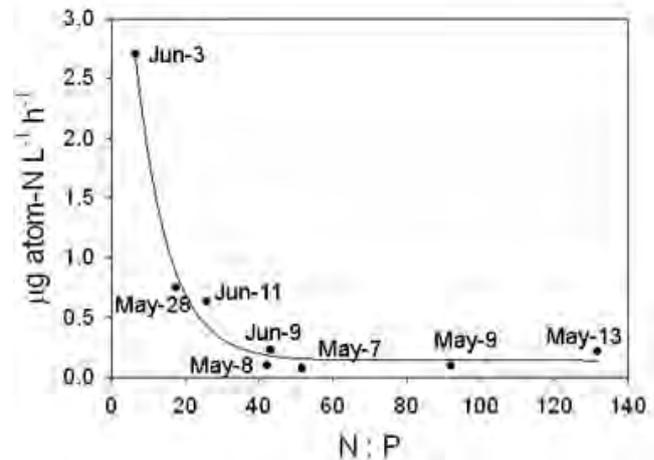
the number of treatments, replication was not possible. The GF/F filters were dried for 24 h at 50 °C and analyzed for isotope enrichment using a Sercon Mass Spectrometer after transport back to Horn Point Laboratory. Rates of  $^{15}\text{N}$  uptake were calculated according to Glibert and Capone (1993). Ambient inorganic nutrients were determined using a Technicon AutoAnalyzer (Lane *et al.* 2000). Dissolved free amino acid (DFAA) was determined by fluorometric analysis according to Lindroth and Mopper (1979), and urea was analyzed using the method of Revilla *et al.* (2005). Samples were also collected onto GF/F filters for pigment analysis by high performance liquid chromatography (HPLC). These analyses were performed by Horn Point Laboratory analytical services according to the method of Van Heuklem and Thomas (2001). The pigment gyroxanthin-diester was used as a diagnostic of *K. mikimotoi* (Stær and Cullen 2003; Richardson and Pinckney 2004).

## Results and Discussion

Experiments in early May were conducted before the peak of the dinoflagellate blooms. At that time, the water was low in total chlorophyll *a* ( $< 2 \mu\text{g L}^{-1}$ ), and had a high ambient N: P ratio ( $\sim 50$ ). Concentration of total N averaged about  $19 \mu\text{mol L}^{-1}$  and  $\text{PO}_4^{3-}$  about  $0.4 \mu\text{mol L}^{-1}$ . From early May through early June, the N: P ratio decreased, approaching Redfield ( $\sim 16$ ), and the blooms developed (Fig. 2). The decrease in the



**Figure 2.** Variation of the molar N:P ratio and in Chl *a* ( $\mu\text{g L}^{-1}$ ) concentration in the ambient water during the study period, 2005.

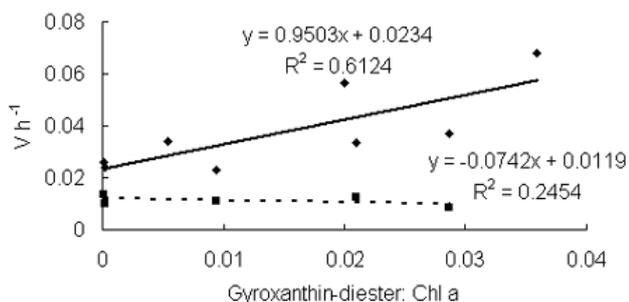


**Figure 3.** The relation between the N:P ratio in the ambient water and the uptake rate of reduced nitrogen (sum of  $\text{NH}_4^+$ , urea, and glycine) during the study period.

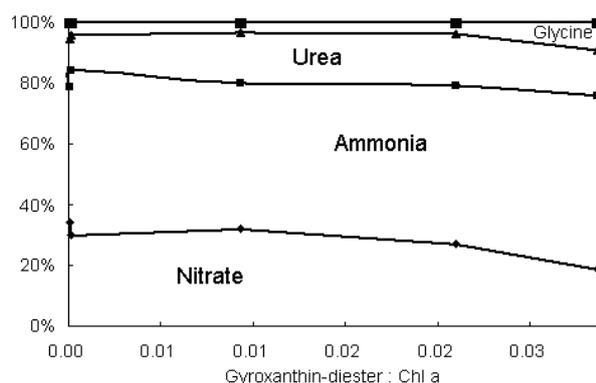
N: P ratio was mostly due to the decrease in concentrations of nitrogen in the water. Chlorophyll *a* was about  $65 \mu\text{g L}^{-1}$  at the peak of the blooms.

Rates of total reduced nitrogen uptake (sum of  $\text{NH}_4^+$ , urea, and glycine) increased with bloom progression and corresponded with the decrease in ambient N: P ratio (Fig. 3). Rates were  $< 0.22 \mu\text{g atom-N L}^{-1} \text{h}^{-1}$  when N: P was  $> 40$ , but increased to  $> 0.5 \mu\text{g atom-N L}^{-1} \text{h}^{-1}$  when N: P was  $< 8$ . These results are suggestive of P limitation due to the high N loading in the early spring, prior to the onset of the blooms.

Gyroxanthin-diester is an indicator of *K. mikimotoi* (Stær and Cullen 2003; Richardson and Pinckney 2004), and the ratio of gyroxanthin-diester:Chl-*a* ratio can be used as an index of the relative abundance of *K. mikimotoi* in the phytoplankton community. As the ratio of gyroxanthin-diester: Chl-*a* ratio in the sample increased, the specific uptake rates of reduced nitrogen increased from  $0.023 \text{ h}^{-1}$  to  $0.068 \text{ h}^{-1}$  and the uptake of  $\text{NO}_3^-$  decreased from  $0.013 \text{ h}^{-1}$  to  $0.008 \text{ h}^{-1}$



**Figure 4.** The relationship between specific nitrogen uptake rate and the Gyroxanthin-diester: Chl *a* ratio for samples collection during the *K. mikimotoi* bloom. Solid line is the trendline of reduced nitrogen ( $\text{NH}_4^+$ , urea, and glycine) and dashed line is the trendline of  $\text{NO}_3^-$ .

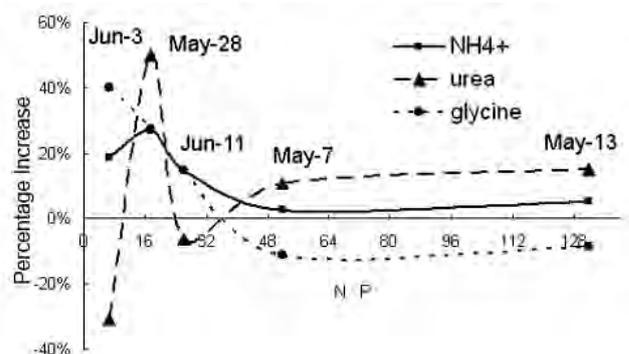


**Figure 5.** The contribution of different nitrogen sources to the total N uptake versus Gyroxanthin-diester: Chl *a* ratio for samples collection during the *K. mikimotoi* bloom.

(Fig. 4). At the peak of the bloom,  $\text{NO}_3^-$  contributed < 30 % of the total N uptake, 50- 60 % of the uptake was as  $\text{NH}_4^+$ , up to 16 % was as urea and 5 % as amino acids (measured only as glycine) (Fig. 5). Thus as the relative abundance of *K. mikimotoi* in the phytoplankton community increased, the relative contribution of  $\text{NO}_3^-$  to total uptake decreased. Reduced nitrogen, especially  $\text{NH}_4^+$  and urea, were preferred and contributed most of the nitrogen uptake.

At high ambient N:P ratios (> 40), the change in N uptake rates when pre-enriched with  $\text{PO}_4^{3-}$  was not large (< 15 %) (Fig. 6). However, when ambient N:P ratio had declined to levels closer to the Redfield ratio, rates of  $\text{NH}_4^+$ , urea and glycine increased 27-50 % over unamended samples when pre-enriched with  $\text{PO}_4^{3-}$ . The lack of a response at high N: P ratios may have been a function of the severity of P limitation, the short period of pre-incubation, or that the P enrichment levels used were insufficient for the cells to overcome their limitation.

The average total nitrogen concentration decreased from  $19.5 \mu\text{g atom-N L}^{-1}$  before the *K. mikimotoi* bloom to  $9.5 \mu\text{g atom-N L}^{-1}$  during the bloom



**Figure 6.** Percentage increase with the rate of uptake of reduced forms of nitrogen ( $\text{NH}_4^+$ , urea, and glycine) in relation to the N:P ratio of the ambient water.

and reached  $5.3 \mu\text{g atom-N L}^{-1}$  at the end of dinoflagellate blooms progression. However,  $\text{PO}_4^{3-}$  only decreased from an average of  $0.32 \mu\text{g atom-P L}^{-1}$  to  $0.20 \mu\text{g atom-P L}^{-1}$  and stayed roughly constant throughout the bloom. Although  $\text{PO}_4^{3-}$  did not become depleted, the high total N availability relative to  $\text{PO}_4^{3-}$  suggested P limitation. The relatively constant  $\text{PO}_4^{3-}$  concentration could suggest more external P supply or internal P regeneration in the bloom area. *Karenia mikimotoi* has been reported to produce alkaline phosphatase (AP) under  $\text{PO}_4^{3-}$  concentrations <  $0.25 \mu\text{g atom-P L}^{-1}$ , and has been shown to have sufficient activity to regenerate dissolved organic phosphorus (DOP) to maintain  $\text{PO}_4^{3-}$  supply for growth (Yamaguchi *et al.* 2004). *Prorocentrum donghaiense* has also been reported to use DOP (Huang *et al.* 2005). Further work on the bioavailability of various P compounds other than  $\text{PO}_4^{3-}$  and improved understanding of the rates of regeneration of these compounds could be helpful to understand the bloom progression.

In summary, the bloom progression was related to the changing N: P composition of the available nutrients. Dinoflagellates bloomed as N: P ratio approached the Redfield ratio. However, even when ambient concentration of N and P were near Redfield proportions, evidence of P limitation was shown by enhanced N uptake rates when pre-incubated with  $\text{PO}_4^{3-}$ . As the dinoflagellate blooms developed, proportionally more of the N was taken up as  $\text{NH}_4^+$  and urea.

### Acknowledgements

We thank Prof. Mingjiang Zhou for the opportunity for Ji Li to participate in the CEOHAB cruises (Project number 2001CB409704). Ji also thanks Horn Point Laboratory Education Committee for the funds for travel to China and to Copenhagen, and the 12th

International Conference on Harmful Algae for student travel support. This is contribution number 4051 from the University of Maryland Center for Environmental Science.

## References

- Chen, Z., Li, J., Shen, H. & Wang, Z. (2001). *Geomorphology* 41: 77-91.
- Glibert, P.M. & Capone, D.G., (1993). In: *Nitrogen Isotope Techniques*, Knowles, R. & Blackburn, T.H. (eds), Academic Press, New York, pp. 243-272.
- Huang, B., Ou, L., Hong, H., Luo, H. & Wang, D. (2005). *Mar. Pollut. Bull.* 51: 838-844.
- Lane, L., Rhoades, S., Thomas, C. & Van Heukelem, L., (2000). *Standard Operating Procedures 2000*. University of Maryland Center for Environmental Science, Technical Report TS-264-00, p. 67.
- Lindroth, P. & Mopper K., (1979). *Anal. Chem.* 51: 667-1674.
- Lu, S., O, M, Lu, D., Zhou, D., Wang, Y., Zhang, C. & Qi, Y., 12th International Conference on Harmful Algae, Poster.
- Revilla, M., Alexander J. & Glibert, P.M. (2005). *Limnol. Oceanogr. Meth.* 3: 290-299.
- Richardson, T.L. & Pinckney J.L., (2004). *J. Phycol.* 16: 315-328.
- Stær, P.A. & Cullen, J.J. (2003). *J. Plankton Res.* 25: 1237-1249.
- Van Heukelem, L., Lewitus, A.J., Kana, T.M. & Craft, N.E. (1994). *Ecol. Progr. Ser.* 114: 303-313.
- Yamaguchi H., Nishijima T., Nishitani H., Fukami K. & Adach M. (2004). *Nippon Suisan Gakkaishi* 70: 123-130.
- Zhou, M., Yan T. & Zou, J., (2003). *China J. Appl. Ecol.* 14: 1031-1038.
- Zhou, M., (2005). COEHAB Cruise Report, unpublished.

## UV responses in three strains of the cyanobacterium *Nodularia spumigena*

V. Lindberg<sup>1</sup>, M. Mohlin<sup>2</sup> and A. Wulff<sup>3</sup>

<sup>1,2,3</sup>Dept of Marine Ecology, Marine Botany, P.O. Box 461, SE 405 30 Göteborg, Sweden,

<sup>2</sup>malin.mohlin@marbot.gu.se, <sup>3</sup>angela.wulff@marbot.gu.se

### Abstract

Toxic summer blooms of cyanobacteria are a problem in the Baltic Sea and *Nodularia spumigena* is one of the most common species. During the blooms, *N. spumigena* cells are often concentrated in the upper water layers where the cells are exposed to high light intensities including both ultraviolet-A (UV-A 320–400 nm) and ultraviolet-B radiation (UV-B 280–320 nm). Three different strains of *N. spumigena* were exposed to PAR (photosynthetically active radiation, 400–700 nm), PAR+UV-A and PAR+UV-A+UV-B for 9 days. Samples were taken for analyses of specific growth rate, photosynthetic capacity and the concentrations of mycosporine-like amino acids (MAAs). Strain-specific differences were found and *N. spumigena* was affected by UV-B and to some extent by UV-A. Exposure to UV-B significantly increased concentrations of MAAs. The specific growth rates were positive in all strains irrespective of treatment and it is concluded that *N. spumigena* is able to withstand quite high intensities of UV-B (0.7–1 W m<sup>-2</sup>) and may therefore have a competitive advantage compared to other less UV-tolerant phytoplankton species.

### Introduction

In the brackish Baltic Sea, cyanobacteria are particularly prominent, forming extensive blooms during spring and summer (e.g. Finni *et al.* 2001). Several of the bloom-forming species produce toxins. One such species is the heterocyst-forming *Nodularia spumigena* Mertens ex Bornet et Flahault, which produces the hepatotoxin nodularin (Stal *et al.* 1999). *N. spumigena* blooms occur in late summer, a period that coincides with strong light, high surface-water temperatures, calm weather, and a stable stratification of the water column (Finni *et al.* 2001; Staal *et al.* 2003). Since *N. spumigena* cells possess gas vesicles, the cells are often concentrated in the upper water layers (Paerl 1988; Staal *et al.* 2003) where they can be expected to be exposed to both ultraviolet-A (UV-A 320–400 nm) and ultraviolet-B radiation (UV-B 280–320 nm). Thus, they require strategies for protection against photodamage caused by high levels of both PAR and UV radiation. Such strategies involve for example production of UV-absorbing compounds, such as mycosporine-like amino acids (MAAs).

The floating and nodularin-producing strains of *N. spumigena* from the Baltic Sea are regarded as belonging to one species (Laamanen *et al.* 2001; Janson and Granéli 2002). Previously, intraspecific variation has been commonly overlooked in studies of toxic microalgae (Burkholder and Glibert 2004), but significant ecophysiological variation does exist among strains, within at least some cyanobacterial species (Karsten and Garcia-Pichel 1996). This emphasizes

the need to include several clones when studying the response of a species to environmental stressors such as UV radiation.

In the present study, the UV tolerance (and particularly the UV-B tolerance) of three strains of *N. spumigena* were isolated from the Baltic Sea and investigated in the laboratory. The working hypothesis was that there is an intraspecific variation in the response of *N. spumigena* to UV-B radiation.

### Material and Methods

Three strains (KAC7, KAC11, KAC71), isolated during 2000, 2001 and 2002 from the Baltic Sea, were obtained in August 2003 from the Kalmar Algal Collection (KAC), Kalmar University, Sweden. The strains were grown in a culture room at 18 °C and a 16:8 L:D cycle of 75 µmol photons m<sup>-2</sup> s<sup>-1</sup>. For each of the 3 strains, 52 quartz bottles were prepared (3 treatments × 4 replicates × 4 samplings). Four bottles were kept outside the experimental area under low light conditions (55 µmol photons m<sup>-2</sup> s<sup>-1</sup>). Before the experiment started, the bottles were put under the experimental PAR light for 3 days. The experiment was run for 9 days. The 3 treatments were PAR (P), PAR+UV-A (PA) and PAR+UV-A+UV-B (PAB). The UV-light tubes were covered with cellulose acetate foil (replaced every second day) to screen out UV-C radiation. Light intensities reaching the bottles varied within the experimental area; PAR 150–180 µmol photons m<sup>-2</sup> s<sup>-1</sup>, UV-A 4–6 W m<sup>-2</sup>, and UV-B 0.7–1 W m<sup>-2</sup>. The bottles were randomly moved every day to

ensure equal light doses during the experiment. The L:D cycle was 16:8 and the UV-B light was on for 4 h in the middle of the light period. Samples were taken on days 0 (initial), 1, 3 and 9. In each replicate, the cell suspension was replaced with 5 ml f/2 medium every second day. For KAC71, 15 ml was exchanged Day 7. Cells were measured and counted in the light microscope, and cell numbers were used to calculate the specific growth rate  $\text{day}^{-1}$ .

The photosynthetic capacity was measured with a pulse amplitude modulated fluorometer (PhytoPAM, Walz, Germany). Cells were dark-adapted for 15 min and the maximum quantum yield of photosynthesis ( $F_v/F_m$ ) was calculated (Walz 1999). For analyses of MAAs, 15 ml of the algal suspension was filtered onto GF/F filters and the cells were extracted in 1.5 ml 25 % MeOH, put in a 45 °C water bath for 2 h, and the extract was analysed in a Shimadzu UV-2401 spectrophotometer. An absorbance spectrum from 280 to 700 nm was recorded. The area under the graph between 310 and 360 nm was calculated and absorbance units per cell were calculated.

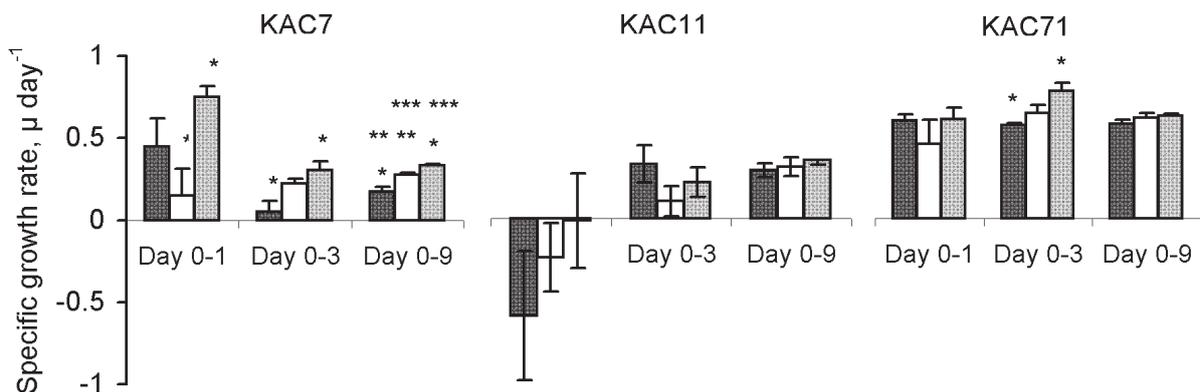
For statistical analyses, 1-way ANOVA was used. Differences were accepted as significant at  $p < 0.05$ . Cochran's test was used to check for homogeneity of variance.

## Results and Discussion

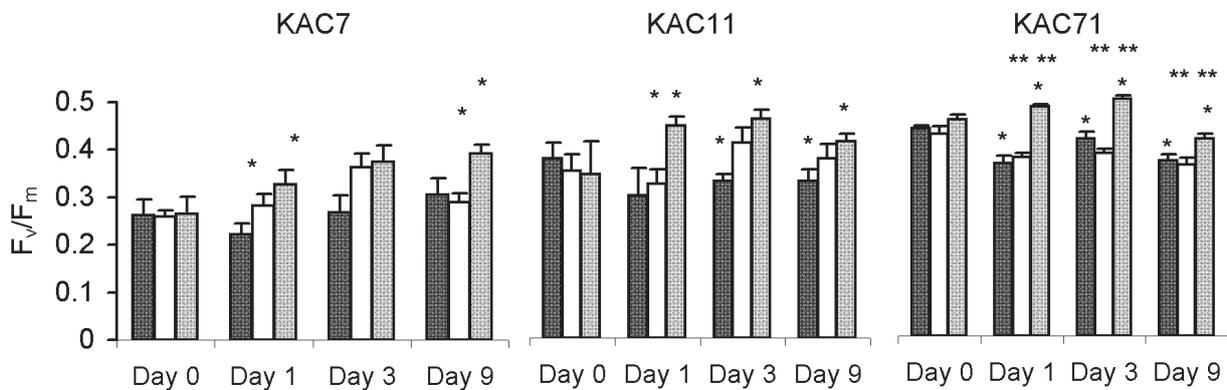
The experimental UV-B intensity was similar to the intensities observed on the Swedish West Coast in midsummer around noon (1-1.2  $\text{W m}^{-2}$ ). The UV-A and PAR intensities, however, were ca 25 % compared with ambient conditions.

A strain-specific difference in cell size was observed, as KAC71 had smaller cells than KAC11 and KAC7. The cells varied in height (KAC71 10  $\mu\text{m}$ , KAC7 and KAC11 17  $\mu\text{m}$ ), but not in width (around 4.3  $\mu\text{m}$  for all strains).

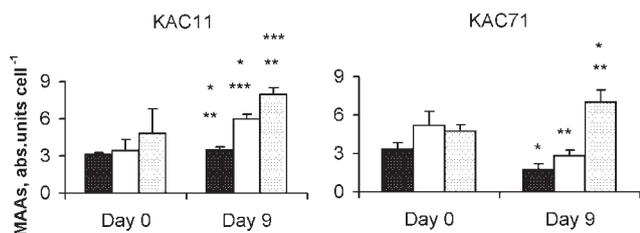
Apart from an initial negative growth rate ( $\mu \text{day}^{-1}$ ) in KAC11, the specific growth rate was positive for all treatments throughout the experimental period (Fig. 1). The specific growth rate was higher in KAC71 than in KAC11 and KAC7 in all treatments (Day 0 to Day 3, and Day 0 to Day 9). Treat-



**Figure 1.** Growth rate,  $\mu \text{d}^{-1}$ , between Day 0 and Day 1, Day 0 and Day 3, and between Day 0 and Day 9, in the 3 different strains of *Nodularia spumigena*. Vertical lines show standard errors,  $n = 4$ , and \* denotes statistical significant treatment differences,  $p < 0.05$ . Treatments are PAR+UV-A+UV-B (black bar), PAR+UV-A (white bar), and PAR (grey bar).



**Figure 2.** The ratio of variable to maximum fluorescence ( $F_v / F_m$ ) in the 3 different strains of *Nodularia spumigena*. Vertical lines show standard errors,  $n = 4$ , and \* denotes statistical significant treatment differences,  $p < 0.05$ . Treatments are PAR+UV-A+UV-B (black bar), PAR+UV-A (white bar), and PAR (grey bar).



**Figure 3.** Relative absorbance units cell<sup>-1</sup> of total mycosporine-like amino acids in the 3 different strains of *Nodularia spumigena*. KAC7 is excluded due to too low concentrations. Vertical lines show standard errors,  $n = 4$ , and \* denotes statistical significant treatment differences,  $p < 0.05$ . Treatments are PAR+UV-A+UV-B (black bar), PAR+UV-A (white bar), and PAR (grey bar).

ment effects were found for KAC7 (Day 0 to Day 9) where the  $\mu$  day<sup>-1</sup> was highest in the P treatment (P>PA>PAB). For KAC11 and KAC71, no treatment effects were observed at the end of the experimental period. The positive growth rate irrespective of treatment and strain suggests that *N. spumigena* is able to withstand high UV-B intensities.

We used the Fv/Fm ratio of the dark-acclimated cells as a marker of damage to the photosynthetic apparatus (PSII), because this ratio has been shown to be a sensitive, non-specific marker of UV-B exposure (Cordi *et al.* 1997). Initially, there were no differences between treatments, but a difference between strains was apparent. KAC7 initially showed Fv/Fm values around 0.25, KAC11 over 0.3 and KAC71 just below 0.4 (Fig. 2). All strains were stressed by the experimental PAR intensities alone, as reflected by a higher Fv/Fm in cells kept under low PAR light outside the experimental area (Fv/Fm 0.5, Day 9). During the experimental period the strains showed different Fv/Fm ratios due to treatments (Fig. 2). In general, however, all strains showed higher Fv/Fm in the P treatment. Although the Fv/Fm was lowered by UV radiation, the photosynthetic capacity was high enough to support a positive growth rate.

MAAs were analysed on Day 0 and Day 9. For PAB, significant treatment effects with higher MAAs per cell were observed for both KAC11 and KAC71 on Day 9 (PAB>PA and P) (Fig. 3). For KAC11, higher concentrations were also found in PA compared with the P treatment. KAC7 showed the same trend with higher concentrations in the PAB treatment but was excluded due to too low concentrations. Increased UV-B radiation has been suggested to alter the specific composition of algal and cyanobacterial communities and favour the best adapted species (e.g. Carreto *et al.* 1989). The algae having some kind of

protection are better adapted to survive high UV intensities and are able to outcompete algae without protection. The production of MAAs is one way for the surface-dwelling cyanobacteria to withstand high UV intensities but possibly at a cost reflected in lower overall fitness. Our results showed that exposure to UV-B is important in the induction/accumulation of MAAs in *N. spumigena*. UV-A treated cells showed a significantly higher amount of MAAs in KAC11, but not in KAC71, indicating that UVAR also could trigger the induction/accumulation of MAAs, but that there are differences between strains. It should be noted, however, that the UV-A intensities were low compared with ambient sea surface conditions.

### Acknowledgements

M. Appelgren is acknowledged for help with analyses. Financial support was provided by The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning; The Foundations Oscar and Lilli Lamm, Magnus Bergvall, Carl Trygger, Lars Hierta, Wilhelm and Martina Lundgren. The Royal Academy of Sciences.

### References

- Burkholder, J.A.M. & Glibert, P.M. (2004). Conference proceedings HAB meeting, Cape Town, p. 78.
- Carreto, J.I., De Marco, S.G. & Lutz, V.A. (1989). In: Red tides: Biology, Environmental Science and Toxicology, Okaichi, T., Nemoto, T. & Anderson, D.M. (eds), Elsevier, New York, pp. 333-336.
- Cordi, B., Depledge, M.H., Price, D.N., Salter, L.F. & Donkin, M.E. (1997). *Mar. Biol.* 130: 41-49.
- Finni, T., Kononen, K., Olsonen, R. & Wallstrom, K. (2001). *Ambio* 30: 172-178.
- Janson, S. & Granéli, E. (2002). *Int. J. Syst. Evol. Microbiol.* 52: 1397-1404.
- Karsten, U. & Garcia-Pichel, F. (1996). *System Appl. Microbiol.* 19: 285-294.
- Laamanen, M.J., Gugger, M.F., Lehtimäki, J.M., Haukka, K. & Sivonen, K. (2001). *Appl. Environ. Microbiol.* 67: 4638-4647.
- Paerl, H. (1988). *Limnol. Oceanogr.* 33: 823-847.
- Staal, M., Stal, L.J., te Lintel, H.S. & Harren, F.J.M. (2003). *J. Phycol.* 39: 668-677.
- Stal, L.J., Staal, M. & Villbrandt, M. (1999). *Aquat. Microb. Ecol.* 18: 165-173.
- Walz, H. (1999). 1. Edition: January 1999. Heinz Walz GmbH, 1999.

## Survival of *Heterocapsa circularisquama* (Dinophyceae) as a pellicle cyst induced by low temperature in the laboratory

Takashi Yoshida, Yuya Takahashi, Kanae Ishikawa, Wang Ming-Kei and Shingo Hiroishi  
 Laboratory of Marine Microbiology, Department of Marine Bioscience, Faculty of Biothchnology, Fukui Prefectural University, 1-1 Gakuen, Obama, Fukui 917-0003, Japan, yoshiten@fpu.ac.jp

### Abstract

Red tide blooms of *Heterocapsa circularisquama* Horiguchi cause mass mortality of bivalves and present a serious problem for shellfish aquaculture in western Japan. Sexual reproduction has not been found in *H. circularisquama*, and the mechanism of overwintering in the populations in Japanese waters is unclear. Dinoflagellate temporary cysts are non-motile and formed from vegetative cell by shedding of the theca (pellicle cyst). However, the pellicle cyst of *H. circularisquama* is probably a resting stage rather than a temporary stage. *H. circularisquama* grew well at  $\geq 17.5$  °C, but poorly at 15 °C and 12.5 °C, and the cells died at 10 °C. A percentage of pellicle cysts in the culture at 12.5 °C were significantly higher than at the other temperatures. Some of these pellicle cysts showed viability after 90 days. Our data suggest that the pellicle cysts of *H. circularisquama* are formed as a response to low temperature and can act as an overwintering population.

### Introduction

Red tide blooms of the marine dinoflagellate *Heterocapsa circularisquama* Horiguchi cause mass mortality of bivalves such as pearl oysters, short-necked clams and oysters, and present a serious problem for shellfish aquaculture in western Japan (Nagai *et al.* 1996; Matsuyama 1999; Yoshida *et al.* 2003).

The life cycle of *H. circularisquama* is poorly known (Iwataki *et al.* 2002). Some dinoflagellates are known to form dormant hypnocyts following sexual reproduction (Anderson 1980; Blackburn *et al.* 1989). However, such cysts have not been found in *H. circularisquama*, and the mechanism of overwintering of the populations in Japanese waters is unclear (Iwataki *et al.* 2002). Dinoflagellate temporary cysts are non-motile and formed from vegetative cells by shedding of the theca (pellicle cyst) (Olli 2004). Recently, temporary cysts have been considered to have ecological roles rather than sampling or culturing artifacts (Garcés *et al.* 2002; Olli 2004). In a bialgal experiment using *H. circularisquama* and *K. mikimotoi*, *H. circularisquama* cells transformed into pellicle cysts (Uchida *et al.* 1999). These cysts were considered to be temporary because the cells resume motility and growth within few days after incubation in fresh medium (Uchida *et al.* 1999).

We propose that pellicle cysts of *H. circularisquama* are transformed by exogenous factors only, and that the pellicle cyst is resting rather than a temporary stage. To test this hypothesis, we investigated pellicle cyst formation in *H. circularisquama* under various

temperatures, and their viability after storage at low temperature.

### Materials and Methods

#### Cultures

*H. circularisquama* OA1 was isolated from Obama Bay, Fukui prefecture, Japan in August 1998 (Yoshida *et al.* 2003). Cultures were maintained in modified SWM3 medium (Okaichi *et al.* 1982) at 20 °C under a 12L:12D cycle at 50  $\mu\text{mol}/\text{m}^2/\text{s}$  with cool white fluorescent bulbs.

#### Effect of temperature on pellicle cyst formation of *H. circularisquama*

Exponentially growing cells of *H. circularisquama* OA1 were inoculated into 300-mL flasks containing 150 mL of SWM3 medium at an initial cell density of  $10^3$  cells/mL, and incubated at 10-25 °C in steps of 2.5 °C. Samples were collected daily for three weeks and fixed by adding formaldehyde to a final concentration of 1%. To measure the cell concentration, fixed cells were observed under the G excitation with epifluorescence microscopy. Pellicle cysts were identified by optical microscopy as round cells that had shed the theca.

#### Viability of pellicle cyst

Pellicle cysts of densities of  $10^3$  cells/ml were inoculated into each of four flasks containing 200 mL of SWM3 medium and inoculated at 10 °C. Two of the flasks were shaded by aluminium foil. 10 mL aliquots were collected from each flask at 7, 14, 30, 60 and 90 days after incubation. The aliquots were serially

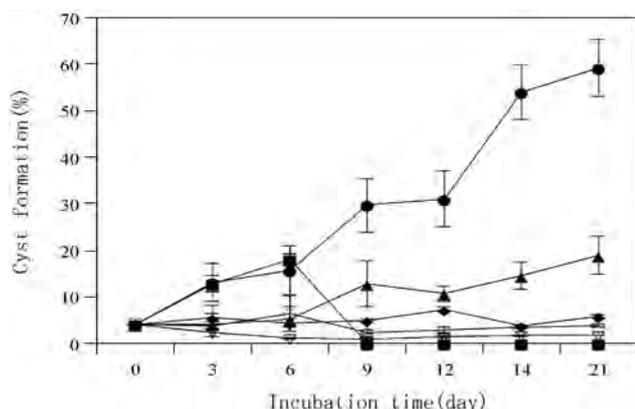
diluted to 100-fold and 1 mL of each diluted aliquot was added to five glass tubes, each containing 9 mL of fresh SWM3 medium. These tubes were incubated at 25 °C for 7-10 days, and cultures in which vegetative cells were observed by optical microscopy were determined as containing viable cells. The number of viable cells was determined by most probable number (MPN) methods (Imai *et al.* 1998).

## Results

### *Effect of temperature on pellicle cyst formation of H. circularisquama*

*H. circularisquama* grew exponentially at 22.5, 20 and 17.5 °C for 9 days but barely grew at 15 °C and 12.5 °C.

The concentration of pellicle cyst was very low (approximately 3 %) and constant when *H. circularisquama* was incubated at 22.5, 20 and 17.5 °C (Fig. 1). At 15 °C, the percentages increased after 3 weeks (19±4.1 %). At 12.5 °C, the percentage after 3 days of incubation was 12 %, and 57.5±1.4 % of the cells transformed into pellicle cyst after 3 weeks.



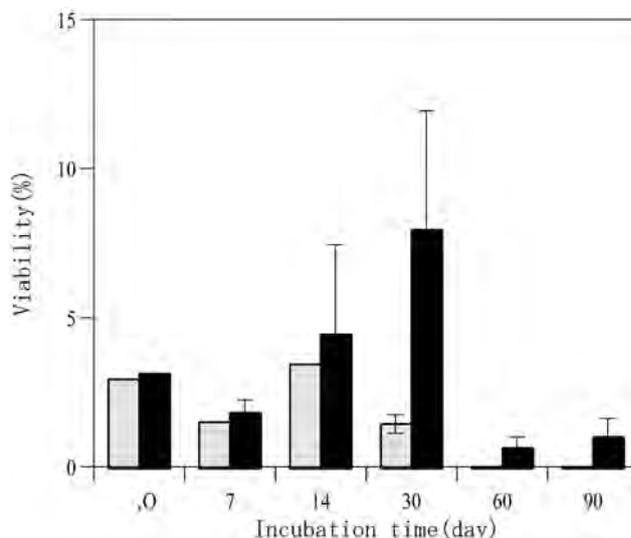
**Figure 1.** Effect of temperature on pellicle cyst formation of *H. circularisquama* OA1. Square, 10 °C; closed circle, 12.5 °C; triangle, 15 °C; diamond, 17.5 °C; open circle, 20 °C; open triangle, 22.5 °C.

### *Viability test of pellicle cyst formed at low temperature*

Pellicle cysts formed at 12.5 °C were transferred into 10 °C (*in situ* winter temperature of *H. circularisquama* habitat in Japan). The cysts were incubated under either a L/D cycle of 12:12 or continuous darkness for 3 months. At the beginning of the experiment, approximately 40 % of cells possessed a theca but all of cells transformed into pellicle cysts within 7 days at 10 °C.

The initial viability was not determined accurately by MPN due to contamination of immotile vegetative-like cells. Within 14 days, there were no

significant differences in the viability of the cells at the two conditions tested (Fig. 2). In contrast, after 30 days incubation, the viability in continuous darkness (10.3±2.4 %) was significantly higher than in the L/D cycle (1.43±0.1 %). After 60 days and 90 days of incubation, the viability percentage of the pellicle cyst exposed to continuous darkness was 0.9 % and 0.8 %, respectively. In contrast, no pellicle cysts incubated at the L/D cycle transformed into vegetative cells.



**Figure 2.** Viability of pellicle cysts of *H. circularisquama* OA1 at 10 °C in relation to incubation time. Pellicle cysts of *H. circularisquama* were incubated under a L/D cycle (light columns) or continuous darkness (dark columns).

## Discussion

Yamaguchi *et al.* investigated the effects of salinity (10-35 psu) and temperature (5-30 °C) on the growth of *H. circularisquama*, and found a high growth rate at relatively high temperature and high salinity (max =1.4 d<sup>-1</sup> at 30 °C and 30 psu) (Yamaguchi *et al.* 1997). Also, they showed that *H. circularisquama* can not grow below 10 °C, or at 15 °C when salinity was increased to 35 psu (Yamaguchi *et al.* 1997). Thus, growth characteristics obtained in our study agree with previous data.

The effect of algae or bacteria is reported to lead to transformation to pellicle cysts in *H. circularisquama* (Nagasaki *et al.* 2000). Our laboratory results indicate that low temperature (12.5 °C) also triggers cyst formation in this species. We did not establish whether cysts formed at low temperature are identical to those formed by other algae or by attack from bacteria. It has been reported that 1 % of a natural population may transform into pellicle cysts and sink out of the water column, although the triggering factors remained obscure (Olli 2004). Pellicle cysts are usually

considered to be temporary cysts that may contribute to bloom maintenance (Garcés *et al.* 2002).

Although viability is very low, the pellicle cysts have an ability to survive at low temperature under the darkness. In agreement with our data, Nagai and Imai (1999) reported that vegetative cells and resting cells of the diatom *Coscinodiscus wailesii* cultured with sediment can survive for 12 months in the laboratory at 10 °C and darkness (Nagai and Imai 1999). When *H. circularisquama* cells were subjected to DAPI staining after incubation at continuous darkness for 90 days, 6.5 % of the cells were seen to possess nuclei and pyrenoids. However DAPI-stained nuclei were not observed in any cells incubated at L/D conditions. Thus, one explanation for the lack of viability in the L/D cycle could be that cells are injured by the light.

At both conditions tested the number of pellicle cysts decreased as a function of incubation time at 10 °C, finally to approximately 50 cell/mL (from the initial 10<sup>3</sup> cells/ml). Unlike dormant hypnocysts, our data indicate that the pellicle cyst is not a dedicated resting stage. Nevertheless, at low temperature and darkness some of the cells survive for at least 90 days, which suffices for overwintering in the coastal waters of western Japan. Thus, although physiologically temporary, they can act ecologically as resting stages. Further studies are necessary to evaluate the apparent correlation of cyst formation with temperature and to search for viable pellicle cysts of *H. circularisquama* in the environment.

#### Acknowledgement

This study was supported by the Research Foundation of Fukui Prefecture for the Promotion of Science.

#### References

- Anderson, D.M. (1980). *J. Phycol.* 16: 166-172.
- Blackburn, S., Hallegraeff, G.M. & Bolch, C.J. (1989). *J. Phycol.* 25: 577-590.
- Garcés, E., Masó, M. & Camp, J. (2002). *J. Plankton Res.* 24: 681-686.
- Imai, I., Kim, M.C., Nagasaki, K., Itakura, S. & Ishida, Y. (1998). *Plankton. Biol. Ecol.* 45: 19-29.
- Iwataki, M., Wong, M.-W. & Fukuyo, Y. (2002). *Fish. Sci.* 68: 1161-1163.
- Matsuyama, Y. (1999). *Japan Agricult. Res. Quart.* 33: 283-293.
- Nagai, S. & Imai, I. (1999). *Plankton Biol. Ecol.* 46: 94-103.
- Nagai, K., Matsuyama, Y., Uchida, T., Yamaguchi, M., Ishimura, M., Nishimura, A., Akamatsu, S. & Honjo, T. (1996). *Aquaculture* 144: 149-154.
- Nagasaki, K., Yamaguchi, M. & Imai, I. (2000). *Bull. Jpn. Soc. Sci. Fish.* 66: 666-673.
- Okaichi, T., Nishino, S. & Imatomi, Y. (1982). In: *Toxic Phytoplankton Occurrence, Mode of Action and Toxins*, Jpn. Soc. Sci. Fish. (eds), Koseisha-Koseikaku, Tokyo, pp. 23-34.
- Olli, K. (2004). *Mar. Biol.* 145: 1-8.
- Uchida, T., Toda, S., Matsuyama, Y., Yamaguchi, M., Kotani, Y. & Honjo, T. (1999). *J. Exp. Mar. Biol. Ecol.* 241: 285-299.
- Yamaguchi, M., Itakura, S., Nagasaki, K., Matsuyama, Y., Uchida T. & Imai, I. (1997). *J. Plankt. Res.* 19: 1167-1174.
- Yoshida, T., Nakai, R., Seto, H., Wang, M-K., Iwataki, M. & Hiroishi, S. (2003). *Microbes Environ.* 18: 216-222.



### 3. MOLECULAR GENETICS



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Species discrimination in the genus *Alexandrium* by Amplified Fragment Length Polymorphism

T.J. Alpermann, B. Beszteri, U. Tillmann, A.D. Cembella and U. John  
 Alfred Wegener Institute, Am Handelshafen 12, D-27570 Bremerhaven, Germany  
 talpermann@meeresforschung.de

### Abstract

Amplified fragment length polymorphism (AFLP) is a molecular technique for genotypic characterization of individuals or clonal strains, genetic differentiation of natural populations and phylogenetic analyses. We applied the AFLP technique for species discrimination by phylogenetic analyses within *Alexandrium*, a marine dinoflagellate genus that contains several PSP toxin-producing species of uncertain taxonomic status. Clonal isolates from three *Alexandrium* spp. - *A. tamutum*, *A. tamarense* and *A. ostenfeldii* - were included in the phylogenetic analysis. Evolutionary genome divergence, expressed as the estimated ratio of nucleotide substitutions per site showed a clear phylogenetic separation of the three species, which is in concordance with previous sequence data analysis. In addition, AFLP showed a separation of several distinct groups within *A. tamutum* and *A. tamarense* and thereby allows a deeper insight into the genetic substructure within these species. Analysis of genotypic variability among clonal isolates by AFLP is a promising tool for investigating correlations between genotypic and phenotypic population markers as well as the genetic causes of phenotypic diversity in biogeographical studies of natural populations.

### Introduction

The dinoflagellate genus *Alexandrium* forms recurrent blooms in coastal regions around the world, and is the main organism causing paralytic shellfish poisoning (PSP). The genus includes more than 28 described species (Balech 1995), of which nine are known to produce the potent neurotoxin saxitoxin and/or some of the >20 naturally occurring derivatives. The taxonomic status of some species within the genus is under continuing debate (Fraga *et al.* 2006).

In addition to classical morphological characteristics, such as thecal plate morphology and pattern, analysis of molecular sequence data has been useful in elucidating phylogenetic relationships among *Alexandrium* spp. (Scholin *et al.* 1994; Montresor *et al.* 2004). However, in some instances, such as in the '*Alexandrium tamarense* species complex', apparently distinct morphospecies (e.g. *A. tamarense*, *A. catenella* and *A. fundyense*) cannot be separated by phylogenetic analysis of molecular sequence data, but rather they fall into ribotypes of different geographical origin, each containing one or several distinct morphospecies (Scholin *et al.* 1994).

To resolve phylogenetic relationships with a fine resolution on the specific or sub-specific level, molecular markers other than the slowly evolving nuclear genes are needed to provide additional support for taxonomic separation of closely related taxa. The amplified fragment length polymorphism (AFLP)

technique has been applied to several groups of organisms at the population level, as well as to apparently closely related species in inter-specific studies. This technique produces a suite of multiple markers by restriction and selective amplification of subsets of genomic DNA restriction fragments, and thereby allows for characterization of individual isolates by their genotypic signature (Vos *et al.* 1995). Among the lower eukaryotes, AFLP has been used for discrimination of isolates at the inter- and intra-specific level (e.g., Mueller and Wolfenbarger 1999 and references therein). While AFLP can distinguish among different geographical populations of *A. tamarense* (John *et al.* 2004; Alpermann *et al.* 2006), its discriminating power at the inter-specific level has not been previously tested for dinoflagellates.

To test the applicability of AFLP in phylogenetic studies, we adopted a mathematical method that estimates the rate of nucleotide substitutions over whole genomes. The underlying assumption is that all AFLP fragments correspond to specific nucleotide sequences of the genome of a common ancestor, namely, the two restriction sites plus the selective bases of the selective primers for PCR amplification of AFLP fragments.

In addition, we investigated the potential of AFLP to describe genetic sub-structuring of populations of different *Alexandrium* spp. by a comparative analysis of AFLP patterns of isolates of *A. tamarense* and *A. tamutum*.

## Methods

Twenty-three clonal cultures of dinoflagellates exhibiting an *Alexandrium*-like habitus under a stereomicroscope were established by single cell micro-pipette isolation from a natural water sample, obtained from coastal waters of the Scottish east coast. DNA was extracted from exponentially growing cultures ( $4\text{--}8 \times 10^4$  cells  $\text{ml}^{-1}$ ) that were treated with antibiotics ( $100 \mu\text{g ml}^{-1}$  penicillin;  $25 \mu\text{g ml}^{-1}$  streptomycin; both final concentrations) to minimize bacterial contamination. In brief, 240 ng of DNA was digested in 50  $\mu\text{l}$  with the restriction endonucleases EcoRI and MseI. Specific adaptors were ligated to one end of each restriction site. Primers for both the MseI and the EcoRI restriction site, containing the adaptor and restriction site motif and one additional selective nucleotide, were used in the pre-amplification PCR to amplify a subset of the ligated DNA fragments containing both restriction sites. In four separate PCR amplification reactions with different combinations of one MseI and one EcoRI primer, which contained two more selective nucleotides, a smaller subset of pre-amplified fragments was amplified. The AFLP alleles were identified after capillary electrophoresis on an ABI 3170 XL automated sequencer with the GeneMapper® v3.7 software (Applied Biosystems, Darmstadt, Germany). Binning of AFLP markers was performed in a size range from 100 bp to 500 bp at a threshold level of 250 for all AFLP primer combinations and subsequent automatic scoring of AFLP fragments was carried out with a threshold of 30.

Evolutionary genome divergence, expressed as the number of nucleotide substitutions per site was calculated using the Dice similarity index with the programme DistAFLP after Mougél *et al.* (2002), where the evolutionary distance between two individuals is corrected to account for unobserved substitutions according to the standard Jukes-Cantor model (Swofford *et al.* 1996). A phylogenetic tree was constructed with PHYLIP (Felsenstein 1993) by the neighbour-joining method (Felsenstein 1985); 1000 bootstrapped replicates were run to confirm tree topology.

Sequencing of the D1/D2 region of the LSU rDNA was performed after PCR amplification with primers DIRF and D2CR (Scholin *et al.* 1994) and subsequent clean-up of PCR products using the forward primer DIR-F in the sequencing reaction. Sequences were compared to the NCBI database by BLAST search to confirm membership of our isolates to one of the species *A. tamutum*, *A. ostenfeldii* or the *A. tamarensis* North American ribotype.

## Results and Discussion

According to their partial rDNA sequences, one of the clonal *Alexandrium* isolates was identified as *A. ostenfeldii*, ten as *A. tamarensis* and twelve as *A. tamutum*. The isolates of all *Alexandrium* spp. gave similar numbers of AFLP fragments - an average of about 25 fragments per clonal isolate per combination of selective primers. Interestingly, the isolates of *A. tamutum* produced a considerably higher number of AFLP loci, of which a greater proportion of loci were unique for the species when compared to *A. tamarensis* (Table 1). This was also expressed in a higher averaged value for the evolutionary genome divergence among isolates of *A. tamutum*, when compared to that among *A. tamarensis* isolates. The average estimated rate of nucleotide substitutions per site (0.089) between isolates of *A. tamutum* and *A. tamarensis* indicates that considerable nucleotide substitution must have occurred in the evolution of the two species. However, since AFLP is a molecular marker that produces alleles irrespective if the DNA region is a coding region or not, these nucleotide substitutions do not necessarily reflect the degree of functional or physiological divergence of the two species from their common ancestor.

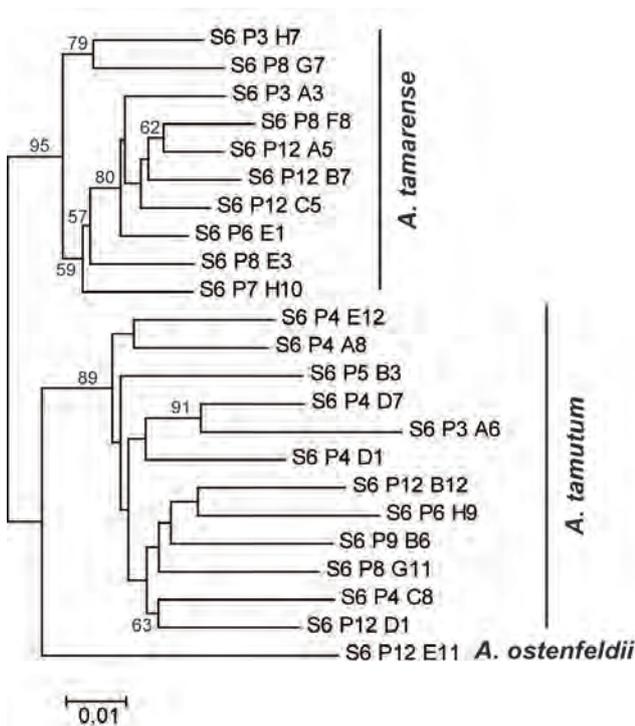
Phylogenetic analysis of AFLP genotypes revealed not only the existence of separated branches for the three species, but also showed that separated groups of genotypes supported by high bootstrap values exist within *A. tamutum* and *A. tamarensis* (Fig. 1). The higher genetic variability within *A. tamutum* is reflected in the comparatively deeper branching within the *A. tamutum* isolates included in this study. An evolutionary explanation for this observation is not readily at hand. The general conclusion that populations of *A. tamutum* are genetically more diverse, or that separated groups of genotypes would persist if more isolates were included in the study, cannot be drawn with certainty. The number of isolates in this study is too small to represent the overall genetic diversity within natural populations of these two species. Additionally, not much is known about the variability and stability of the genotypic composition of natural populations of *Alexandrium* species during the different phases of bloom development and decline. Different physical and biotic factors may lead to the promotion of certain genotypes, which might be expressed in shifts of the genotypic composition of a population over a relatively short time. Whether or not neutral genetic markers such as AFLP can reflect such processes at all is crucial for these considerations.

In this study, we showed the usefulness of AFLP for species discrimination within the genus *Alexan-*

**Table 1.** Comparative characteristics of AFLP data for *A. tamutum* (12 isolates) and *A. tamarensis* (10 isolates) generated with 4 different pairs of PCR primers and a total of 481 alleles produced for isolates from these two species.

	<i>A. tamutum</i>	<i>A. tamarensis</i>
# of loci*	417	300
# of unique loci*	181	64
Average # of loci*	104 (S.D. 23.0)	116 (S.D. 22.1)
Average $\hat{f}$ <i>A. tamutum</i>	0.063	0.089
Average $\hat{f}$ <i>A. tamarensis</i>	0.089	0.041

\*refers only to those loci for which AFLP fragments were scored in any isolate of the respective species.



**Figure 1.** Phylogenetic relationships of North Sea isolates of *Alexandrium* spp. inferred from evolutionary genome divergences determined by AFLP analysis. The dendrogram was generated by the neighbour-joining method with the estimated ratio of nucleotide substitution over whole genomes. Branching robustness is expressed as the percentage reliability after 1000 bootstrap resamplings corrected for fragment dependences (only bootstrap values above 55 % are indicated). The bar indicates the rate of base substitutions.

*drium*. Previous work using the same protocol, e.g. John *et al.* (2004), has shown the high reproducibility of AFLP band patterns in *Alexandrium*; we did not therefore specifically test this property in the inter-specific comparison. Each of the multilocus genotypes of *A. tamutum* and *A. tamarensis* isolates represents a biological replicate in our analysis. Furthermore, the large number of markers generated with AFLP substantially advances the robustness of analyses based upon individual multilocus genotypes (Hollingsworth and Ennos 2004). At a time when the taxonomic and evolutionary significance of morphological characters for species discrimination among the lower eukaryotes must be reconciled with often discordant taxonomic and phylogenetic inferences based on molecular markers of single genes (e.g., LSU and SSU rDNA, ITS, etc.), AFLP provides additional support for molecular phylogenies. In prokaryotes, the potential of AFLP to replace the obligate genetic marker for species definition (DNA-DNA hybridization) has already been shown (Mougel *et al.* 2002). For eukaryotic microalgae with unstable or indistinguishable morphologies, AFLP may prove to be a valid phylogenetic criterion, as well. Further applications of AFLP, e.g., for quantitative trait mapping or candidate gene identification with cDNA-AFLP, also seem to be auspicious for functional diversity studies.

## Conclusions

This is the first published work showing the utility of AFLP for discrimination of members of the genus *Alexandrium*. Although the genetic differentiation of the three species was already shown by comparison of the LSU rDNA sequences, the novelty of the AFLP approach is supported by the possibility of gaining deeper insight into the inter-specific genetic differentiation at the species and population level at the same time. The universal application of AFLP to genetic diversity and species differentiation in dinoflagellates opens new avenues to study the evolution and functional ecology of marine microalgae.

## Acknowledgements

Thanks to N. Jaeckisch for maintaining algal cultures, and to A. Luedeking for valuable discussions.

## References

- Alpermann, T.J., John, U., Tillmann, U., Evans, K.M., Nagai, S., Anderson, D.M. & Cembella,

- A.D. (2006). In: Abstracts, 12th International Conference on Harmful Algae, Copenhagen, Denmark, 4-8 September 2006, pp. 53-54.
- Balech, E. (1995). Sherkin Island Marine Station Publication, Sherkin Island, Co, Cork, Ireland.
- Felsenstein, J. (1985). *Evolution* 39: 783-791.
- Felsenstein, J. (1993). PHYLIP: Phylogenetic Inference Package, version 3.5c. U. Washington, Seattle, Wash.
- Fraga, S., Figueroa, R. I., Bravo, I., Sampedro, N., Franco, J. M., Penna, A., Ramilo, I. & Fernández-Villamarín, A. (2006). In: Abstracts, 12th International Conference on Harmful Algae, Copenhagen, Denmark, 4-8 September 2006, p. 64.
- Hollingsworth, P.M. & Ennos, R.A. (2004). *Heredity* 92: 490-498.
- John, U., Groben, R., Beszteri, B. & Medlin, L. (2004). *Protist* 155: 169-179.
- Montresor, M., John, U., Beran, A. & Medlin, L.K. (2004). *J. Phycol.* 40: 398-411.
- Mougel, C., Thioulouse, J., Perrière, G. & Nesme, X. (2002). *Int. J. Syst. Evol. Microbiol.* 52: 573-586.
- Mueller, U.G. & Wolfenbarger, L.L. (1999). *Trends Ecol. Evol.* 14: 389-394.
- Scholin, C. Herzog, A.M., Sogin, M. & Anderson, D.M. (1994). *J. Phycol.* 30: 999-1011.
- Swofford, D.L., Olsen, G.J., Waddell, P. J. & Hillis, D.M. (1996). In: *Molecular Systematics*, D.M. Hillis, C. Moritz & B.K. Mable (eds), Sinauer Sunderland, Mass., pp. 407-514.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandele, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995). *Nucleic Acids Res.* 23: 4407-4414.

## Phylogeography of Atlantic Coast *Karlodinium veneficum* strains: a genetic marker correlate of toxin type

Tsvetan R. Bachvaroff, Jason E. Adolf and Allen R. Place  
 UMBI Center of Marine Biotechnology, 701 E. Pratt St., Baltimore, MD 21202;  
 place@umbi.umd.edu

### Abstract

*Karlodinium veneficum* is a potentially ichthyotoxic bloom-forming dinoflagellate in estuarine systems throughout the world. A culture collection from the U. S. Atlantic coast (25 unialgal strains) was used to identify genetic correlates of karlotoxin types in *K. veneficum*. Differences in genotype were determined using DNA sequences isolated from a library of enriched repeat sequences. One marker, named F4, yields a 485-bp fragment for U.S. Chesapeake Bay strains producing karlotoxin 1, while the same locus yields a 283-bp fragment for the U.S. strains producing karlotoxin 2. This marker is a promising tool for application to field samples and archived DNA samples.

### Introduction

Along the United States Atlantic Coast *Karlodinium veneficum*-associated fish kills have been reported from South Carolina (Kempton *et al.* 2002) and Chesapeake Bay (Goshorn *et al.* 2004). The toxins associated with *K. veneficum* are easily released and exhibit broad-spectrum lytic activities on a diverse array of cell types (Deeds *et al.* 2002). The lytic activity appears to be based on sterol-mediated interactions with cellular membranes such that the dinoflagellate itself is protected by its membranes containing 4-methyl sterols and lacking des-methyl sterols (Deeds and Place 2006). The toxins function both to deter grazing on *Karlodinium* (Adolf *et al.* 2006) and to enhance feeding by *Karlodinium* (Adolf *et al.* this volume); however they have the additional impact of killing fish in blooms of high density ( $10^4$  -  $10^5$  cells / mL).

Previously a distinct geographic break was shown between karlotoxins from field samples or cultures from south of the Chesapeake and from north of the Chesapeake Bay outlet (Deeds *et al.* 2004). The toxin found south of the Chesapeake, karlotoxin 2 (KmTX 2), has an UV absorption maximum at 235 nm whereas the toxins found within the Chesapeake have UV absorption maxima at 225 nm (KmTX 1). Both groups are lytic to rainbow trout erythrocytes and have fish killing activity (Deeds *et al.* 2006).

The toxin type was characterized from a total of 25 strains derived from the Atlantic Seaboard of the United States, with 9 strains from south of the Chesapeake including examples from every state; the remaining 16 strains were from the Chesapeake or Coastal Bays of Maryland and Delaware (Fig. 1). Toxin type was determined using HPLC with a haemolytic activity assay from cultured material grown under common

laboratory conditions in f/2 media at a salinity of 15. Genetic markers derived from a microsatellite repeat-enriched library were used to correlate toxin type with genetic differences.

### Methods

#### Sources of strains

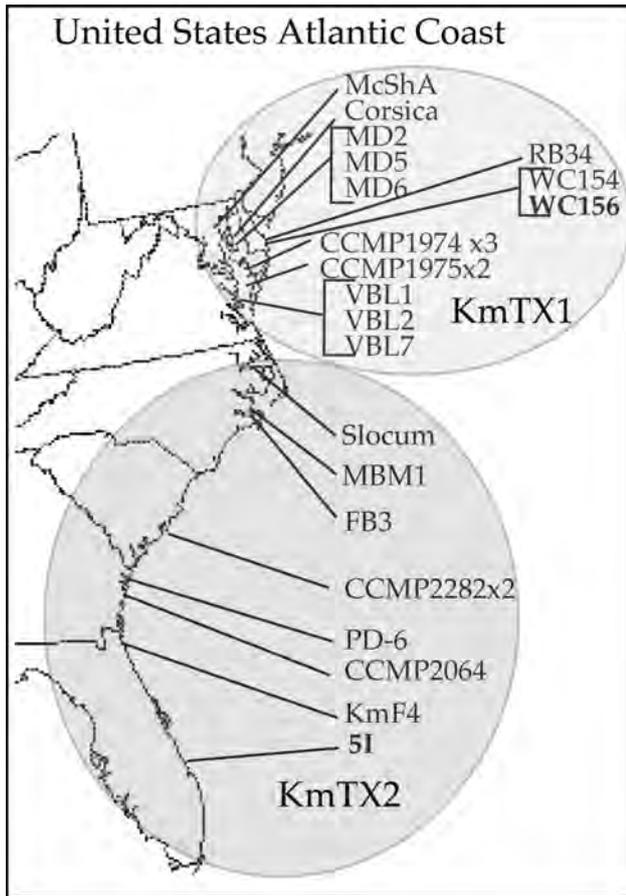
Cultures were acquired from the Center for Culture of Marine Phytoplankton (CCMP), Steven Kibler of NOAA at Charleston S.C., Gabriella Smalley of Ryder University, Vince Lovko of Virginia Biological Labs, Matt Johnson of Horn Point Labs, or isolated by the authors. Additional replicates of CCMP1974, 1975 and 2282 were acquired from Diane Stocker of Horn Point Labs and from Wayne Coats of Smithsonian Estuarine Research Center. Cultures were grown at 100  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  and 20 °C, under a 14:10 L:D cycle. The medium was from 1- $\mu\text{m}$  filtered natural seawater diluted to a salinity of 15 and supplemented with f/2 nutrients (Andersen *et al.* 1997).

#### Toxin analysis

Cultures were harvested in stationary phase and filtered across a GF/F filter prior to toxin purification and analysis by HPLC and activity assay (Deeds *et al.* 2002; Kempton *et al.* 2002).

#### Marker cloning

A repeat-enriched library was prepared (Zane *et al.* 2002) using CCMP2282 total DNA as the starting material. Primers were prepared for clones with priming sites flanking repeats, and PCR was used to screen six to eight representative strains for amplification. If products were successfully amplified from a subset of strains, sequencing was used to determine differences between amplicons. All PCR reactions were



**Figure 1.** Map of the United States Atlantic Coast with state boundaries showing the sources of the cultures used in this survey. The cultures from north of the Chesapeake mouth all contained KmTX1 and those from south of the Chesapeake all contained KmTX2. The two strains in bold lettering, WC156 and 5I, did not yield a PCR product with any F4 primer combination.

performed using 100 ng genomic DNA based on 260-nm absorption and used in a 20- $\mu$ L PCR reaction in the following buffer: 3 mM MgCl<sub>2</sub>, 500 mM Tris HCl pH 8.3, 500  $\mu$ g/mL BSA, 2mM dNTPs (final concentrations) with 4 pmol of each primer for 35 cycles and 15 s denaturation at 94 °C, and 50-65 °C annealing for 15 s, followed by 30 s extension at 72 °C. Sequencing on an ABI 3130xl followed the manufacturer's protocol.

## Results

All strains from north of the Chesapeake Bay outlet contained KmTX1 type toxins with absorbance maxima at 225 nm (Fig. 1). Strains from south of the Chesapeake all contained KmTX2 based on retention time and an absorbance maximum of 235 nm.

Of 48 repeat-containing clones, 11 were rejected because of unsuccessful amplification, and 24 were re-

jected because the resulting products had nearly identical sequence. Interestingly, the ITS region was also identical between these strains. Thirteen primer pairs produced PCR products variable at the DNA sequence level, and one pair produced a PCR product, which segregated based both on size and sequence according to culture source (Fig. 2). This product contained 12 base repeat units, which could be multiplied into larger units of 24 or 36 bases (Fig. 3) using repeat finder (Benson 1999). Based on these sequences a second set of primers was designed to amplify specifically one or the other amplicon (Table 1). This redesign allowed for a slightly longer forward primer where the sequence is particularly AT rich. For KmTX2 strains

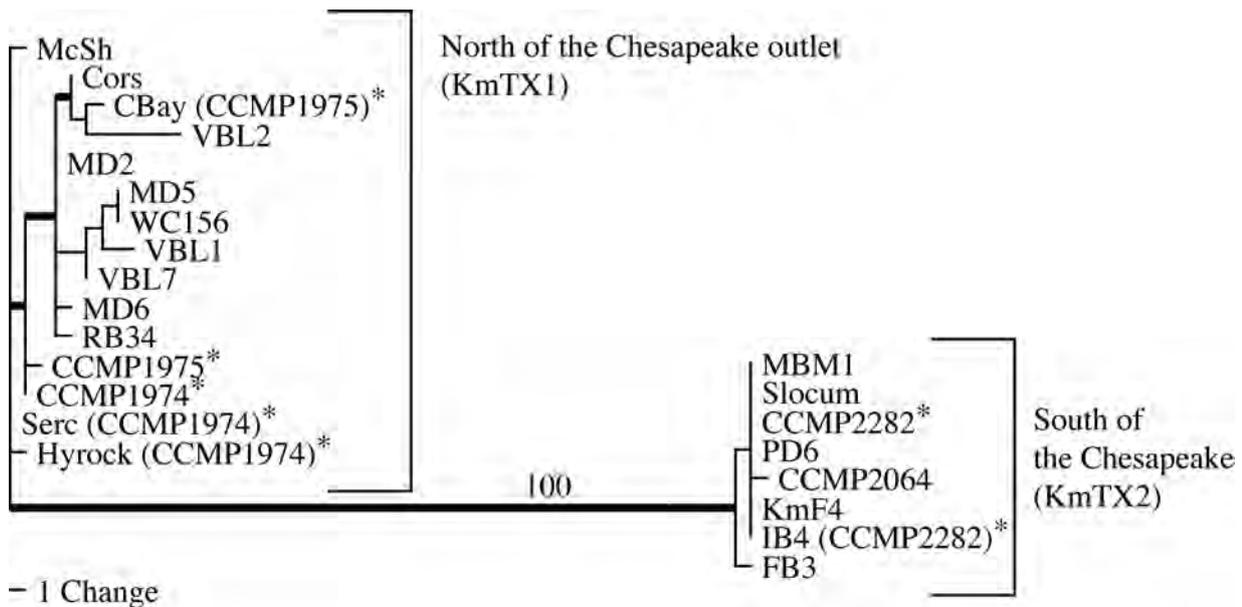
**Table 1.** Primers Used

Name	Sequence
Kmf4_077F	TTTGGGATAAAAATTGCTTACATA
Kmf4_077R	CTGGAGCTGCAACACGTC
KmF4Tx1F	TTTGGGATAAAAATTGCTTACATAGA
KmF4Tx2F	TTTGGGATAAAAATTGCTTACATAGT
KmF4Tx1R	CTGCAACACGTCCCAGGT

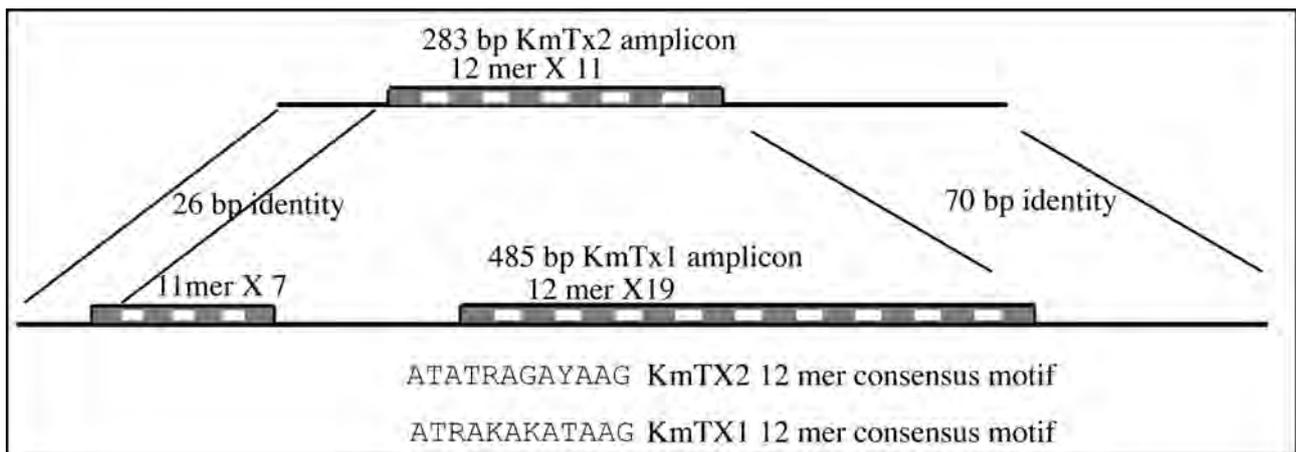
the reverse primer remained the same. A number of single nucleotide polymorphisms were also found between the different strains (GenBank accession #s EF193786-EF193808).

## Discussion

The differences between KmTX 1 and KmTX 2-containing *K. veneficum* strains, confirmed here with the discovery of a genetic marker distinguishing KmTX1 and 2 toxin phenotypes, suggests a genetic basis for the production of different toxins. Our results indicate little or no gene flow between the *K. veneficum* populations within the Chesapeake Bay and south of the bay. *K. veneficum* along the U.S. Atlantic Coast thus forms a metapopulation with at least two subpopulations, with a boundary between the Chesapeake Bay outlet and Cape Hatteras. The boundary could be more clearly marked by determining the sequence for the F4 marker from field samples in this region. This marker was not amplified from several strains, suggesting that it is broadly applicable to United States Southern Atlantic *K. veneficum* populations. Thus, while significant single-nucleotide polymorphism differences exist between individual isolates, the primer sites are sufficiently conserved to allow amplification. However, no products were found with these prim-



**Figure 2.** A parsimony tree (one of 56 equal length trees) based on the F4 marker sequence for the strains discussed in the text. Thicker branches were found in >70 % of the consensus trees, and the single branch with bootstrap support is indicated. Strains marked with an asterisk (\*), were derived from the same parent clone (marked with parentheses), but were maintained in different laboratories for several years.



**Figure 3.** A schematic diagram of the F4 marker in KmTX 1 and KmTX2-producing strains indicating the repeat motif differences between the two PCR amplicons (to scale). The differences between the KmTX1 and KmTX2 repeats are due to different patterns of degeneracy.

ers when used on strains from outside of the United States (data not shown).

Another intriguing result was obtained when several cultures grown from the same initial clonal isolate were compared. For the CCMP1974 and 1975 cultures maintained at the CCMP facility and other labs, changes at the sequence level for the F4 marker were found (Fig. 3). However, within the KmTX 2 group, two cultures derived from CCMP2282 had exactly the same sequence, implying that DNA sequence change in this locus occurs at different rates in these strains.

### Acknowledgements

The authors thank G. Smalley, V. Lovko, M. Johnson, W. Coats, D. Stocker, and S. Kibler for cultures; E. Whereat and M. Farestad (Delaware Citizen's monitoring group), E. Humphries (Delaware Department of Natural Resources) and P. Tango (Maryland Department of Natural Resources) provided field samples for culture isolation. D. Krupatkina and H. Nonagaki provided technical assistance. This work was funded in part by grants from National Oceanic and Atmospheric Administration Coastal Ocean Program under

ECOHAB award #NA04NOS4780276 to University of Maryland Biotechnology Institute, and Grant # U50/CCU 323376, Centers for Disease Control and Prevention and the Maryland Department of Health and Mental Hygiene. This is contribution # 06-155211 from the UMBI Center of Marine Biotechnology, and XXXXX from the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) programme.

### References

- Adolf, J.E., Bachvaroff, T.R., Krupatkina, D.N., Nonagaki, H., Brown, P.J.P., Lewitus, A.J., Harvey, H. R. & Place, A. R. (2006). *Afr. J. Mar. Sci., Harmful Algae* 2004, 28: 415-421.
- Andersen, R. A., Morton, S.L. & Sexton, J.P. (1997). *J. Phycol.*33 (Suppl.): 1-75.
- Benson, G. (1999). *Nucleic Acids Res.* 27: 573-580.
- Deeds, D.E., Terlizzi, J.R., Adolf, J.E., Stoecker, D. & Place, A.R. (2002). *Harmful Algae* 1: 169-189.
- Deeds, J.R., Kibler, S.R., Tester, P.A. & Place, A.R. (2004). In: *Harmful Algae 2002*, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A., (eds), Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, pp. 145-147.
- Deeds, J.R. & Place, A.R. (2006). *Afr. J. Mar. Sci., Harmful Algae* 2004, 28: 421-427.
- Deeds, J.R., Reimschuessel, R. & Place, A.R. (2006). *J. Aquat. Anim. Health* 18: 136-148.
- Goshorn, D., Deeds, J.R., Tango, P., Poukish, C., Place, A.R., McGinty, M., Butler, W. Lucket, C. & Magnien, R. (2004). In: *Harmful Algae 2002*, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. and Vargo, G.A., (eds), Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, pp. 361-363.
- Kempton, J.W., Lewitus, A.J., Deeds, J.R., Law, J.M. & Place, A.R. (2002). *Harmful Algae* 1: 233-241.
- Zane, L., Bargelloni, L. & Patarnello, T. (2002). *Mol. Ecol.* 11: 1-16.

## Genetic characteristics of non-toxic subclones obtained from toxic clonal culture of *Alexandrium tamarense* (Dinophyceae)

Cho Y.<sup>1</sup>, Hiramatsu, K.<sup>1</sup>, Ogawa, M.<sup>1</sup>, Omura, T.<sup>2</sup>, Ishimaru, T.<sup>3</sup> and Oshima, Y.<sup>1</sup>

<sup>1</sup>Laboratory of Bioorganic Chemistry, Graduate School of Life Sciences, Tohoku University, Sendai 981-8555, Japan, oshimay@mail.tains.tohoku.ac.jp, <sup>2</sup>University of Tokyo, Asian Natural Environmental Science Center, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8675 Japan, <sup>3</sup>Tokyo University of Marine Science and Technology, 4-5-7, Konan, Minato-ku, Tokyo, 108-8477, Japan

### Abstract

A non-toxic subclone obtained from a toxic clonal culture was confirmed to be the common *Alexandrium tamarense* found in Japan by analysis of 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2). The parent culture, OF935-AT6 was suggested to be heterogeneous in terms of toxicity, although the OF935-AT6 was established from a single cell from a natural population. The most significant difference between the non-toxic and a toxic subclone identified by subtractive hybridization of cDNA, pertains to gene fragments homologous with mitochondrial cytochrome c oxidase polypeptide three and cytochrome b.

### Introduction

Genetic diversity among *Alexandrium* species from distinct areas can be very large even within the same species (Taroncher-Ordenburg 2000). A non-toxic and a toxic subclone, both derived from a toxic clonal strain of *Alexandrium tamarense*, may be expected to have identical genetic profile and are excellent materials for the search of genes active in toxin synthesis. In 1997 Omura *et al.* (2003) performed a series of mutation experiments using three toxic strains of *Alexandrium* species (*A. tamarense*, *A. catenella* and *A. minutum*). Non-toxic clones were obtained from only *A. tamarense*. Furthermore, the original strain of *A. tamarense*, OF-935-AT6 proved co-existence of non-toxic mutational cells after five years of culture. In this study, we aimed to find more evidence that the non-toxic mutational event could occur in a short period of time and to show that the non-toxic cells are not contamination of other non-toxic species similar to *A. tamarense*. Further to present the major difference between the genes of the toxic and non-toxic subclones obtained from the toxic clonal culture of *A. tamarense*, OF935-AT6 strain. Since the previous report was described in Japanese, some results are summarized here.

### Materials and Methods

#### Origin of material

All subclones used in this study were derived from strain OF935-AT6, which was established from a single cell isolated from natural sea water at Ofunato Bay, Japan in 1993 by Dr Ogata of Kitasato University.

#### Culture conditions

All subclones were maintained at a 12:12h L:D photocycle with light provided by cool white bulbs (100-150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 15 or 17 °C in modified T1 medium (Ogata *et al.* 1987; Omura *et al.* 2003) without nitrilotriacetic acid and with two-fold EDTA and 20 nM of selenic acid.

#### Preparation of subclones

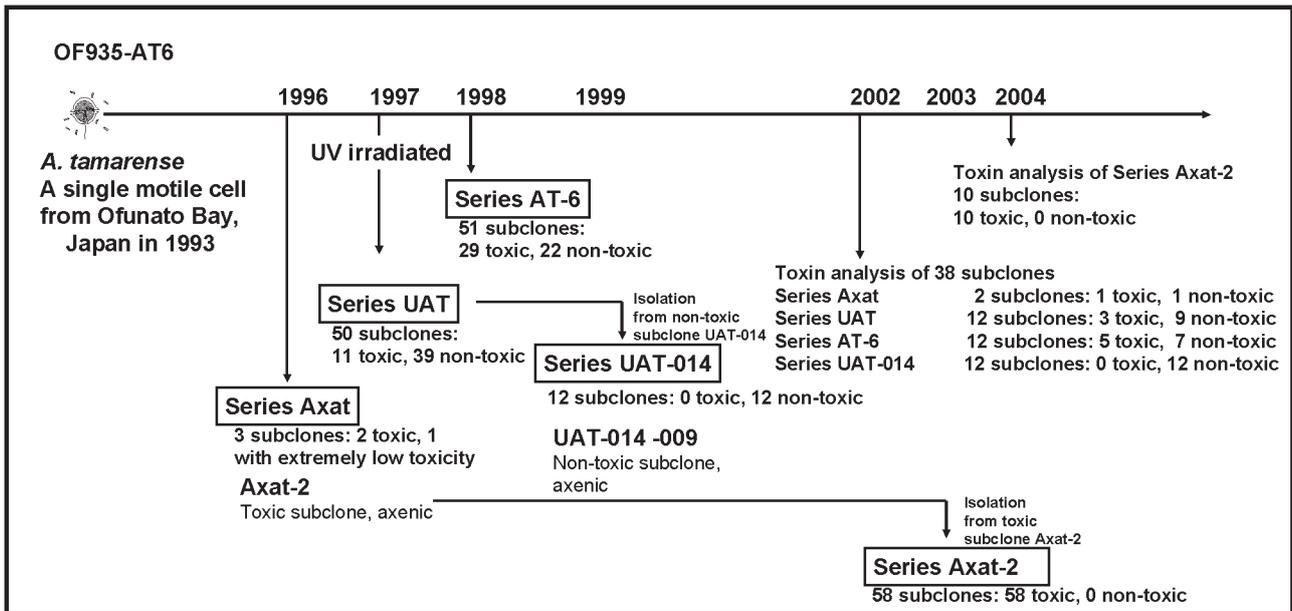
Single cells isolated from strain OF935-AT6 using a capillary pipette were inoculated into 1 ml of modified T1 medium in 24- or 48-well plates. After an adequate cell density was achieved, the cells were transferred to test tubes or culture flasks. Mutation experiments were carried out with strain OF935-AT6. One millilitre of culture was exposed to UV light (865  $\mu\text{W}\cdot\text{cm}^{-2}$ ) for 2 min using a UV germicidal lamp. Surviving cells were isolated and inoculated as described above.

#### Toxin analysis

HPLC-FL for all subclones was carried out according to the Oshima method (Oshima 1995). A mouse bioassay for subclone UAT-014-009 was performed according to the AOAC method (AOAC 1995). The same extracts utilized for HPLC analysis were injected in the mouse after appropriate dilution.

#### Ribosomal RNA genes

Total genomic DNA of toxic (Axat-2) and non-toxic (UAT-014-009) subclones were used to amplify the 5.8S ribosomal RNA gene (rDNA), as well as the flanking internal transcribed spacers 1 and 2 (ITS1-ITS2 regions) with primers ITSa and ITSb (Adachi *et al.* 1994; Usup *et al.* 2002). DNA sequences (519 bp)



**Figure 1.** Origin and process of subcloning.

were aligned with the multiple-sequence-alignment tool Clustal W. The phylogenetic tree is shown in Fig. 2.

#### *Subtractive hybridization*

Two axenic subclones (toxic Axat-2 and non-toxic UAT-014-009) were used for subtractive hybridization of cDNA. Double-stranded cDNA (ds cDNA) was synthesized and amplified using a SMART PCR cDNA Synthesis kitR (BDBiosciences Clontech, Tokyo, Japan). Pools of subtracted cDNA libraries were created using a PCR-Select cDNA Subtraction Kit (BDBiosciences Clontech, Tokyo, Japan), according to the protocol of the manufacturer.

### **Results and Discussion**

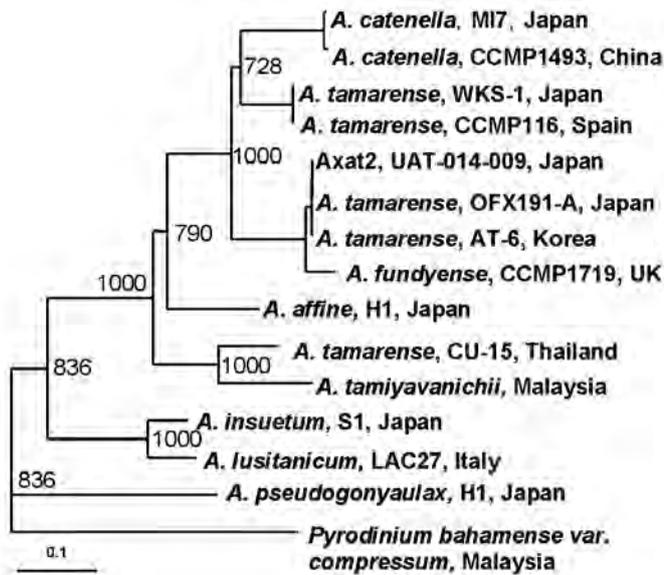
#### *Presence of non-toxic subclones in a series of re-isolates from OF935-AT6 strain*

The origin and the process of subcloning are summarized in Fig. 1. Series-Axat was prepared from OF935-AT6 in 1996 to obtain an axenic strain. The three subclones were all toxic, including one with extremely low toxicity. Series UAT was the subclones derived from mutation experiments carried out in 1997 on OF935-AT6 strain. Since non-toxic mutants were obtained at a greater frequency (39 out of 50 subclones) than expected, the presence of non-toxic cells in the parent culture was suspected. Series AT-6 was established from OF935-AT6 strain without UV irradiation in 1998. Fifty one subclones were analysed. Twenty nine subclones were toxic and twenty two subclones were non-toxic; thus, it confirmed the presence of non-

toxic cells in the original culture. Series UAT-014 was established from the non-toxic subclone UAT-014 in 1999 to provide an axenic non-toxic clone by pipette washing. All twelve subclones were non-toxic.

In 2002, toxin analysis was performed of all subclones from OF935-AT6 which had been maintained for six to three years. Totally, thirty eight subclones (two subclones in Series Axat, twelve subclones in Series UAT, twelve subclones in Series AT-6 and twelve subclones in Series UAT-014) survived until 2002. One subclone in Series Axat with extremely low toxicity in 1996 was found to be non-toxic. In Series UAT, three subclones were toxic and nine subclones were non-toxic. In Series AT-6, five subclones were toxic and seven subclones were non-toxic. Two previous toxic subclones in this series were found to be non-toxic. In Series UAT-014, all twelve subclones were non-toxic as in 1999. It is significant that three out of nine toxic subclones of OF935-AT6 isolates not exposed to UV irradiation (Series-Axat and AT6) lost their toxicity over a relatively short time period.

In 2003, series Axat-2 was prepared from the toxic subclone Axat-2 to investigate whether the mixture of toxic and non-toxic subclones of OF935-AT6 was due to short term mutations. Fifty eight subclones were analysed for toxicity one month after re-isolation and found to be toxic. Ten subclones were maintained for one year and re-analysed. All subclones were toxic and the toxicity among them was found in narrower range than that among the subclones from OF935-AT6 strain.



**Figure 2.** Phylogenetic tree inferred from 5.9S rDNA and ITS

Numbers on the branches indicate branch frequency from 1000 bootstrap samples for neighbor-joining. Sequence for the dinoflagellate *P. bahamense var. compressum* was used as the outgroup. GenBank accession No. of rDNA gene nucleotide sequence data used in this figure were as follows. *A. tamarensis* (OFX191 identical with that of FK-788 according to Adachi 1996: AB006993, AT-6: AF374228, CCMP116: AJ005047, WKS-1: AB006991, CU-15: AB006992), *A. fundyense* (CCMP1719: AJ005049), *A. catenella* (MI7: AB006990, CCMP1493: AJ005048), *A. affine* (H1: AB006995), *A. tamiyavanichii* (AF145224), *A. lusitanicum* (LAC27: AJ005050), *A. insuetum* (S1: AB006996), *A. pseudogonyaulax* (H1: AB006997), *Pyrodinium bahamense var. compressum* (AF145225)

The high frequency of mutations resulting in a loss of toxicity noticed among subclones of OF935-AT6 seems to contradict the limited natural abundance of non-toxic *A. tamarensis* found around Japan. It also contrasts with the fact that the subcloned strains from the toxic strain Axat-2 were all toxic and kept their toxicity over time. Further investigation is required to explain these findings.

#### *Toxicity of non-toxic subclone, UAT-014-009 and toxic subclone, Axat-2*

Extracts equivalent to  $1.4 \times 10^6$  cells of subclone UAT-014-009 were injected into each mouse. No symptoms or unusual behaviour were observed for up to 3 days after injection into three mice. The same extract was analyzed by HPLC and no PSTs were detected. The subclone Axat-2 primarily contained C1, C2, GTX4, GTX1, and neoSTX toxins. The overall toxicity and toxin composition produced by toxic subclone Axat-2 did not vary significantly during maintenance. The

difference in toxin content between the toxic and non-toxic subclones was calculated to be more than 104-fold from the detection limit and the toxin contents of toxic subclone.

#### *Phylogenetic analysis*

The non-toxic subclone UAT-014-009 could not be distinguished from the toxic subclone Axat-2 by rDNA sequences. They were different from known non-toxic *A. tamarensis* strains, WKS-1 (Kushimoto, Japan) and CU-15 (Gulf of Thailand) reported by Adachi *et al.* (1996), but identical to known toxic strains of *A. tamarensis* (OFX191) from Ofunato Bay, Japan based on the 5.8S rDNA and ITS (Fig. 2).

#### *Difference of genes between UAT-014 and Axat-2*

Ten candidate genes were found to be expressed differently between the non-toxic subclone UAT-014-009 and toxic subclone Axat-2. Six gene fragments showed high homology with either cytochrome c oxidase III (cox3) of *Gonyaulax polyedra* or cytochrome b (cob) of *A. tamarensis*. The deduced amino acid sequence of one gene fragment showed 87% homology with cytochrome c6 of *Heterocapsa triquetra*. Three had no homologous gene in the data base by NCBI BLAST. Whether the genetic difference found between the two subclones is peculiar of subclones from OF935-AT6 is ambiguous. It might be universal for cox3 and cob of dinoflagellates, since the mitochondrial genome of other dinoflagellates are also suggested to contain numerous gene fragments (Chaput *et al.* 2002).

#### **References**

- AOAC Official Method 959.08, (1995) In: Cunniff, P. (ed.), AOAC international, 16th edition, 2, 35, 21 – 22.
- Adachi, M., Sako, Y. & Ishida, Y. (1994). J. Phycol. 30: 857- 863.
- Chaput, H., Wang, Y. & Morse, D. (2002). Protist 153: 111-122.
- Ogata, T., Ishimaru, T. & Kodama, M. (1987). Mar. Biol. 95: 217-220.
- Omura, T., Onodera, H., Ishimaru, T. & Oshima, Y. (2003). La Mer 41: 86-93.
- Oshima, Y. (1995). J. AOAC Int. 78: 528 – 532.
- Taroncher-Ordenburg, G. & Anderson, D. M. (2000). Appl. Environ. Microbiol. 66: 2105-2112.
- Usup, G., Pin, L.C., Ahmad, A. & Teen, L.P. (2002). Harmful Algae 1: 59–68.

## Molecular tools for the identification of *Pseudo-nitzschia calliantha* and *P. delicatissima* in the Ebre Delta, Spain

L. Elandaloussi<sup>1,2</sup>, R. Venail<sup>1,2</sup>, S. Quijano-Scheggia<sup>2,3</sup>, M. Fernández-Tejedor<sup>1,2</sup>, E. Mallat<sup>1,2</sup>, J. Diogène<sup>1,2</sup>, E. Garcés<sup>1,2,3</sup>, J. Camp<sup>2,3</sup> and K. Andree<sup>1,2</sup>

<sup>1</sup>IRTA-Centre d'Aqüicultura, Crta. Poble Nou, Km 5.5, 43540-Sant Carles de la Ràpita, Spain, Laurence. elandaloussi@irta.es, <sup>2</sup>CRA, Centre de Referència en Aqüicultura, CIRIT-Generalitat de Catalunya, Spain,

<sup>3</sup>Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar-CMIMA, CSIC, P. Marítim de la Barceloneta, 37-43, E08003 Barcelona, Spain, quijanosonia@gmail.com

### Abstract

Six non-axenic clonal cultures of *Pseudo-nitzschia* from Alfacs and Fangar Bay in the Ebre delta in the NW Mediterranean were characterised in terms of morphology and rRNA sequences. Based on scanning and transmission electron microscopy, the isolates were identified as *P. delicatissima* (Cleve) Heiden and *P. calliantha* Lundholm, Moestrup et Hasle. The identity of the species was supported by analyses of the ITS1, 5.8S and ITS2 of the nuclear-encoded rRNA sequences. We report the initial results in the development of *P. calliantha*- and *P. delicatissima*-specific probes targeting the rRNA for the identification of *Pseudo-nitzschia* spp. by fluorescent *in situ* hybridisation (FISH) techniques. The *P. delicatissima*-specific probe was shown to react with three out of the four clones of *P. delicatissima* tested whereas *P. calliantha*-specific probe reacted with only one out of the two *P. calliantha* clones tested.

### Introduction

Diatoms of the genus *Pseudo-nitzschia* include species associated with the production of domoic acid, the toxin responsible for amnesic shellfish poisoning (ASP). Identification of this genus can be performed by light microscopy; however, discrimination amongst species often requires observation of morphological characters using electron microscopy. Molecular methods are valuable tools for rapid identification of harmful algae, and species-specific probes have already been developed for the identification of *Pseudo-nitzschia* species in North America (Scholin 1996).

### Materials and Methods

#### Cultures

*P. calliantha* and *P. delicatissima* were isolated from field samples collected in Alfacs and Fangar Bays in the Ebre Delta, Spain during winter 2006. Other cultures used were *P. pungens* clone PO3, *P. multistriata* clone 115 and *P. fraudulenta* clone AR3, all established from field samples collected along the Catalan coast in 2005.

#### Microscopy

*Pseudo-nitzschia* isolates were cleaned (Lundholm *et al.* 2002) and examined morphologically in a Hitachi S-3500N Scanning Electron Microscope. For Transmission Electron Microscopy, drops of cleaned material were placed on Formvar-coated copper grids, dried and studied with a Hitachi H800 microscope.

#### DNA extraction, cloning and sequencing

DNA extractions were performed using the DNeasy® Plant Kit from Qiagen. ITS1, 5.8S and ITS2 of the nuclear-encoded rDNA were amplified using the primers MicroSSU (5'-GTGAACCTGCG-GAAGGATC-3') and Dino E (5'-CCKSTTCAYT-CGCCRTTAC-3'). The resulting amplicons were cloned and sequenced.

#### Sequence analysis

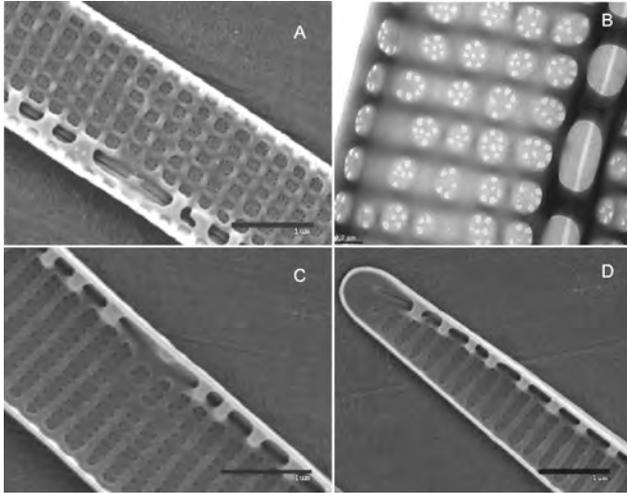
*Pseudo-nitzschia* sequences deposited in GenBank and those obtained in this study were used for phylogenetic analysis (for accession numbers, see Fig. 2). Sequences were aligned using clustalW and BioEdit software. Non-homologous sequences present at the 5' and 3' ends were truncated leaving 709 nucleotides of homologous sites for phylogenetic analyses. Modelgenerator 0.83 was used to determine the model that best fits the data according to the Akaike Information Criterion. The programme TreePuzzle was used to reconstruct phylogenetic trees using the Maximum Likelihood (ML) approach. Ten thousand puzzling steps were applied using the general time reversible (GTR) model of substitution. For neighbour joining (NJ) and maximum parsimony (MP) 1000 bootstrap replicates were calculated using PHYLIP 3.65 program.

#### Probes

Oligonucleotide probes were designed to recognise species-specific regions of the rRNA of *P. delicatissima* (Pdel1) and *P. calliantha* (Pcal1) by analysing alignments of *Pseudo-nitzschia* sequences using clustalW and Bioedit software.

### Whole-cell hybridisation

The probes Pcal1 (5'-TGGCTACTGGAGCAGC-3'), Pdel1 (5'-CCAACG-AAACCCAAGCAAAG-3') and Proret (5'-GCACAGTTTCACACAGAATTTTCGC-3', this probe targeting ITS2 rRNA of *Protoceratium reticulatum*, unpublished) were 5'-end labelled with fluo-



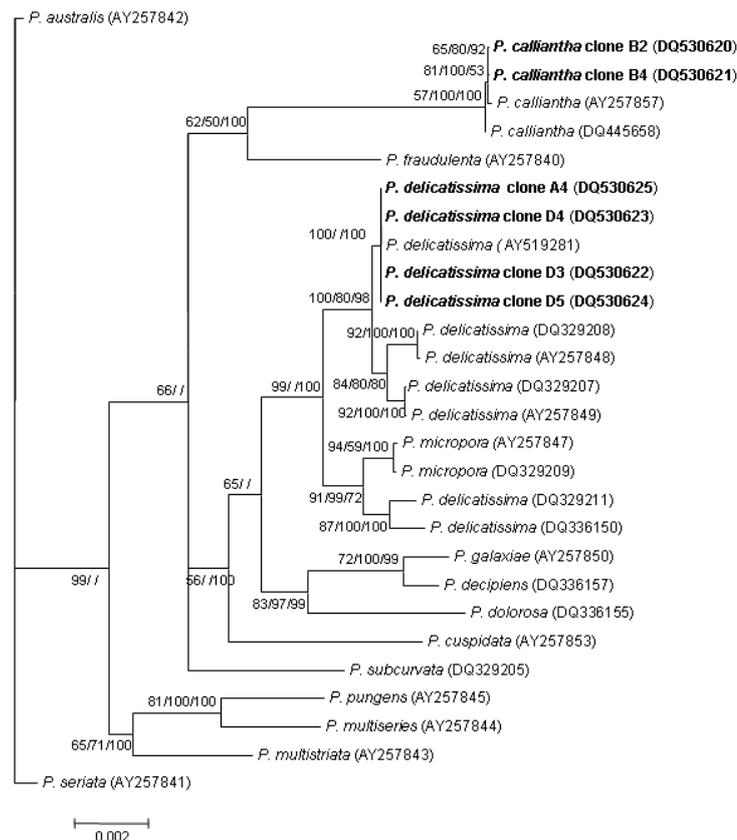
**Figure 1.** Scanning electron micrographs of *P. delicatissima* (C, D) and *P. calliantha* (A). TEM of *P. calliantha* (B). Scale bars are indicated on the photos.

orescein for use in a whole-cell FISH assay (Scholin *et al.* 1996). Clonal cultures of *P. delicatissima* and *P. calliantha* were hybridised with Pdel1, Pcal1 or Proret (negative control) and visualised using epifluorescence microscopy. The two species-specific probes, Pdel1 and Pcal1 were also applied to *P. pungens* clone PO3, *P. multistriata* clone 115 and *P. fraudulenta* clone AR3 in a FISH assay.

### Results

Six clonal and non-axenic cultures of *Pseudo-nitzschia* were obtained and maintained in F/2 medium with silicate. Based on electron microscopy, four of these isolates were identified as *P. delicatissima* (Lundholm *et al.* 2006) and two as *P. calliantha* (Fig. 1). Amplification of the ITS region by PCR generated an unambiguous sequence with an average length of 879 nucleotides. The identity of the species was supported by analysis of these sequences.

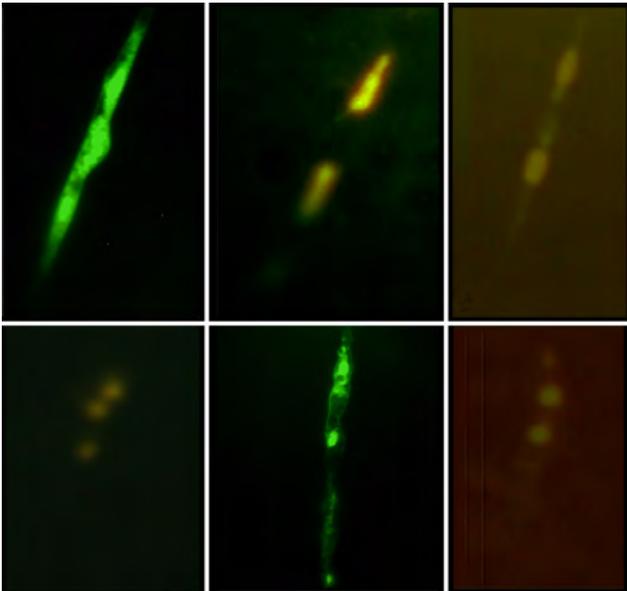
A maximum likelihood unrooted tree with a log likelihood value of -13468.64 is shown in Fig. 2. The clade containing the isolates of *P. calliantha* from Spain (clones B2 and B4), Vietnam (AY257857) and USA (DQ445658) was highly supported by bootstrap



**Figure 2.** Maximum likelihood unrooted tree reconstructed from the ITS1, 5.8S and ITS2 of the nuclear-encoded rRNA of the genus *Pseudo-nitzschia*. GenBank accession numbers are indicated in brackets and those generated during this study are written in bold. The values indicated on the branches are reliability percentages (10000 quartet puzzling steps, before slash), distance matrix in neighbour joining (1000 replicates, between slashes) and parsimony (1000 replicates, after slash).

values in NJ and MP analysis (100 %). The four Spanish isolates of *P. delicatissima* (A4, D3, D4 and D5) clustered with an isolate from Italy (# AY519281) with high bootstrap values using MP (100 %) and reliability percentage in ML analysis of 57 %.

Species-specific probes for identification of *P. delicatissima* and *P. calliantha* from the Ebre Delta were developed. Of the four *P. delicatissima* clones tested, one (clone D3) did not hybridise with the Pdel1 probe and none cross-reacted with the Pcal1 probe. Of the two *P. calliantha* clonal cultures tested, only clone B4 hybridised with the Pcal1 probe. However, positive FISH tests did not result in high and homogeneous fluorescence signals. Nevertheless, no fluorescent labelling could be observed when *P. delicatissima* and *P. calliantha* clones were treated with the negative probe. Images of FISH tests performed on two of these isolates are shown in the Fig. 3. The two probes, Pdel1 and Pcal1, did not stain any cells of *P. pungens* clone PO3, *P. multistriata* clone 115 or *P. fraudulenta* clone AR3 (data not shown).



**Figure 3.** FISH images of hybridisation tests of *P. delicatissima* clone A4 (row A) and *P. calliantha* clone B4 (row B) with the *P. delicatissima*-specific probe (column 1), the *P. calliantha*-specific probe (column 2) or with the *Protoceratium reticulatum*-specific probe (column 3) as a negative control.

### Discussion

Of the 5 species of *Pseudo-nitzschia* recorded so far in the Catalan coast, *P. pungens*, *P. fraudulenta*, *P. multistriata*, *P. delicatissima* and *P. calliantha*, we were able to establish a culture of the last two from the Ebre Delta during winter 2006.

In the phylogenetic analyses, *P. delicatissima* showed a higher degree of genetic diversity than *P.*

*calliantha*. As previously reported (Lundholm *et al.* 2006), *P. delicatissima* and *P. micropora* formed a clade. It comprised two subclades of *P. delicatissima* and one clade of *P. micropora*. Phylogenetic analyses supported the grouping of Mediterranean isolates of *P. delicatissima*.

Species-specific oligonucleotide probes targeting *P. delicatissima* and *P. calliantha* were developed and shown to hybridise with their respective targets. The weak signal observed indicates that the probes do not have easy access to their target or that the cells contain a low amount of rRNA. It also cannot be excluded that the affinities of binding of the probes to their targets are weak. The rRNA contents of our cultured *Pseudo-nitzschia* might be low, thus rendering detection by fluorescent labelling difficult. In addition, a small decrease in the amount of rRNA in cells from different cultures will appear significant in an originally poor label and could explain the lack of detectable labelling observed for some of the strains when hybridised with their specific probes. Nevertheless, improved binding efficiency of the probes and/or increased membrane permeability could be achieved by altering hybridisation conditions. In conclusion, the protocol for *in situ* hybridization still needs to be optimised in terms of efficiency of probe binding, and the molecular probes have also to be tested on a larger number of *Pseudo-nitzschia* species in both culture and field samples as well as on unrelated microalgae to determine cross-reactivity. Sequencing of additional *Pseudo-nitzschia* clones from Catalan waters is currently underway to assess their molecular diversity and develop specific probes to detect a wider variety of *Pseudo-nitzschia* species.

### Acknowledgements

This project was funded by project RTA-2005-00109-00-00, INIA, Ministry of Education and Science, Spanish Government/ Centre de Referència en Aqüicultura, DURSI, Generalitat de Catalunya, /ACA, Agència Catalana de l'Aigua, /Xarxa de Vigilància de Fitoplàncton Tòxic o Nociu.

### References

- Lundholm, N., Hasle, G.R., Fryxell, G.A. & Hargraves, P. E. (2002). *Phycologia* 41: 480-97.
- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. & Miller, P. (2006). *J. Phycol.* 42: 464-481.
- Scholin, C.A., Buck, K.R., Britschgi, T., Cangelosi, G. & Chavez, F. P. (1996). *Phycologia* 35: 190-197.

## Genomic characterization of the spirolide-producing dinoflagellate *Alexandrium ostenfeldii* with special emphasis on PKS genes

N. Jaeckisch<sup>1</sup>, R. Singh<sup>2</sup>, B. Curtis<sup>2</sup>, A. Cembella<sup>1</sup> and U. John<sup>1</sup>

<sup>1</sup>Alfred-Wegener-Institut, Am Handelshafen 12, 27570 Bremerhaven, Germany, njaeckisch@awi-bremerhaven.de, <sup>2</sup>Institute for Marine Biosciences, National Research Council, 1411 Oxford Street, Halifax, Canada B3H 3Z1

### Abstract

The dinoflagellate *Alexandrium ostenfeldii* produces toxic macrocyclic imines known as spirolides. Spirolides are derived via polyketide biosynthetic pathways therefore synthesis is almost certainly mediated by polyketide synthase (PKS) genes. At the genomic level, our research focuses on identification and characterization of genes involved in spirolide biosynthesis, specifically PKS genes. Genomic characterization of *A. ostenfeldii* was conducted by generating an Expressed Sequence Tag (EST) databank, based on a normalized cDNA library. About 5,300 ESTs were sequenced from strain AOSH1 from Nova Scotia, Canada, which produces a distinctive spirolide profile. Several putative PKS genes were detected, and further studied by phylogenetic analysis. The results provide the first steps to gain significant insights into the general genomic organisation of *A. ostenfeldii* and the relationship with toxin-producing genes.

### Introduction

Marine eukaryotic microalgae produce a great variety of novel secondary metabolites, including phycotoxins. Among these phycotoxins are the spirolides, macrocyclic imines belonging to a group of highly potent 'fast-acting toxins'. The marine dinoflagellate *Alexandrium ostenfeldii* is the only known primary source of spirolides (Cembella *et al.* 2000). Stable isotope labelling of *A. ostenfeldii* has confirmed that spirolides are derived via polyketide biosynthesis (MacKinnon *et al.* 2006, in press), and thus production is likely to be regulated by one or more polyketide synthases (PKS). Due to the pharmaceutical importance of polyketides, PKS genes and the biosynthetic pathways to polyketides have been studied mostly in bacteria and fungi (Staunton and Weissman 2001). In contrast, there is very little information about the biosynthesis of protist-derived polyketides at the genomic level. In the case of dinoflagellates, this is partly because of the large size, structural complexity, and regulatory particularities of the dinoflagellate genome relative to that of typical eukaryotes. For example, nucleotide substitution of a large percentage of hydroxyl-methyluracil for thymine is known from dinoflagellates. This group exhibits very large genomes of up to 200 pg DNA per nucleus, which probably contain large introns. The generation of ESTs is therefore a promising approach to gain insights into the assemblage of expressed genes in *A. ostenfeldii* and serves as a tool to identify putative PKS genes.

### Methods

*Alexandrium ostenfeldii* strain AOSH1 was grown in K medium (Keller *et al.* 1987) in 10-L glass flasks on a L:D cycle of 14:10 h at 15 °C and a photon flux of 90  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Cultures were harvested in the mid-exponential growth phase.

Total RNA of *A. ostenfeldii* was extracted with peqGOLD RNAPure (PEQLAB Biotechnology, Erlangen, Germany) and cleaned with peqGOLD Optipure following manufacturer's instructions. A normalized cDNA library was constructed in cooperation with BASF Plant Science (Ludwigshafen, Germany) and prepared by Vertis Biotechnologie AG (Freising, Germany). Normalization was performed to reduce redundant DNA. About 5,300 clones of the resulting cDNA library were sequenced from the 5' end. The contigs were compared to several gene libraries by Basic Local Alignment Search Tool (BLAST) searches. The detected putative PKS sequences were screened for ketoacylsynthase (KS) domains and compared to KS domain sequences of apicomplexans, fungi, bacteria and to animal fatty acid synthase (FAS) sequences by BLAST searches and alignment using ClustalX version 1.83 (Thompson 1997). A detailed phylogenetic tree was constructed based on amino acid sequences with MEGA version 3.1 (Kumar *et al.* 2004), applying neighbour-joining and bootstrap as test of phylogeny. The bacterial Type II FAS sequences Z99112 and AE009073 were used as outgroups.

## Results

After vector and quality clipping, 3,538 sequences remained and formed 2,634 unique contig sequences with an average length of 613,7 bp. An automated annotation based on BLAST searches resulted in 784 contig sequences (30 % of all unique contig sequences) with homology to known proteins. For 105 (12 %) of these ESTs, the annotation could be verified manually (Fig. 1). The vast majority fell into the functional main categories (according to COGs, clusters of ortho-logous groups of proteins) cellular processes (6 %) and metabolism (5 %), whereas the remaining (1 %) showed similarity to genes of the categories information storing and processing, stress, defence and toxicity, cell structure and general function prediction only. Within the category stress, defence and toxicity, three putative PKS genes were found. The number of annotated ESTs for each sub-category are illustrated in Fig.1. Members of almost all expected functional categories were present.

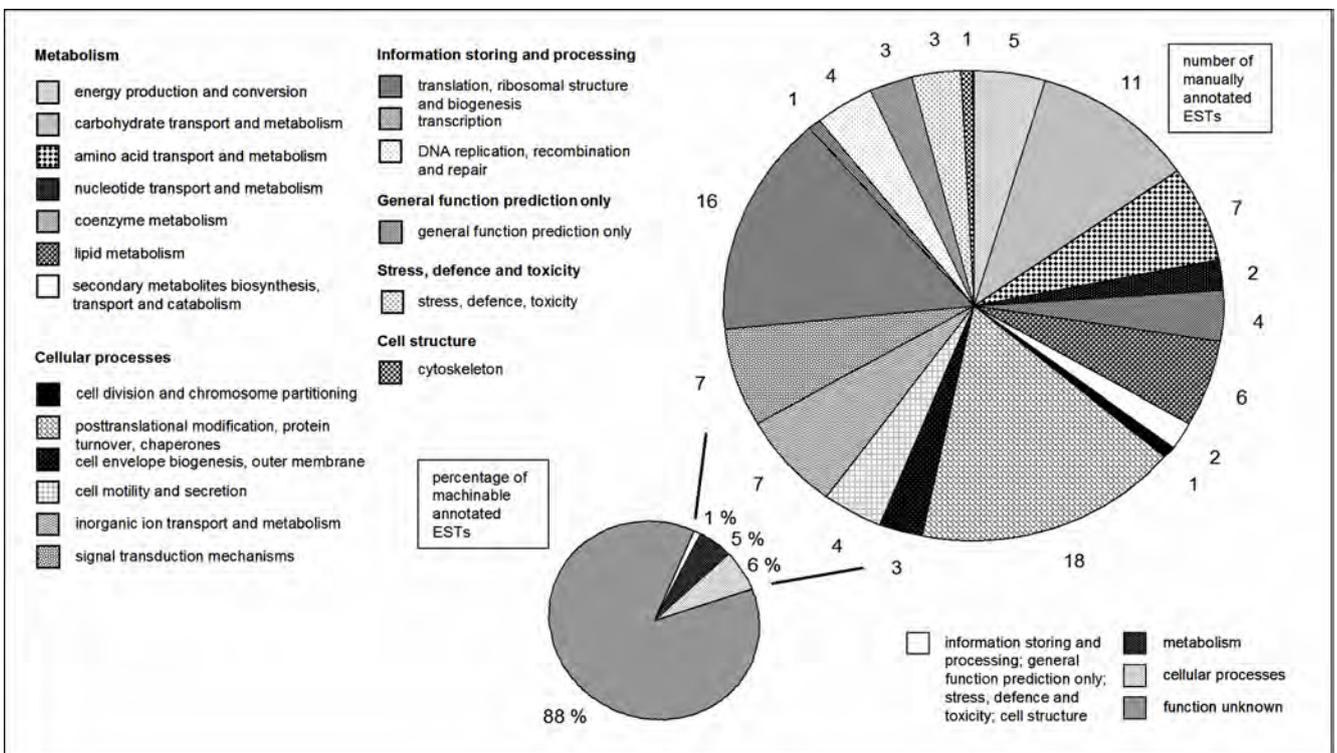
Phylogenetic analysis of the putative PKS sequences confirmed these to be PKS gene fragments. Phylogenetic placement in terms of evolutionary relationships was only possible with one PKS sequence (Fig. 2) which contained a ketoacyl synthase (KS) module that is known to be quite conserved among different organisms. Our sequence was nested into the apicomplexan clade between two *Cryptosporid-*

*ium parvum* sequences, AAC-99407.1 (FAS) and AAN60755.1 (PKS). The alignments of these sequences were among the best BLAST hits with e-values of  $1e-33$  (PKS) and  $1e-40$  (FAS), respectively.

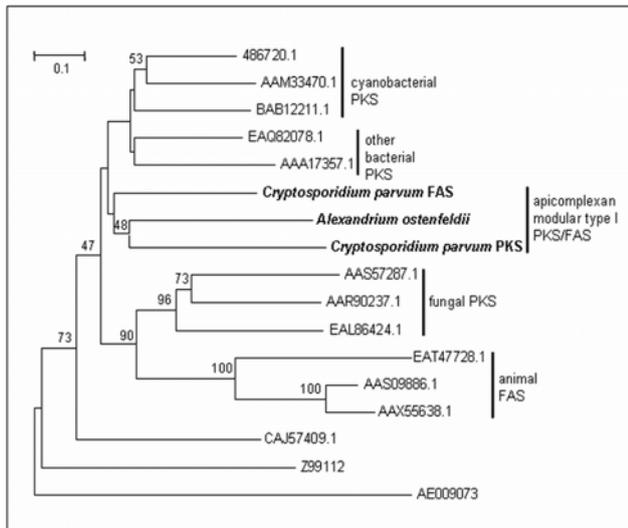
## Discussion

The high sequence diversity (2,634 unique contig sequences) found in our normalized cDNA library is congruent with results for other dinoflagellate species, e.g. *Lingulodinium polyedrum* (1,519 unique sequences) and *Amphidinium carterae* (3,380 unique sequences; Bachvaroff *et al.* 2004). The finding that only a minority (30 % in our automated annotation) of the *A. ostensfeldii* unique sequences could be identified via the database searches is typical for dinoflagellate studies. Only about 29 % of 5,280 *Karenia brevis* ESTs were found to have similarity to known sequences in other organisms (Lidie *et al.* 2005) and even less could be annotated using BLAST search for *Alexandrium tamarense*, about 20 % of 6,723 unique sequences (Hackett *et al.* 2005). Recent EST projects of other algae groups yielded more identifiable genes, for instance 45 % of 5,108 unique sequences in the diatom *Phaeodactylum tricorutum* (Montsant *et al.* 2005) and 36 % of 1,523 unique sequences in the coccolithophorid *Emiliana huxleyi* (Wahlund *et al.* 2004).

As our cDNA library was created from a culture grown under a single optimized condition it is



**Figure 1.** Distribution of ESTs of the normalized cDNA library by functional categories according to COGs.



**Figure 2.** Phylogenetic analysis of KS domain sequences which are 178 to 222 amino acids in length. The bacterial Type II FAS sequences Z99112 (*Bacillus subtilis*) and AE009073 (*Agrobacterium tumefaciens*) served as outgroups.

possible that many genes are not represented in the dataset. On the other hand, the cDNA library was normalized. Normalization is known to improve the rate of gene discovery because the amount of highly redundant cDNAs is reduced whereas low copy cDNAs are amplified. Another methodological constraint is the possibility that the generated EST sequences did not extend deep enough into the coding region of the transcript. This is probable because in 28.5% of our sequences a poly-A tail was detected, and untranslated regions (UTRs) of more than 500 bp are known to occur in some eukaryotic species. Another explanation for the low amount of identifiable genes is the paucity of dinoflagellate sequences in the current databases for comparison and the possible strong distinctions compared to known protein sequences. These distinctions could be due to the highly unusual genome structure and regulation in dinoflagellates (as mentioned before) or that the unidentifiable sequences encode novel dinoflagellate-specific proteins involved in unknown metabolic pathways.

In the phylogenetic analysis, our sequence showed highest similarity to PKS and FAS sequences of the apicomplexan *Cryptosporidium parvum* and clustered together with bacterial PKS. The relatively low bootstrap values in this part of the tree are probably due to the shortness of the included sequences (178–222 amino acids). Nevertheless, the analysis supports the

assumption that our sequence is consistent with an apicomplexan modular PKS. This is perhaps not surprising given the close phylogenetic relationship between apicomplexans and dinoflagellates. Such modular Type I PKSs are large multifunctional enzymes with several catalytic domains located on a single protein. In summary, the phylogenetic tree displays the expected picture except for sequence CAJ57409.1 (bacterial PKS Type I), which for unknown reasons did not fall into a distinct clade.

To detect and further analyse toxin-related genes, particularly those involved in spirolide biosynthesis, the generation of a fosmid library is in progress. This method (insert size of about 40 kb) offers the potential to obtain a full-length PKS gene. Identification and characterization of PKS genes from dinoflagellates provide the first steps for confirmation of polyketide biosynthetic pathways and regulatory processes related to this pharmaceutically and phylogenetically interesting enzyme group.

## References

- Bachvaroff, T.R., Conception, G.T., Rogers, C.R., Herman, E.M. & Delwiche, C.F. (2004). *Protist* 155: 65-78.
- Cembella, A.D., Lewis, N.I. & Quilliam, M.A. (2000). *Phycologia* 36: 67-74.
- Hackett, J.D., Scheetz, T.E., Yoon, H.S., Soares, M. B., Bonaldo, M.F., Casavant, T.L. & Bhattacharya, D. (2005). *BMC Genomics*: 6.
- Keller, M.D., Selvin, R.C., Claus, W. & Guillard, R. R.L. (1987). *J. Phycol.* 23: 633-638.
- Kumar, S., Tamura, K. & Nei, M. (2004). *Briefings in Bioinformatics* 5: 150-163.
- Lidie, K.L., Ryan, J.C., Barbier, M. & Van Dolah, F.M. (2005). *Mar. Biotechnol.* 7: 481-493.
- Montsant, A., Jabbari, K., Maheswari, U. & Bowler, C. (2005). *Plant Physiol.* 137: 500-513.
- MacKinnon, S.L., Cembella, A.D., Burton, I.W., Lewis, N., LeBlanc, P. & Walter, J.A. (2006). *J. Org. Chem.*, in press.
- Staunton, J. & Weissman, K.J. (2001). *Nat. Prod. Rep.* 18: 380-416.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). *Nucl. Acids Res.* 25: 4876-4882.
- Wahlund, T.M., Hadaegh, A.R., Clark, R., Nguyen, B., Fanelli, M. & Read, B.A. (2004). *Mar. Biotechnol.* 6: 276-290.

## Differences within the nodularin synthetase gene cluster between toxic *Nodularia spumigena* and non-toxic *Nodularia harveyana*

T. Krüger<sup>1</sup>, R. Oelmüller<sup>2</sup>, S. Hiller<sup>1</sup> and B. Luckas<sup>1</sup>

<sup>1</sup>Friedrich-Schiller-University, Institute of Nutrition, Food Chemistry, Dornburger Str. 25, 07743 Jena, Germany, thomas.krueger.1@uni-jena.de

<sup>2</sup>Friedrich-Schiller-University, Institute of General Botany, Plant Physiology, Dornburger Str. 159, 07743 Jena, Germany

### Abstract

In the Baltic Sea, planktonic *Nodularia spumigena* with the capability to form gas vacuoles has been described as the only toxin-producing cyanobacterium. Baltic Sea populations of benthic *Nodularia harveyana* lack gas vacuoles and the ability to produce the hepatotoxin nodularin. The nodularin synthetase gene cluster (nda) of *Nodularia* strains *N. spumigena* Huebel 1988/306 and *N. harveyana* Huebel 1983/300 were analysed. Polymerase chain reaction (PCR) of DNA and cDNA indicated the absence of ndaE and ndaF in the non-toxic *N. harveyana* strain Huebel 1983/300. Southern analysis validated the characteristic genetical differences between these two closely related *Nodularia* strains and demonstrated that ndaE and ndaF are not present in *N. harveyana*, whereas the nodularin-producing *N. spumigena* strain Huebel 1988/306 contained the nda genes as expected. Our results provides simple molecular techniques as an advanced diagnostic tool for detection and risk assessment of potential nodularin-producing *Nodularia* mass developments, especially in the Baltic Sea.

### Introduction

In the Baltic Sea, mass occurrences of the filamentous heterocystous cyanobacterial genus *Nodularia* are an annual phenomenon in late summer and may cover areas of 60.000 km<sup>2</sup> (Laamanen *et al.* 2001). They are hazardous health risks for humans and animals. *Nodularia* species are toxic via the production of the cyclic pentapeptide hepatotoxin nodularin, a potent inhibitor of eukaryotic protein phosphatases 1 and 2A (Yoshizawa *et al.* 1990; Lethimäki 2000). With exception of the benthic strain PCC7804 originating from a thermal spring (Dax, France), which produces [L-Har2]nodularin (Beattie *et al.* 2000; Saito *et al.* 2001), the planktic *Nodularia spumigena* has been confirmed as the only nodularin-producing species so far.

Recently, the complete nodularin synthetase gene cluster was sequenced in *N. spumigena* NSOR10 and biosynthesis of the hepatotoxic and tumour-promoting cyclic pentapeptide was characterized (Moffitt and Neilan 2004). The 48-kb gene cluster responsible for the toxin production consists of nine open reading frames (ORFs), ndaA to ndaI, which are transcribed from a bidirectional regulatory promotor region and encode nonribosomal peptide synthetase modules (NRPS), polyketide synthase modules (PKS) and tailoring enzymes.

Nodularin has been suggested as the most extreme structural variant of microcystins (Rantala *et al.* 2003). Sequence analysis of related microcystin (mcy) and nodularin synthetase genes (nda) suggests

that a common ancestor of the genes existed very early in the evolutionary history. In general, hepatotoxin synthetase genes are the candidate genes used in approaches for the discrimination of toxic and non-toxic genotypes (Dittmann and Börner 2005).

The aim of this study was to investigate differences in the genome between the toxic *Nodularia spumigena* (Huebel 1988/306) and the non-toxic *Nodularia harveyana* (Huebel 1983/300) concerning the nodularin synthetase gene cluster.

### Material and Methods

The *Nodularia* strains were cultivated in BG-11 medium with addition of 6 g NaCl and adjusted to pH 9.0. Cultures were grown at 25 °C with continuous illumination of 80 µmol m<sup>-2</sup> s<sup>-1</sup>.

For extraction of total genomic DNA, cells were damaged in lysis buffer (7 M urea, 0.3 M NaCl, 1% lauryl sarcosin, 20 mM Na<sub>2</sub>EDTA, 50 mM Tris-HCl, pH 8.0) via homogenisation with glass beads, extracted with phenol/chloroform/ isoamyl alcohol (25:24:1) and subsequently precipitated with isopropanol. The pellet was washed twice with 80 % ethanol and finally vacuum-dried as well as resuspended in pure water with addition of RNase.

Total RNA isolation was performed after a modified protocol of Chomczynske and Sacchi (1987) using aliquots of exponentially growing *Nodularia* cultures which were lysed via homogenisation with sterile glass beads.

The cDNA synthesis was performed by the cDNA first strand synthesis kit (Fermentas) following the instruction manual.

A 944-bp fragment of the whole *ndaE* (o-methyltransferase) gene was amplified with primers *ndaE*-LP (5'-AGCTTTGGAAAATGTTG-GC-3') and *ndaE*-RP (5'-CAGGAAATCTAATCT-GG-3'). A 858-bp fragment of *ndaF* was amplified with *ndaF*-LP (5'-GGGAGAAATTCCCGTATTT-GA-3') and *ndaF*-RP (5'-CTGAGAGTATTGTCC-CCCACA-3'). PCR amplification of *cpcBA* (phycocyanin gene B and A) was performed with primer pair *cpcBA*-LP (5'-AACCTACCAAGCTCTAGGA-AC-3') and *cpcBA*-RP (5'-GTCAAAGCACGAGC-AGCTT-3') as a genus-specific positive control. Thermal cycling was performed in MWG Primus 96 plus PCR machines using primer annealing temperatures at 50 °C (*ndaE*) and 55 °C (*ndaF*).

Genomic Southern analysis was performed as previously described (Sambrook and Russell 2001) using several endonuclease restriction enzymes (*EcoRI*, *HindIII*, *MluI*, *PvuI*, *PvuII*, *SacII*, *XbaI*, all Fermentas) as well as upward capillary transfer and 20 x SSPE transfer buffer (3.6 M NaCl, 20 mM Na<sub>2</sub>EDTA, 362 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 44 mM NaH<sub>2</sub>PO<sub>4</sub>). Probes of *ndaE* and *-F* were <sup>32</sup>P radio-labelled (Rediprime™ II random prime labelling kit) following the instruction manual. Hybridised radioactive probes were detected with Phosphor-Imager System Storm 820 (Molecular Dynamics). The *nda* probes were subsequently removed from the membranes by dehybridisation. The

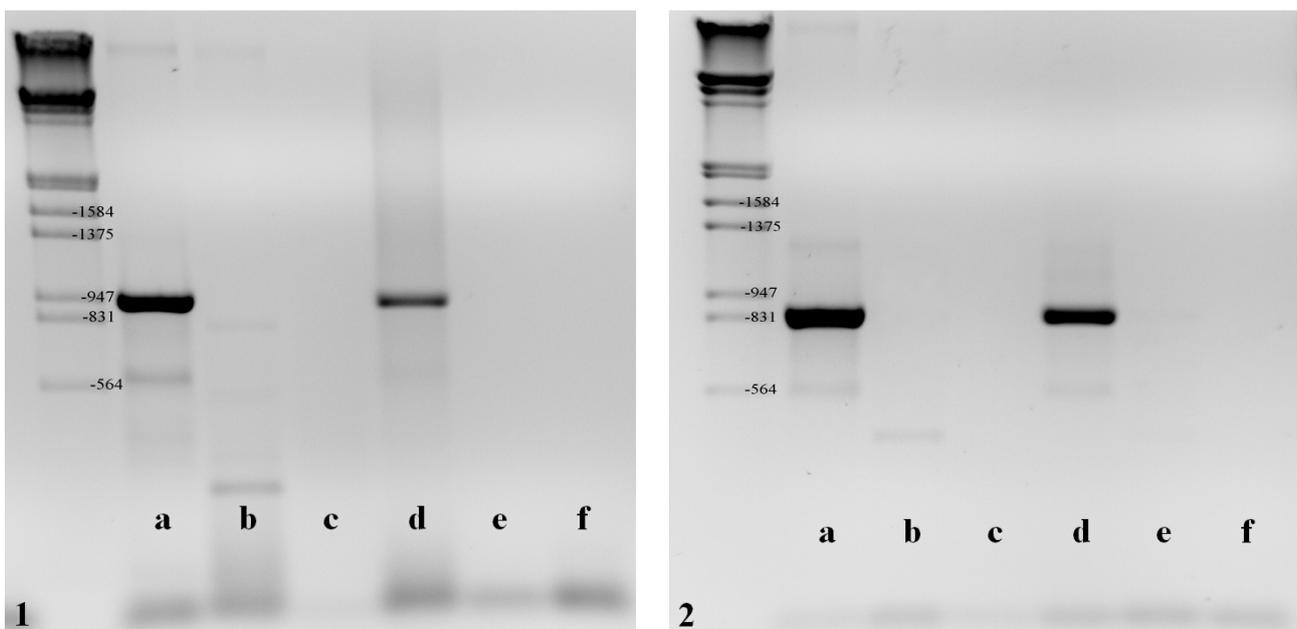
hybridisation steps were repeated with *cpcBA* probes as positive control.

## Results and Discussion

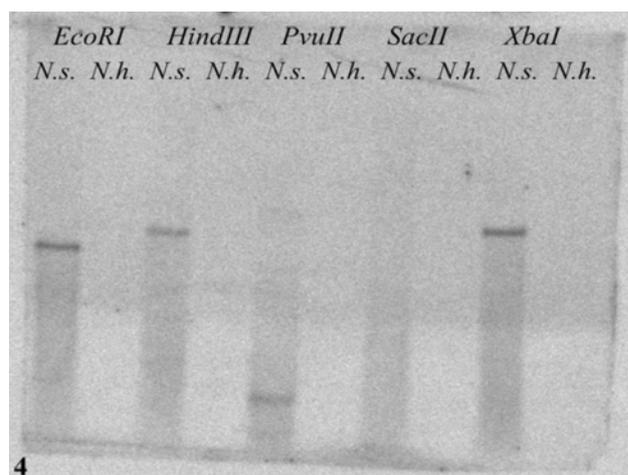
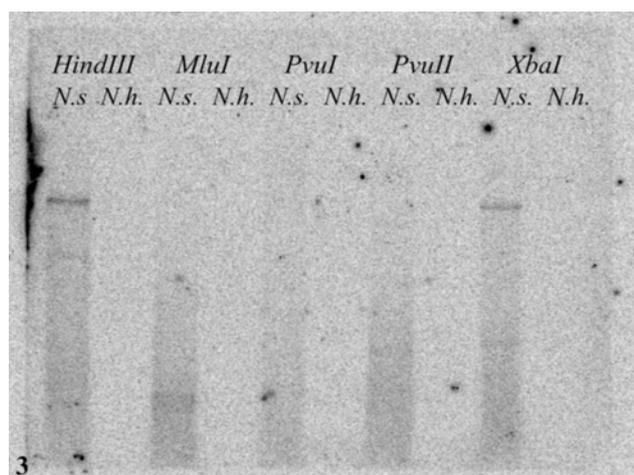
PCR experiments indicated the lack of *ndaE* (Fig. 1) and *ndaF* (Fig. 2) in the non-toxic strain consistent with recent findings concerning *ndaF* (Lyra *et al.* 2005; Surakka *et al.* 2005). Southern analysis validated these results. The toxic *N. spumigena* strain contained both nodularin synthetase genes as expected, whereas hybridisation failed to detect the *ndaE* (Fig. 3) and *ndaF* (Fig. 4) genes in *N. harveyana*. Hybridisation with a *cpcBA* DNA fragment, which was used as control, onto the identical membranes demonstrated that this gene is present in both strains (Figs 5 and 6).

The O methyltransferase *NdaE* contains a glycine-rich motif I, which is required for S adenosyl-L-methionine (SAM) binding (Kagan and Clarke 1994). In addition, the hybrid PKS-NRPS enzyme complex *NdaF* catalyses the final round of polyketide extension and the biosynthesis of Adda completed via the aminotransferase domain. Adda is essential for the toxicity of microcystins and nodularin (An and Carmichael 1994). Hence, we have to assume that the lack of at least two genes within the *nda* cluster interrupts the biosynthetic pathway of nodularin and leads to the inability of *N. harveyana* to produce the toxic cyclic pentapeptide.

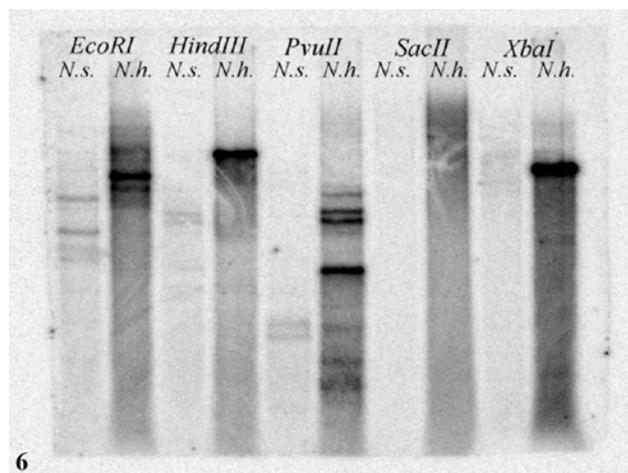
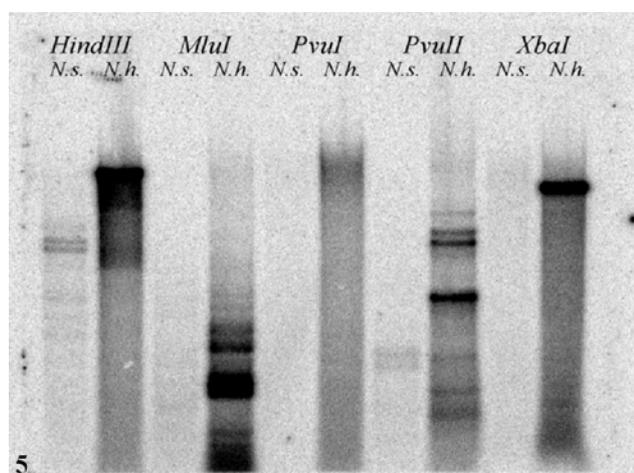
Moreover, according to recent additional PCR analysis we consider that the complete nodularin synthetase gene cluster might be missing in the benthic



**Figures 1, 2.** PCR amplification of *ndaE* (1) and *ndaF* (2). DNA of *N. spumigena* (a), *N. harveyana* (b) and negative DNA control, *Arabidopsis thaliana* (c). cDNA of *N. spumigena* (d) and *N. harveyana* (e) and negative water control (f).



**Figures 3, 4.** Southern hybridisation of *ndaE* (3) and *NdaF* (4). N.s. is *Nodularia spumigena*, N.h. is *Nodularia harveyana*.



**Figures 5, 6.** Southern hybridisation of *cpcBA* (5 and 6).

strain. However, we need to confirm our data (not shown), also by southern analysis, before drawing conclusions. More strains need to be tested to understand the sporadic distribution of nodularin among different *Nodularia* strains and to verify the explanatory power of molecular biological risk determinations. Whether the absence of the complete *nda* gene cluster in *N. harveyana* Huebel 1983/300 is typical for all non-toxic *N. harveyana* strains or only for some of them is currently a topic of discussion, particularly with regard to the determination of *N. harveyana* as the phylogenetically most diverse species within the genus *Nodularia* (Lyra *et al.* 2005).

Strains have been described for all microcystin and nodularin producing genera which contain or lack the corresponding toxin synthetase genes and thus the ability to produce the hepatotoxins (Dittmann and Wiegand 2006). Neilan *et al.* (1999) amplified a 758-bp PCR product of a *mcvB* gene orthologue with primers originally designed for *Microcystis*.

DNA amplification of strains PCC73104 (Canada), HEM and BY1 (Baltic Sea), NSOR10 (Australia) of nodularin-producing *Nodularia spumigena* resulted in PCR products, homologous to *mcvB*. The tested non-toxic *Nodularia sphaerocarpa* did not possess a *mcvB* gene orthologue. Moffitt and Neilan (2001) supposed that non-toxic *Nodularia* strains differ from toxic strains in the peptide synthetase and polyketide synthase sequences. Lyra *et al.* (2005) hypothesized that the benthic *Nodularia* species lost their gas vacuoles and nodularin synthetases once they adopted the benthic habitat.

In the future, simple and powerful molecular detection techniques of nodularin synthetase genes could assist chemical measurements as additional risk assessment tools, allowing quantification of the hepatotoxin synthetase gene copy number via quantitative real-time PCR (Kurmayer and Kutzenberger 2003; Vaitomaa *et al.* 2003) as well as studying population dynamics of toxic cyanobacterial blooms (Dittmann and Wiegand 2006).

## Acknowledgements

We are grateful to the donor of the strains, Dr. Norbert Wasmund (IOW, Rostock, Germany). We also thank Dr. Bationa Shahollari, Paul Hein and Yvonne Venus (University of Jena, Germany) for helpful advice.

## References

- An, J. & Carmichael, W.W. (1994). *Toxicon* 32: 1495-1507.
- Beattie, K.A., Kaya, K. & Codd, G.A. (2000). *Phytochemistry* 54: 57-61.
- Chomczynski, P. & Sacchi, N. (1987). *Anal. Biochem.* 162: 156-159.
- Dittmann, E. & Börner, T. (2005). *Toxicol. Appl. Pharmacol.* 203: 192-200.
- Dittmann, E. & Wiegand, C. (2006). *Mol. Nutrition Food Res.* 50: 7-17.
- Kagan, R.M. & Clarke, S. (1994). *Arch. Biochem. Biophys.* 310: 417-427.
- Kurmayer, R. & Kutzenberger, T. 2003. *Appl. Env. Microbiol.* 69: 6723-6730.
- Laamanen, M.J., Gugger, M.F., Lehtimäki, J.M., Haukka, K. & Sivonen, K. (2001). *Appl. Env. Microbiol.* 67: 4638-4647.
- Lehtimäki, J. (2000). Academic dissertation in microbiology. Helsinki.
- Lyra, C., Laamanen, M., Lehtimäki, J.M., Surakka, A. & Sivonen, K. (2005). *J. Syst. Evol. Microbiol.* 55: 555-568.
- Moffitt, M.C. & Neilan B.A. (2001). *FEMS Microbiol. Lett.* 196: 207-214.
- Moffitt, M.C. & Neilan B.A. (2004). *Appl. Env. Microbiol.* 70: 6353-6362.
- Neilan, B.A., Dittmann, E., Rouhiainen, L., Bass, R.A., Schaub, V., Sivonen, V. & Börner, T. (1999). *J. Bacteriol.* 181: 4089-4097.
- Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T. & Sivonen, K. (2004). *Proc. Natl Acad. Sci.* 101: 568-573.
- Saito, K., Konno, A., Ishii, H., Saito, H., Nishida, F., Toshihiko, A. & Chen, C. (2001). *J. Nat. Prod.* 64: 139-141.
- Sambrook, J. & Russell, D.W. (2001). *Molecular Cloning. A Laboratory Manual. Third Edition.* Cold Spring Harbor Laboratory Press, New York.
- Surakka, A., Sihvonen, L.M., Lehtimäki, J.M., Wahlsten, M., Vuorela, P. & Sivonen, K. (2005). *Env. Toxicol.* 20: 285-292.
- Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. & Sivonen, K. (2003). *Appl. Env. Microbiol.* 69, 7289-7297
- Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K., Ichihara, A., Carmichael, W.W. & Fujiki, H. (1990). *J. Cancer Res. Clin. Oncol.* 116: 609-614.

## Using distinct ribotype groups in recognizing dinoflagellate HAB species

Wayne Litaker<sup>1</sup>, Patrice Mason<sup>2</sup>, Hae Jin Jeong<sup>3</sup>, Wolfgang Vogelbein<sup>2</sup>, Mark Vandersea, Steven Kibler and Patricia Tester<sup>1</sup>

<sup>1</sup>NOAA, Coastal Fisheries and Habitat Research, 101 Pivers Island Rd., Beaufort, NC 28516, USA, Wayne.Litaker@noaa.gov, Steve.Kibler@noaa.gov, Mark.W.Vandersea@noaa.gov, Pat.Tester@noaa.gov

<sup>2</sup>Virginia Institute of Marine Science, P.O. Box 1346, Gloucester Point, Virginia 23062, USA, ptrice@vims.edu, wolf@vims.edu, <sup>3</sup>School of Earth and Environmental Sciences, College of Natural Sciences, Seoul National University, Seoul 151-747, Republic of Korea, hjeong@snu.ac.kr

### Abstract

Molecular methods developed over the past ten years allow for accurate and cost-effective sequencing of rDNA genes. Analysis of the resulting sequences from known species has revealed that the sequences often cluster in distinct groups that correlate with the morphologically defined species. Because the rDNA sequence variation within species is generally less than that between species, newly acquired sequences can be potentially identified as belonging to either a known species or as originating from a species for which sequence data are not yet available. This paper presents a brief case study examining the ITS rDNA ribotypes found within the dinoflagellate genus *Luciella* and discusses the potential use of molecular data in helping recognizing new or cryptic dinoflagellate HAB species.

### Introduction

A major issue addressed during the 12th International HAB conference session on taxonomy and phylogeny concerned the use of molecular data in defining species. Opinions expressed ranged from sole reliance on morphological criteria to describe species to formally describing each genetically distinct unit as a different species. Within this spectrum, the predominant opinion seemed to favour using only morphological criteria despite the fact that the International Code of Botanical Nomenclature (ICBN) accommodates molecular-based species definitions (Reynolds and Taylor 1991). Clearly, a definitive resolution to this issue is beyond the scope of this paper. Instead, we briefly explore how distinct ribotype groups may prove helpful in recognizing HAB species. The data presented here were obtained for phenotypic and genotypic characterization of heterotrophic dinoflagellates related to *Pfiesteria piscicida*. Morphologically, a subgroup of these isolates could be assigned to two morphologically well-defined species, *Luciella masanensis* and *L. atlantis*. When the internal transcribed spacer (ITS) rDNA regions from these isolates were sequenced and aligned, they fell into 5 disjunct ribotype groups, one of which was associated solely with *L. atlantis* and four with *L. masanensis*. The results shown below indicate that all four of the *L. masanensis* ribotypes exhibited ITS divergences typical of distinct species (Mason *et al.* 2007). The *L. masanensis* ribotypes also exhibited subtle morphological differences that were statistically significant. Distinct ribotype groupings from morphologically similar isolates have been pos-

tulated to represent cryptic species (Montresor *et al.* 2003; Leblond *et al.* 2006). As more sequence data become available from multiple isolates, it is likely that additional distinct ribotype groups will be observed. Their existence raises an interesting question as to how these ribotypes might be used in identifying morphologically similar species.

### Materials and Methods

Single cell isolates of small, thecate, heterotrophic dinoflagellates having the Kofoidian plate formula of Po, cp, X, 4', 2a, 6'', 6c, PC, 5+s, 5''', 0p, and 2'''' were established as described in Litaker and Tester (2002). These isolates were assigned to either *L. masanensis* or *L. atlantis*. Multiple ITS rDNA region sequences were obtained from each isolate, aligned, and phylogenetic relationships between the ribotypes were estimated using MrBayes (Litaker *et al.* 2007). The phylogenetic results were plotted as an unrooted phylogram. Uncorrected pairwise ("p") distance values were also calculated to estimate the relatedness among the distinct ribotype groups revealed in the phylogenetic analysis. Previously, p values exceeding 0.04 substitutions per site were shown to be indicative of the genetic distance between different species (Litaker *et al.* 2007). Representative isolates from *L. masanensis* ribotypes 1, 3 & 4 were grown under the same conditions and preserved for SEM analysis (Mason *et al.* 2003). The ribotype 2 culture was lost before SEM analysis could be performed. The resulting SEM micrographs were scaled to the same magnification and subjected to a detailed morphometric analysis.

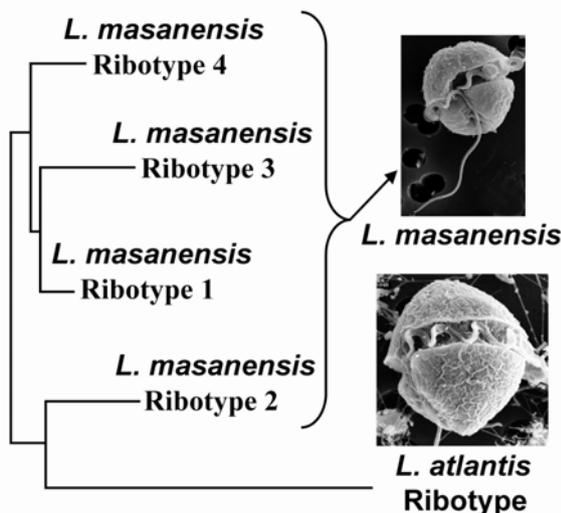
This was done to determine if any significant size or plate shape differences existed between ribotypes.

## Results

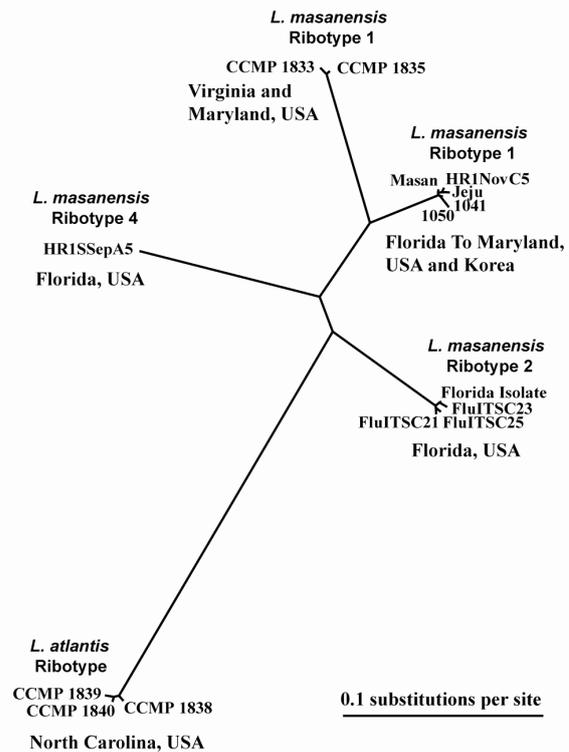
Morphologically, *L. atlantis* was found to be approximately twice as large as *L. masanensis* and to have hypotheca and epitheca that were distinctly more conical (Fig. 1). A phylogenetic analysis of the ITS/5.8S sequences from the *L. atlantis* and *L. masanensis* isolates fell into five distinct ribotype groups, one of which was associated with *L. atlantis* and the other four with *L. masanensis* (Fig. 2). The between-ribotype group p values for all 5 ribotypes exceeded 0.04 substitutions per site and were indicative of separate species (Table 1, Litaker *et al.* 2007). A detailed SEM analysis of the three *L. masanensis* ribotypes revealed statistical differences in cell length and width as well as in the surface area of the 1a, 2a and epicone surface areas (Table 2).

## Discussion

The genetic distances between the five *Luciella* ribotypes are consistent with species-level differences observed in the ITS/5.8S regions for 81 dinoflagellate species belonging to 14 different genera (Table 1, Litaker *et al.* 2007). For *L. atlantis*, a clearly distinctive morphology, associated with a single unique ribotype, can be interpreted as a new species (Fig. 1). The four *L. masanensis* ribotypes are more problematic. Interestingly, each ribotype exhibits morphological differences in plate size that were statistically significant. These combined data would suggest that the ribotypes represent distinct, morphologically cryptic species. Many taxonomists, however, would argue



**Figure 1.** Morphology of the various ribotypes. The *L. masanensis* ribotypes were morphologically similar except for subtle measurements shown in Table 2.



**Figure 2.** An unrooted phylogeny for the ITS/5.8S/ITS rDNA sequences illustrating the 5 different ribotype groups along with locality of collection.

**Table 1.** Uncorrected genetic distances between the five ribotype groups ranged from 0.07 to 0.17 substitutions per site.

	<i>L. masanensis</i> Ribotype 2	<i>L. masanensis</i> Ribotype 3	<i>L. masanensis</i> Ribotype 4	<i>L. atlantis</i> Ribotype
<i>L. masanensis</i> Ribotype 1	0.10	0.07	0.10	0.15
<i>L. masanensis</i> Ribotype 2	-----	0.10	0.10	0.14
<i>L. masanensis</i> Ribotype 3		-----	0.10	0.17
<i>L. masanensis</i> Ribotype 4			-----	0.14

that because of the similar overall morphology, the four ribotypes, despite significant differences in plate sizes, likely represent clonal variants of the same species. This conclusion is bolstered by the widely held belief that most dinoflagellates reproduce asexually and the observed ribotypes are likely due to divergent asexual lineages that have evolved over long periods of time. Recent research indicates that sexual reproduction in dinoflagellates is much more common than previously believed (Figuroa 2005). Sexual repro-

**Table 2.** Results for Student's t-test for cell length and width, as well as the 1a, 2a and epicone surface areas for *L. masanensis* ribotypes 1, 3 and 4. The culture of ribotype 2 was lost before it could be analyzed.

	Ribotype 3	Ribotype 4
	(N=22)	(N=25)
Cell Length	p<	p<
Ribotype 1 (N=20)	0.043	0.001
Ribotype 4	0.001	-----
Cell Width	(N=22)	(N=25)
Ribotype 1 (N=19)	0.697	0.001
Ribotype4	0.001	-----
Plate 1a Surface Area	(N=7)	(N=5)
Ribotype 1 (N=7)	0.045	0.004
Ribotype 4	0.002	-----
Plate 2a Surface Area	(N=7)	(N=5)
Ribotype 1 (N=9)	0.035	0.002
Ribotype 4	0.002	-----
Epicone Surface Area	(N=8)	(N=8)
Ribotype 1 (N=10)	0.489	0.001
Ribotype 4	0.002	-----

duction would tend to homogenize ITS ribotypes in a given geographical region suggesting that the identical ribotypes from different parts of the world represent distinct species rather than isolated species from asexual lineages (Fig. 2, Logares 2006).

Because a primary function of taxonomy is to provide classification to a level that is useful to the greater scientific community, there is a desire to avoid describing more species than is necessary. In the case of the *L. masanensis* ribotypes, no evidence exists that the ribotypes have different ecologies or vary toxicologically. Hence an argument for recognizing them as a single species, even if they represent genetically distinct species, can be made. In contrast, when similar species-level ribotype differences indicate potentially new HAB species, combining these ribotypes into a single species may prove less prudent without first establishing whether they vary in toxicologically, morphologically or in ecologically significant ways.

We suggest that when significant ribotype differences are first observed in data obtained from single-cell isolates or field isolated cells, that those cells be examined in detail morphologically. New species can

be established when both sets of data unequivocally support doing so. In other cases, genetically distinct ribotypes might be found that exhibit slight, but morphologically significant differences that are more difficult to classify. We advocate initially assigning these ribotypes to separate species or subspecies. We realize that this approach may represent undue species "splitting" in the opinion of many investigators. However, as more and more researchers rely on molecular tools for routine identification of HAB species, how species boundaries are decided will profoundly affect which particular genetic sequences are selected as a basis for "species-specific" assays. If the species boundaries are drawn too broadly, important genetically distinct species are likely to go unrecognized and any ecological or toxicological differences under appreciated.

The success of using genetic data will therefore depend largely on how well the within versus between-species variation is characterized (Litaker *et al.* 2007). If applied judiciously, and in conjunction with careful morphological study, it is likely that this approach will prove successful in identifying new species. As with any morphological approach, there will be instances where the genetic data fail to properly identify species or, as additional data become available, where species designations may need to be combined. These potential shortcomings, however, should not be used as justification for failing to explore the best methods for combining morphological and genetic data, including ribotype and other relevant gene sequences, in recognizing HAB species.

## References

- Figuroa R. I. (2005). Ph.D. Department of Ecology, Limnology and Marine Biology, Lund.
- Litaker, R.W. & Tester, P.A. (2002). Manual of Environmental Microbiology, 2nd Ed., Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J. & Stetzenbach, L.D. (eds), ASM Press, Washington, D.C., pp. 342-353.
- Litaker, R.W. *et al.* (2003). *J. Phycol.* 39: 754-761.
- Litaker, R.W. *et al.* (2007). *J. Phycol.* 43: 344-355.
- Logares, R.E. (2006). *Ecología Austral* 16: 85-90.
- Mason, P.L., Vogelbein, W.K., Haas, L.W. & Shields, J.D. (2003). *J. Phycol.* 39: 253-258.
- Mason, P.L. *et al.* (2007). *J. Phycol.* 43: 799-810.
- Montesor, M., Sgrosso, S., Procaccini, G. & Koistra, W.H.C.F. (2003). *J. Phycol.* 42: 56-70.
- Reynolds, D.R. & Taylor, J.W. (1991). *Taxon* 40: 311-315.
- Vacquier, V.D. (1998). *Science* 281: 1995-1998.

## 4. HAB MONITORING



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Monitoring PSP toxicity and *Alexandrium* hotspots in Scottish waters

E. Bresnan, E. Turrell and S. Fraser  
 Fisheries Research Services Marine Laboratory (FRS),  
 375 Victoria Road, Aberdeen, Scotland  
 E.bresnan@marlab.ac.uk

### Abstract

A programme of monitoring the Scottish coast for the presence of potential shellfish toxin-producing phytoplankton, in fulfilment of the European Shellfish Hygiene Directive (91/492/EEC), was operated 1996 - 2005. The programme identified a number of regional 'hotspots' for the presence of *Alexandrium* cells, particularly the Orkney and Shetland Islands. Over the monitoring period, decreased *Alexandrium* cell densities were recorded during early summer, correlating with lower PSP toxin concentrations in farmed mussels (*Mytilus edulis*).

### Introduction

The presence of *Alexandrium* species and paralytic shellfish poisoning (PSP) toxicity in *Mytilus edulis* in Scottish waters has been extensively documented (Bresnan *et al.* 2005; Howard *et al.* 2001, 2002, 2003). A monitoring programme in fulfilment of the EU 'Shellfish Hygiene Directive' 91/492/EEC has been in operation since 1990 to detect the presence of PSP toxicity in mussels and potential toxic phytoplankton species since 1996. During this time, considerable spatial and temporal variation has been observed in the occurrence of the potential toxin-producing *Alexandrium* species. Previous research has identified a range of species in Scottish waters, e.g. *A. tamarense* (North American strain) (Higman *et al.* 1997), *A. minutum* (FRS, unpublished data), *A. ostenfeldii* (John *et al.* 2000) and *A. tamutum* (Alpermann *et al.* this volume). This report summarises the data collected during the phytoplankton and PSP toxicity monitoring programme from its conception until 2005.

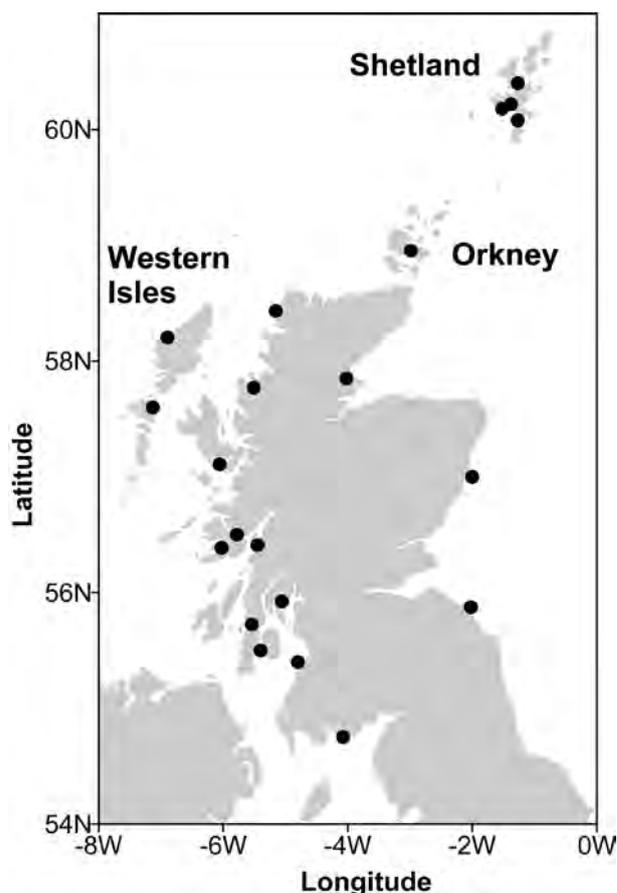
### Materials and Methods

Sampling sites included in the monitoring programme are shown in Fig. 1. Sites were chosen on the basis of their geographic location and relevance to shellfish harvesting areas.

Water samples for phytoplankton analysis were collected by volunteers around the Scottish coast. A sampling frequency of monthly from November to February and weekly from March to October was requested. Only sites showing a minimal annual sample return of 30 samples per year and a weekly return during the summer months were used to calculate monthly and annual means.

Samples were collected using a 10-m integrated tube sampler. Water samples were preserved in 0.5 % acidic Lugol's iodine solution. A 50-ml subsample was analysed on an inverted microscope using a modified Utermöhl technique (Kelly and Fraser 1998). *Alexandrium* cells were identified to genus level only.

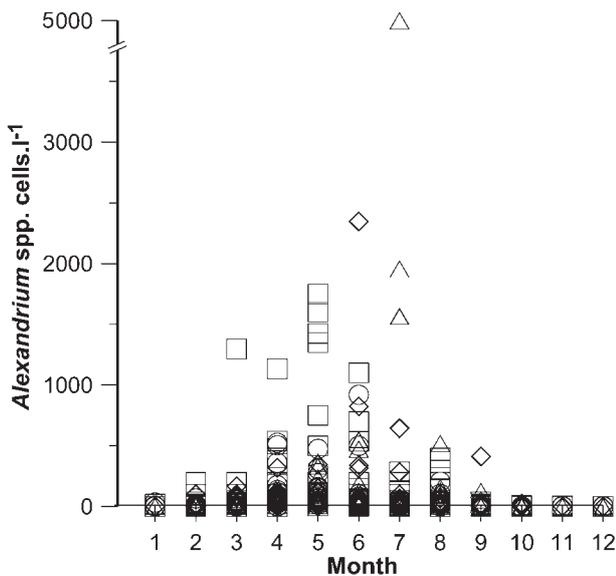
The level of PSP toxicity in *M. edulis* was determined by the AOAC mouse bioassay (Anon. 1990).



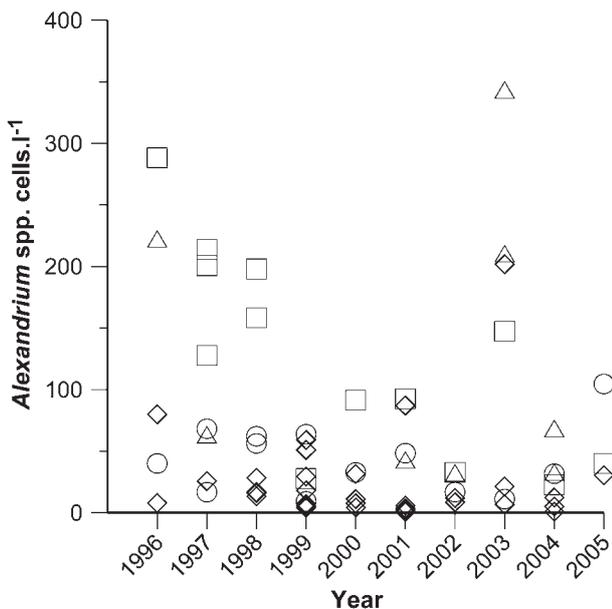
**Figure 1.** Location of phytoplankton sampling sites included in the toxic phytoplankton monitoring programme.

## Results

The monthly average of cells of *Alexandrium* spp. observed during the monitoring programme is shown in Fig. 2. A difference in the seasonality of occurrence of *Alexandrium* spp. is apparent with high numbers of cells observed in the spring and early summer months (April/May) particularly in the Orkney islands, whereas increased numbers were recorded in Shetland during July. *Alexandrium* spp. can be particularly toxic in Scottish waters with PSP toxicity  $>80 \mu\text{g STX eq. } 100 \text{ g}^{-1}$  recorded when *Alexandrium* concentrations



**Figure 2.** Monthly averages of cell concentrations of *Alexandrium* species along the east coast (○) west coast (◇), Orkney (□) and Shetland (△).



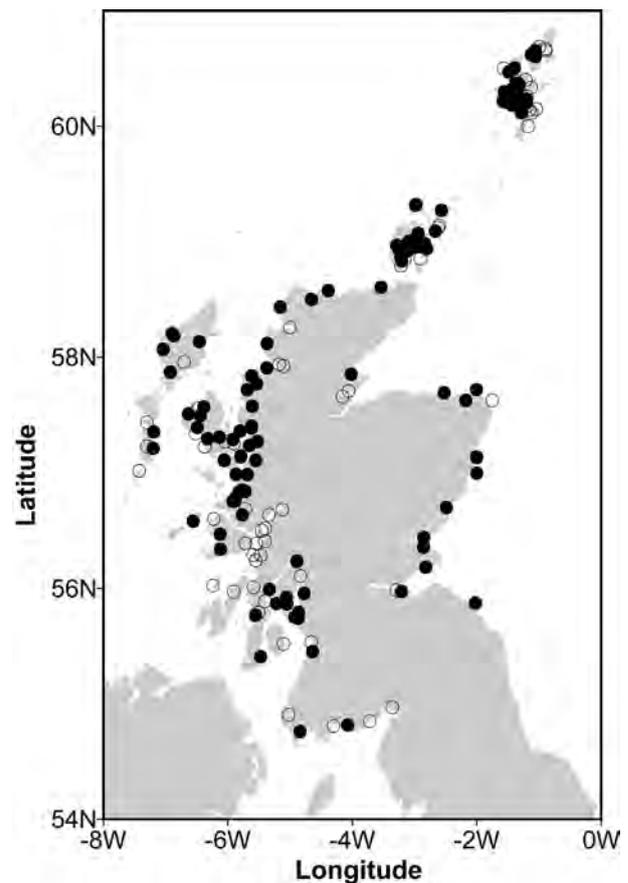
**Figure 3.** Annual averages of cell concentrations of *Alexandrium* species along the east coast (○), west coast (◇), Orkney (△) and Shetland (□).

of approximately 1,000 cells L<sup>-1</sup> have been observed (Kelly and Fraser 1998). A regionally high individual cell concentration was observed in the Western Isles during June 2003 (5,000 cells L<sup>-1</sup>) and Shetland in July 2003 where the highest individual concentration of *Alexandrium* cells recorded in the programme was observed (18,000 cells L<sup>-1</sup>).

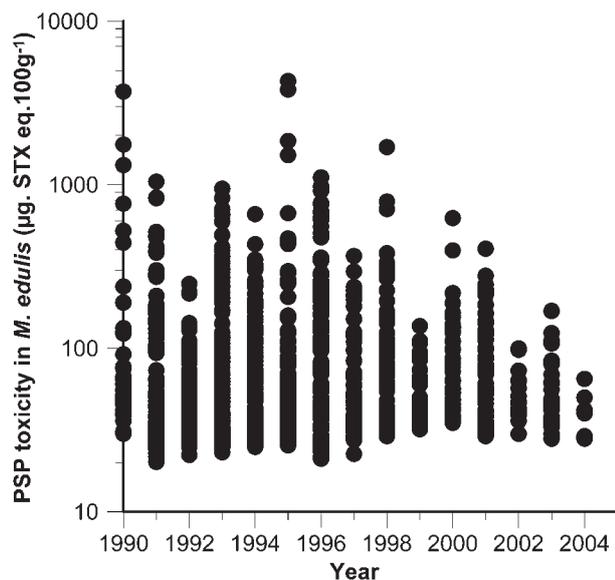
The annual average of *Alexandrium* cell concentrations, using data from all months, showed a general decline from 1996 to 2002, particularly in the Orkney Islands and along the Scottish east coast (Fig. 3). High cell concentrations were again observed during 2003.

PSP toxicity was detected in *M. edulis* around the Scottish coast since routine monitoring began in 1990 (Fig. 4), although in a localised area in the central west coast (56 °N) these toxins were apparently absent or below the detection limit of the mouse bioassay (approx. 40  $\mu\text{g STX eq. } 100 \text{ g}^{-1}$  shellfish flesh). The range of concentrations of PSP toxicity is shown in Fig. 5.

The downward trend of decreased *Alexandrium* cells observed during the monitoring period (certainly up to 2002) is reflected in the PSP data, which show a decrease in the concentrations of PSP toxicity in *M.*



**Figure 4.** Detection of PSP toxicity in *M. edulis* from 1990 to 2005. (●) PSP toxins detected, (○) PSP toxins not detected.



**Figure 5.** Log-transformed PSP toxicity ( $\mu\text{g STX eq. } 100 \text{ g}^{-1}$ ) in *M. edulis*.

*edulis* from Scottish waters (Fig. 5). Unfortunately, shellfish samples were not available for toxicity testing from the sites where elevated *Alexandrium* cell counts were recorded during 2003. Hence it is unknown if the increased cell concentrations resulted in elevated levels of PSP toxicity in shellfish. Elevated concentrations of *Alexandrium* and PSP toxicity in shellfish were once more reported during 2006 (Food Standards Agency Scotland, [www.food.gov.uk/scotland](http://www.food.gov.uk/scotland)).

Shellfish harvesting area closures were not enforced in 2004 due to PSP toxin levels that exceeded the regulatory limit, which contrasts sharply with widespread closures imposed during the early 1990s (Megginson and Bresnan 2005).

## Discussion

The Orkney and Shetland Islands emerge as 'hotspots' for *Alexandrium* species, with high cell concentrations being consistently detected. *Alexandrium tamarense* cyst beds have been observed in Orkney (Joyce 2004), as well as along the east coast of Scotland (Lewis *et al.* 1995; Joint *et al.* 1997). The cyst beds could act as seed populations for the observed *Alexandrium* events. One curiosity is the lack of detectable PSP toxicity in *M. edulis* from the Loch Linnhe area ( $56^\circ\text{N}$ ). Examination of the sediments for any *Alexandrium* cysts in this area would provide useful information for the future development of shellfish aquaculture.

Overall a general warming of Scottish coastal waters has been observed since the 1980s (Hughes 2004), however without longer-term studies of individual *Alexandrium* species dynamics the real influ-

ence of environmental variables on the PSP toxicity of Scottish shellfish is difficult to monitor. It is possible that the downward trend in the detection of PSP toxicity in Scottish shellfish 1990-2005 could be due to a change in the dominance of different *Alexandrium* species. A survey of the *Alexandrium* population in the Shetland and Western Isles using fluorescent in situ hybridisation probes (FISH) during the spring and autumn of 2005 highlighted the diversity within *Alexandrium* populations with *A. minutum*, *A. ostenfeldii* and *A. tamarense* (North American strain) detected (Turrell *et al.* 2006). The presence of *A. tamarense* (North American strain) was coincident with the presence of PSP positive Jellet rapid test phytoplankton kits during this study, suggesting that this species is associated with PSP toxicity in Scottish waters. The advent of molecular methods such as FISH and real time PCR means that these studies are now a real possibility on a regional scale.

## Acknowledgements

The monitoring programmes were funded by the Scottish Executive 1990-2000 and the Food Standards Agency Scotland 2001-2005. The authors acknowledge the contributions of Marie Kelly, Elspeth MacDonald, Nikki Smith, Lyndsay Brown, Godfrey Howard, John Turriff, Shona Kinnear, Dave Watson, Bob Andersen and Joyce Petrie.

## References

- Alpermann, T.J., Beszteri, B., Tillmann, U., Cembella, A.D. & John, U. (2008). Proc. 12th Int. Conf. Harmful Algal Blooms, this volume.
- Anonymous (1990). Official methods of Analysis of AOAC International. 15th ed. AOAC International, Arlington VA sec 959.08.
- Bresnan, E., Fryer, R., Hart, M. & Percy, L. (2005). FRS Contract Report 04/05, 58 pp.
- Higman, W.A., Stone, D.M. & Lewis, J.M. (2001). *Phycologia* 40: 256-262.
- Howard, G., Bresnan, E. & Petrie, J. (2003). *Shellfish News* 15: 39-42.
- Howard, G., Bresnan, E. & Petrie, J. (2002). *Shellfish News* 13: 34-46.
- Howard, G., Bresnan, E. & Petrie, J. (2001). *Shellfish News* 11: 36-38.
- John, U., Cembella, A., Hummert, C., Elbrächter, M., Groben, R. & Medlin, L. (2003). *Eur. J. Phycol.* 38: 25-40.
- Joint, I., Lewis, J., Aiken, J., Proctor, R., Moore, G., Higman, W. & Donald, M. (1997). *J. Plankton*

- Res. 19: 937-956.
- Hughes, S.L. (2004). FRS Internal Report 12/04, 50 pp.
- Joyce, L.B. (2004). *Bot. Mar.* 47: 173-183.
- Kelly, M.C. & Fraser, S. (1998). *Fish. Res. Services Report* 06/98, 43 pp.
- Lewis, J., Higman, W. & Kuenster, S. (1995). In: *Harmful Marine Algal Blooms*, Lassus, P., Arzul, G., Erard, E., Gentesn, P. & Marcaillou, C. (eds), Lavoiser, Intercept Ltd., pp. 175–180.
- Meggison, C. & Bresnan, E (2005). *Shellfish News* 19: 29–30.
- Turrell, E., Bresnan, E., Collins, C., Brown, L., Graham, J. & Grieve, M. (2006). FRS Internal Report 26/06, 112 pp.

## The monitoring programme for harmful algal blooms in shellfish production areas in Catalonia. Long term data and impact on aquaculture

J. Diogène<sup>1,2</sup>, M. Fernández<sup>1,2</sup>, E. Cañete<sup>1,2</sup>, A. Caillaud<sup>1,2</sup>, E. Mallat<sup>1,2</sup>, M. Delgado<sup>3</sup> and D. Furones<sup>1,2</sup>

<sup>1</sup>IRTA, Sant Carles de la Ràpita, Ctra. de Poble Nou, Km 5,5. E-13540 Sant Carles de la Ràpita (Tarragona), Spain, jorge.diogene@irta.es

<sup>2</sup>XRAq, Xarxa de Referència en Aqüicultura, CIRIT-Generalitat de Catalunya, Spain

<sup>3</sup>Institut de Ciències del Mar (CMIMA, CSIC). Passeig Marítim de la Barceloneta, 37-49, E-08003 Barcelona. Spain

### Abstract

The monitoring programme for harmful algal blooms (HABs) in shellfish production areas in Catalonia focuses mainly on shellfish harvesting areas in sandy sediments along the shore (mainly natural production sites of clams) and production areas within the semi-confined areas (mainly oyster and mussel cultures). HAB incidences in production areas include recurrent events with presence of toxins in shellfish leading to DSP (e.g., *Dinophysis sacculus*), PSP (e.g., *Alexandrium minutum*) and to a much lesser extent ichthyotoxic events (*Karlodinium* spp.). An estimate of the percentage of closure periods indicates that annually these figures have increased since 1989, DSP events being the major cause for administrative closures. For the period 2002-2005 closure time in shellfish production areas reached maxima of 23 % (84 days in Alfacs Bay during 2002 for DSP) and 17 % (62 days in Alfacs Bay during 2005 for DSP). The economic impact of closures is irregular and depends on the time of the year closures occur.

### Introduction

The Catalan coast comprises approx. 450-580 km of coast along the NW Mediterranean Sea. Major shellfish harvesting sites include sandy beaches and coastal embayments. The monitoring programme for Harmful Algal Blooms (HABs) in Catalonia was established in 1989 and has developed in response to: i) changes in legal regulations, ii) scientific advancement, iii) technical advancement and iv) descriptive studies and long-term analysis of local characteristics. We present long-term data to evaluate the impact of marine toxins on aquaculture. The data contribute to the understanding of shellfish production areas and are needed to define efficient monitoring programmes.

### Material and Methods

Main parameters evaluated within the monitoring programme for HABs include water temperature, salinity, oxygen, chlorophyll-*a* content, density of phytoplankton species, toxin content in shellfish (PSP, DSP, ASP toxins). PSP and DSP in bivalves are evaluated using the AOAC mouse bioassays for PSP (AOAC 1995), DSP (Yasumoto 1978) and the HPLC method (UV detection) for ASP toxins (Lawrence *et al.* 1991). Sample location and sampling frequency are adapted to shellfish harvesting area characteristics. In major production areas, evaluation of PSP and DSP and phytoplankton samples are taken weekly. ASP content in shellfish is conducted every 15 days (Furones *et al.* 2006).

### Results

*HABs in shellfish harvesting sites within semi-confined areas (Alfacs and Fangar Bay)*

The major shellfish production in Catalonia is located in the Alfacs and Fangar Bays in the Ebre Delta at the southern end of the Catalan coastline. These coastal embayments are shallow water bodies (max. 6 m deep) influenced by Mediterranean climate, NE-SW offshore dominant currents and freshwater runoff from agriculture. Within the bays high production of shellfish includes mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) in rafts, and clams (*Ruditapes philippinarum* and *Ruditapes decussatus*) in sandy sediments. Confinement reduces water exchange, increases water residence time and can lead in summer to high water temperatures (30-32 °C maxima) and oxygen depletion. Examples of HAB species observed are *Alexandrium minutum*, *A. catenella* (PSP), *Dinophysis sacculus*, *D. caudata* (DSP) and *Pseudo-nitzschia* spp. (ASP). Ichthyotoxic species (*Karlodinium* spp.) are also present. HAB population dynamics is influenced by environmental parameters, offshore advection, presence of cysts and shellfish translocation.

*Shellfish harvesting areas along the Catalan coastline. Sandy sediments*

These areas are mainly dominated by the production of clams (*Ruditapes decussatus*, *Callista chione*, *Donax trunculus*), and cockle (*Cerastoderma edule*).

Examples of HAB species observed are *Alexandrium minutum*, *A. catenella* (PSP), *Dinophysis sacculus*, *D. caudata* (PSP) and *Pseudo-nitzschia* spp. (ASP). The presence of harbours along the Catalan coastline may lead to increased densities of HAB species exported along the Catalan coastline southward by NE-SW off-shore dominant currents (Furones *et al.* 2006).

#### HAB incidences in production areas

These include recurrent DSP (e.g., *Dinophysis sacculus*), PSP (e.g., *Alexandrium minutum*) and to a much lesser extent ichthyotoxic events (*Karlodinium* spp.). As an example, maxima for Alfacs Bay are presented in Table 1.

**Table 1.** Maximum cell densities and month of year for selected harmful algae in Alfacs Bay from 1999-2005 (cells/L).

	<i>A. minutum</i>		<i>D. sacculus</i>		<i>Karlodinium</i> spp.	
	Cell Density	Month	Cell Density	Month	Cell Density	Month
1999	11 375	May	2920	May	233 870	Dec.
2000	18 880	April	14 440	May	942 400	Feb.
2001	3 200	March	17 200	June	10 400	April
2002	28 400	April	700	Nov.	2 000	May
2003	11 200	May	15 600	May	1 112 360	April
2004	5 200	March	140	April	15 600	June
2005	4 000	May	6 800	May	61 600	June

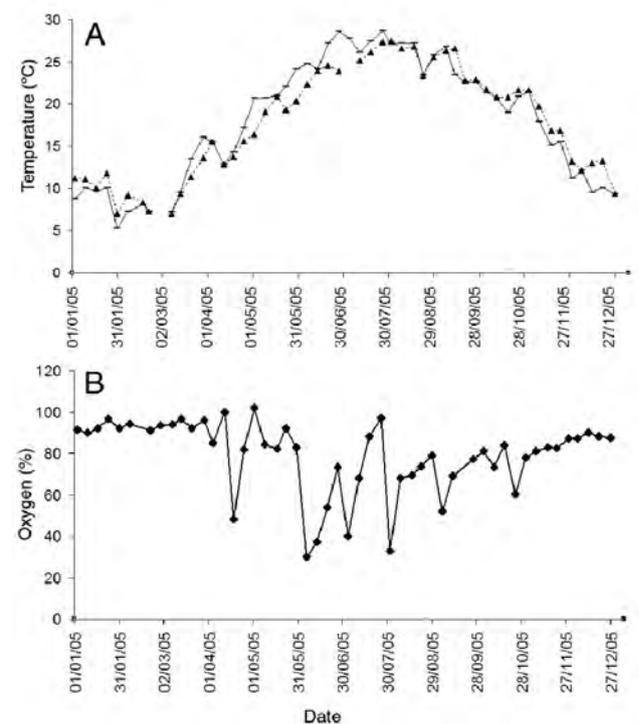
During the period 1999-2005, maximum or high densities for *A. minutum* occurred in January-June, for *D. sacculus* in April-November and for *Karlodinium* spp. in November-July. The percentage of closure periods indicates that annually these figures have increased since 1989, DSP events being the major cause for administrative closures (Table 2). PSP events are less frequent, but have occasionally caused important episodes such as the *A. catenella* bloom of 2001 that affected mainly production areas south of Barcelona. In 2002-2005 closure time in shellfish production areas reached maxima of 23 % (84 days in Alfacs Bay during 2002 for DSP) and 17 % (62 days in Alfacs Bay during 2005 for DSP). No closures for ASP have been ever recorded in Catalonia, and no domoic acid has been detected in shellfish.

#### Mediterranean climate influence on shellfish

One of the characteristics of the Mediterranean climate that influences HAB population dynamics and shellfish harvesting areas is a wide surface water temperature difference of approximately 20 °C between

**Table 2.** Number of closures of shellfish harvesting areas for toxins in Catalonia from 1989 to 2005 and months of closures.

	ALFACS BAY		FANGAR BAY		REST OF THE COAST	
	PSP	DSP	PSP	DSP	PSP	DSP
1989-2002	2 May Jan.	5 Jan. Mar. Jul. Aug. Sep. Nov.	1 Nov.	1 Jul.	3 Jun. Jul.	0
2003	0	1 May. Jun.	0	2 Aug. Oct.	1 May	0
2004	0	1 Dec.	0	2 Aug Nov.	0	0
2005	0	3 Jan. Feb. Jul. Dec.	0	0	0	3 Jun. Jul. Aug.



**Figure 1.** Alfacs Bay : A) water temperature at 0,5 m (solid line) and at 6 m depth (dotted line) at central station and B) oxygen minima records in 2005.

winter and summer, reaching maxima of about 30 °C in early summer. Mussel mass mortalities as a consequence of summer high temperatures (above 30 °C) have been recorded in 2001, 2003 and 2006. Oxygen minima are also present in summer (Fig. 1).

**Table 3.** Interactive approach between the Catalan monitoring programme for HAB in shellfish-harvesting areas and applied and fundamental research.

HAB monitoring specific objectives	Major parameters evaluated and tasks	OUTPUT 1: Management of shellfish production areas	OUTPUT 2: Link with research projects
Describe environmental variables	- Temperature - Salinity - Oxygen content - Stratification	- Optimize location of production sites. - Management of shellfish production (e.g. overcome temperature stress).	- Description of harvesting areas: structures and processes. - Define physical conditions that may correlate with HABs (e.g. stratification, autecology of key HAB species).
Record densities of major phytoplankton species with particular emphasis on harmful microalgae and bloom forming species.	- Chlorophyll- <i>a</i> - HAB microalgal identification and density estimation.	- Management of shellfish harvesting area: Issue warnings and eventually preventive closure of harvesting areas due to high densities of harmful microalgae.	- HAB population dynamics and historical series. - Key information for the presence of key HAB species in order to obtain cultures or individuals from the field (e.g. for toxin, physiological and phylogenetic studies) - Field and laboratory exposure of shellfish to HAB in order to obtain toxic material.
Identify and quantify toxins present in shellfish.	- Evaluate PSP, DSP and ASP toxin content in shellfish. - Accomplish ongoing regulations using reference methods.	- Manage administrative closures of shellfish harvesting areas. Issue warnings and closures according to ongoing regulations for toxins.	- Identify toxins, toxin derivatives and metabolic transformations in shellfish. - Predictive models to study toxin kinetics in shellfish: incorporation and release of toxins.
Incorporate innovative and complementary methodologies and approaches	- Current actions (Sept 2006): LC-MS and HPLC toxin determination. Dissolved lipophilic toxins in water. Additional toxins in shellfish. Develop field and laboratory methodologies.	- Current actions (Sept 2006): Obtain toxin profiles in shellfish. Confirmation of dubious results in conflictive samples. Toxicity risk assessment from dissolved toxins in water.	- Improve toxin studies for HAB and shellfish. - Apply specific information to understand local ecosystems and eventually extend it to other ecosystems.

## Discussion

The economic impact of closures is irregular and depends also on the time of the year. For example, due to unbalanced commercial demand along the year, closures before Christmas affect mainly oyster producers. In regard to mussel production in the Ebre Delta coastal embayments (3000 - 4500 Tm /year), high summer temperatures have led to 100 % mortality. Closures in spring strongly affect mussel producers who plan to harvest all the production before summer. Analysis of the local characteristics is therefore crucial for the design and implementation of the monitoring program and also for the management of shellfish harvesting areas. The information generated by the monitoring program since 1989 is in that sense communicated to the aquaculture sector in order to optimize productivity of the system and reduce the impact of HABs. Additionally, the HAB monitoring program stands as a unique platform to interact with scientific research projects as well as to improve techniques and experimental approaches (Table 3).

## Acknowledgements

This project was funded by: /DGPiAM, Fisheries Department, Generalitat de Catalunya/IFOP Program, European Union /INIA. We kindly acknowledge the collaboration and data exchange with ICM-CSIC, ACA, Generalitat de Catalunya and support received by technical staff from IRTA.

## References

- AOAC (1995). Association of Official Analytical Chemists, sec 959.08. Chapter 35: 21-22.
- Furones, D., Vila, M., Garcés, E., Sampedro, N., Arin, L., Masó, M., Camp, J., van Lenning, K., Quijano-Scheggia, S., Delgado, M., Fernández, M., Mallat, E., Cañete, E., Caillaud, A. & Diogène J. (2006). In: Henswood, K., Deegan, B., McMahon, T., Cusack, C., Keaveney, S., Silke, J., O'Conneide, M., Lyons, D. & Hess, P. (eds) Proc. 5th Int. Conf. Molluscan Shellfish Safety, Galway, Ireland, June 14th-18th, 2004, pp. 197-205
- Lawrence, J.F., Charbonneau, C.F. & Menard, C. (1991). J. Assoc. Off. Anal. Chem. 74: 68-72.
- Yasumoto, T., Oshima, Y. & Yamaguchi, M. (1978). Bull. Jap. Soc. Sci. Fish. 44: 1249-1255.

## Phytoplankton community composition observed by autonomous underwater vehicles

G.J. Kirkpatrick<sup>1</sup>, D.F. Millie<sup>2</sup>, M.A. Moline<sup>3</sup>, S.E. Lohrenz<sup>4</sup> and O.M. Schofield<sup>5</sup>

<sup>1</sup>Mote Marine Laboratory, Sarasota, FL, 34236, gkirkpat@mote.org, <sup>2</sup>Florida Institute of Oceanography, St. Petersburg, Florida, 33701, david.millie@myfwc.com, <sup>3</sup>California Polytechnic State University, San Luis Obispo, CA, 93407, mmoline@calpoly.edu, <sup>4</sup>University of Southern Mississippi, Stennis Space Center, MS, 39529, steven.lohrenz@usm.edu, <sup>5</sup>Rutgers University, New Brunswick, NJ, 08903, oscar@marine.rutgers.edu.

### Abstract

Laboratory and field studies have demonstrated the feasibility of detecting *Karenia brevis* blooms in the eastern Gulf of Mexico utilizing light absorbance spectra. Development of this technique has aimed at providing more timely access to data and information on the initiation, transport, and effects of *K. brevis* blooms. Management efforts to mitigate the harmful effects of blooms will require temporal and spatial monitoring of phytoplankton community taxonomic composition and dynamics. To achieve taxonomic discrimination, laboratory cultures of 12 species of microalgae representing five taxonomic classes were used to develop a library of target classes. A fitting routine involving multiple least-squares analyses was applied to BreveBuster absorbance spectra to determine the 'best fit' estimates of chlorophyll a concentration contributed by each class in both laboratory culture mixes and natural mixed populations. A 10-day deployment of a BreveBuster on an autonomous underwater vehicle (AUV) off the west coast of Florida in September and October 2004 detected a *Karenia brevis* population associated with cyanobacteria and diatom populations which had not been observed by manual sampling. Multiple transects across the shelf by the AUV over this period illustrated the spatial and temporal dynamics of the phytoplankton community.

### Introduction

Harmful algal blooms in the Eastern Gulf of Mexico are most often composed of toxic dinoflagellates from the genus *Karenia*. Though much has been learned about the organism and its blooms, little is known about the transitions in phytoplankton community structure during the very early and late stages of the blooms.

Mitigation of some of the harmful effects of the blooms depends on an ability to detect the initiation stage and forecast development and transport. For instance, shellfish aquaculture operations can effectively utilize a few days early warning of a developing bloom to harvest or move their crop. An understanding of species succession sequences leading to and following a mono-specific *Karenia* bloom may uncover the processes necessary to form a bloom and hence a means to forecast the bloom and its demise. However, acquiring this knowledge requires continuous presence in areas of potential bloom development and continuous evaluation of the phytoplankton community structure.

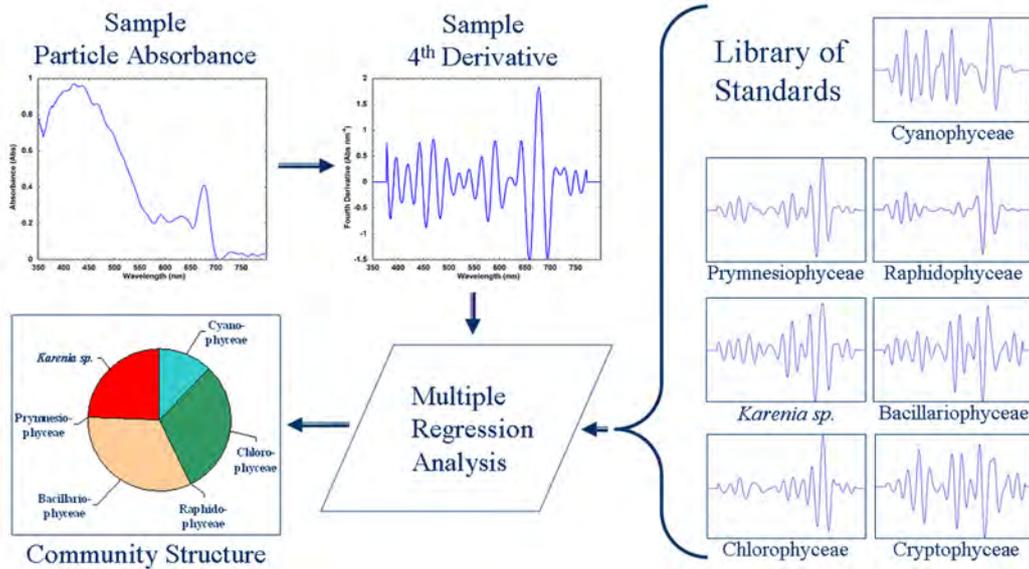
Recent advances in automation of optical instrumentation have provided a means to continuously estimate the phytoplankton community class structure on fixed and mobile underwater platforms. This pa-

per reports results from two field deployments of the optical phytoplankton discriminator referred to as the BreveBuster (due to its origins as a *Karenia brevis* detector) on two types of autonomous underwater vehicles.

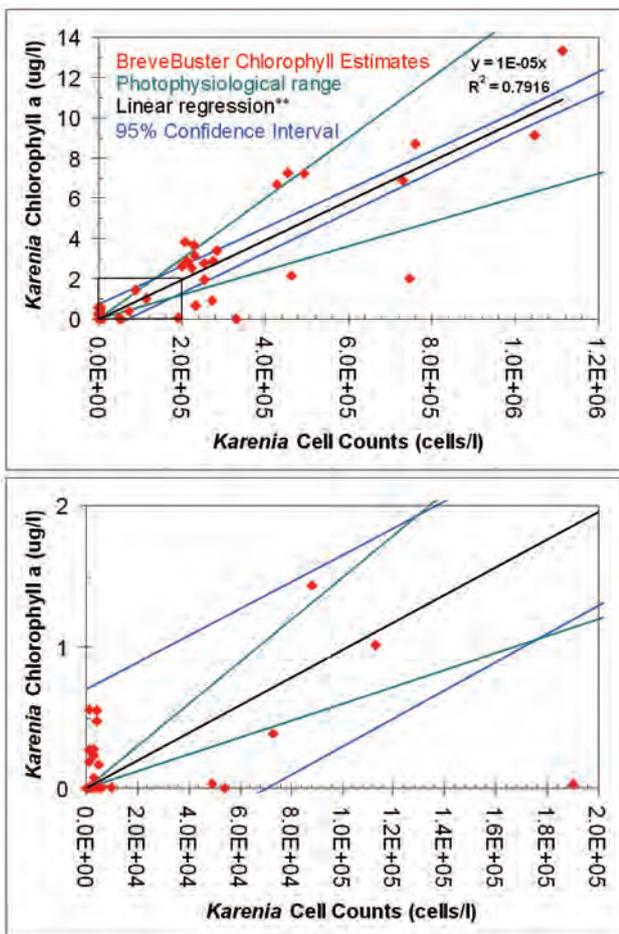
### Methods

Field deployments of the BreveBuster (Kirkpatrick *et al.* 2000; Robbins 2006) were conducted using autonomous underwater vehicles. A buoyancy propelled Slocum glider (Webb Research Corp) repeatedly traversed a cross-shelf survey between 28 September and 12 October 2004. On 21 January 2005 a propeller-driven REMUS (Hydroid, Inc.) conducted two circuits of a 5-km long survey line at two depths 15 km offshore from Sarasota, Florida in an area known to contain moderate-high concentration of *Karenia brevis*.

The class discrimination algorithm used by the BreveBuster fits a set of known 'standard' absorbance fourth derivative spectra to sample fourth derivative spectra collected during the deployments using multiple regression analysis (Fig. 1). The resulting set of regression coefficients provided estimates of the chlorophyll biomass of each taxonomic class present in the sample. When applied to natural phytoplankton



**Figure 1.** Flowchart of taxonomic class discrimination algorithm used by the BreveBuster.



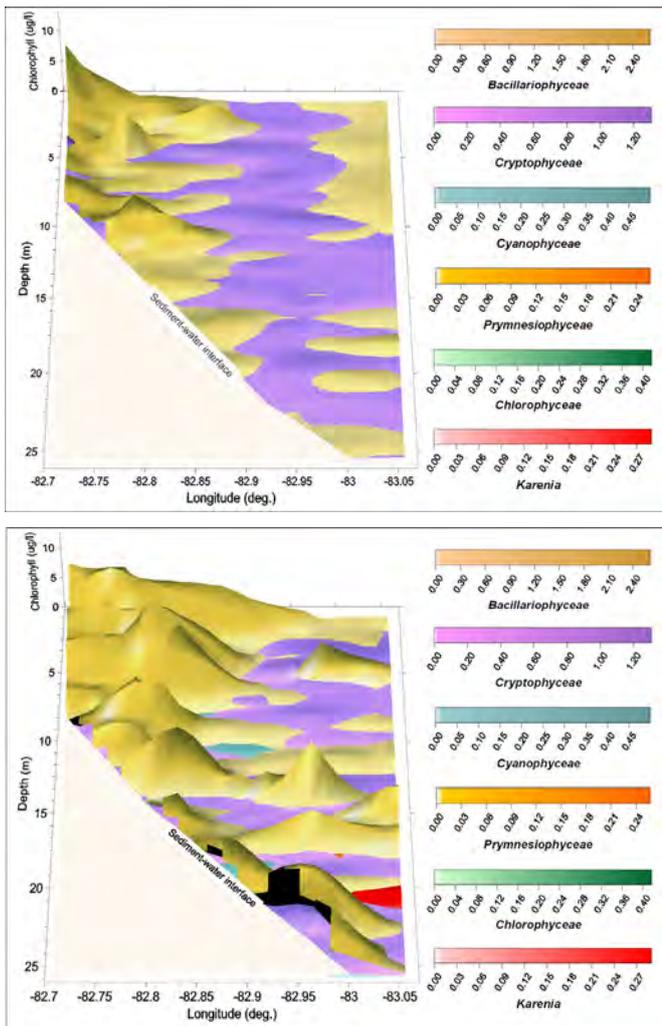
**Figure 2.** The relationship between BreveBuster estimated *Karenia* sp. chlorophyll a and the *Karenia* sp. cell count. A significant linear regression is indicated by the black line and the equation in the upper-right corner of the upper panel. The green lines indicate the typical range of cellular chlorophyll content for *Karenia brevis*. The bottom panel is an expanded view of the cell count range from 0 to 200,000 cells per liter.

communities, the taxonomic class discrimination algorithm successfully estimated the concentration of *Karenia* sp. compared to microscopic enumeration. The amount of chlorophyll a per *Karenia* sp. cell was estimated at 10 pg/cell using this method (Fig. 2), the value commonly cited as the average for *K. brevis* (Shanley and Vargo 1993; Tester *et al.* 1998; Higham *et al.* 2004).

## Results

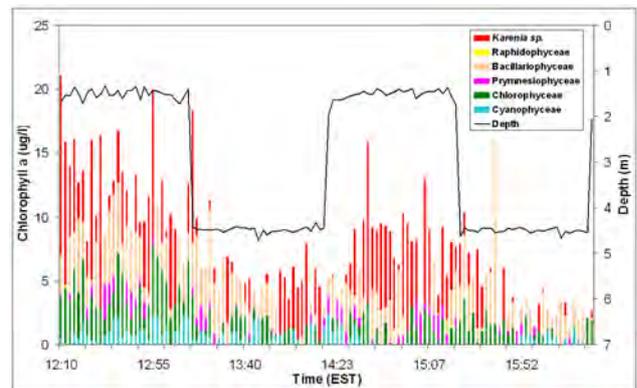
The Slocum Glider mission in 2004 yielded eight days of spectral absorbance data for taxonomic class discrimination analysis. The fitting routine produced 145 fits with coefficients of determination above 0.6 for the first 2-day transit 50 km offshore. Fits with c.o.d. less than 0.6 were not used in this evaluation. Six of the seven taxonomic classes in the library of standards were detected during the first and second transits (Fig. 3). During the first transit, diatoms dominated most of survey area, with the exception of the central portion of the transit where Cryptophyceae dominated. *Karenia* sp. were only found at low concentration in the lower water column in the outer half of the transit. This community distribution was similar during the second 2-day transit across the shelf with the exceptions that diatoms were less prevalent offshore and that *Karenia* sp. were detected higher in the water column nearshore as well as near the bottom offshore.

The REMUS mission conducted in January 2005 produced 120 useable class distributions (Fig. 4). The BreveBuster community structure analysis indicated that the chlorophyll biomass was highest along the near-surface (1.5 m) track. This agrees with the chlorophyll fluorometer findings of Robbins *et al.* (2006).



**Figure 3.** Three-dimensional perspective cross sections of chlorophyll a biomass of the taxonomic classes detected by the BreveBuster. The BreveBuster was carried in the payload section of a Slocum glider as it undulated between 2 meters below the surface and 2 meters above the bottom. Panel A depicts results from the first transit of a survey line across the inner shelf off Sarasota, Florida, 28-30 September 2004. Panel B depicts the second transit, 1-3 October 2004. The color bars represent chlorophyll a concentration for each class and have the units of micrograms per liter.

Although there were indications of high *Karenia* sp. biomass during the first pass along the surface track the community was composed of a variety of taxonomic classes. However, the second pass along the surface track found a phytoplankton community structure dominated by *Karenia* sp. These findings can be explained through a combination of water mass advection (Robbins *et al.* 2006) and vertical migration by *Karenia* sp.



**Figure 4.** The taxonomic class distribution observed by a BreveBuster carried in a REMUS autonomous underwater vehicle operating in a *Karenia brevis* bloom off Sarasota, Florida on 21 January 2005. The solid black line represents the depth of the REMUS.

### Acknowledgements

The authors wish to recognize the essential contributions provided by those who prepared the equipment and conducted the deployments. These results would not exist if not for the dedication and perseverance of Jim Hillier, Liz Creed, Brad Pederson, John Kerfoot, Ian Robbins, Barb Berg, Shelley Blackwell, Cory Boyes, Augie Kotlewski, and the late Captain Paul Rocshe. Funding support was provided by the U.S. National Oceanic and Atmospheric Administration, the Florida Fish and Wildlife Conservation Commission, the U.S. National Science Foundation, and the U.S. Office of Naval Research.

### References

- Higham, C.J., Kirkpatrick, G.J., Pederson, B.A. Berg, B.A. & Millie, D.F. (2004). In: Harmful Algae 2002, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), FFWCC, FIO, and IOC UNESCO, St Petersburg, pp. 417-419.
- Robbins, I.C., Kirkpatrick, G.J., Blackwell, S.M., Hillier, J., Knight, C.A. & Moline, M.A. (2006). Harmful Algae. doi:10.1016/j.hal.2006.03.005.
- Kirkpatrick, G.J., Millie, D.F., Moline, M.A. & Schofield, O.M. (2000). *Limnol. Oceanogr.* 45: 467-471.
- Shanley, E. & Vargo, G.A. (1993). In: Toxic Phytoplankton Blooms in the Sea, Smayda, T. J., Shimizu, Y. (eds), Elsevier, New York, pp. 919-923.
- Tester, P.A., Stumpf, R.P. & Steidinger, K.A. (1998). In: Harmful Algae, Reguera, B., Blanco, B., Fernandez, J. & Wyatt, T. (eds), UNESCO, Paris, pp. 149-151.

## Monitoring a bloom of *Pyrodinium bahamense* var. *compressum* in El Salvador and the southern coast of Mexico (November 2005 - March 2006)

S. Licea<sup>1</sup>, A. Navarrete<sup>2</sup>, J. Bustillos<sup>3</sup> and B. Martínez<sup>4</sup>

<sup>1</sup> Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México (UNAM), 04510 México D. F. Apartado Postal 70-305, licea@mar.icmyl.unam.mx; <sup>2</sup>CENSALUD Universidad de El Salvador, Final 25 Av. Norte, Cd. Universitaria, San Salvador, armando\_microalgas@yahoo.com; <sup>3</sup>Northwest Biological Research Center POB 128, La Paz B.C.S. Mexico 23000, jose04@cibnor.mx; <sup>4</sup>Centro de Ciencias de la Atmósfera, UNAM, benmar@troposfera.atmosfcu.unam.mx

### Abstract

The Pacific coast of Central America is often impacted by blooms *P. bahamense* var. *compressum*, resulting in human illnesses and deaths, and affecting the local sea turtle populations through food web interactions. During a bloom in November 2005-March 2006 in El Salvador, 3 persons died and 7 became ill, while 206 turtles (mostly *Lepidochelis olivacea* and *Chelone mydas*) died, and large quantities of jellyfish (*Stomolophus meleagris*) were found on the beaches. Environmental conditions at the time showed a positive temperature anomaly of 1.5 °C between Southern Mexico and Costa Rica. Chlorophyll-*a* concentrations were 0.5-1.0 mg m<sup>-3</sup> and numbers of *P. bahamense* var. *compressum* ranged from 48,900 cell ml<sup>-1</sup> in December 2005 to a minimum of 15 cells ml<sup>-1</sup> in March 2006. The concentration of saxitoxin was 27.9-627.8 µg STX eq/100 g in turtles, 11.7-12.2 µg STX eq/100 g in live jellyfish, and 60.4 to >4000 µg STX eq/100 g in shellfish, all measurements from Salvadorian beaches. Unpublished data from Mexico collected between December 1989 and February 2002 revealed blooms of *P. bahamense* var. *compressum* at the same time (winter/spring) associated with the increased surface water temperature.

### Introduction

The Pacific coast of Central America is often impacted by blooms of *Pyrodinium bahamense* var. *compressum* during autumn and winter, forcing closure of the shellfish industry. In spite of the effects on human health, economic activities and the environment, there is still a lack of information on the subject, apart from sporadic observations during notable blooms. Recent blooms in Central America (Mata *et al.* 1990; Vargas-Montero and Freer 2003), occurred off the Guatemala coast in July-August 1987 (Rosales-Loessener *et al.* 1989) and in El Salvador in August 2001-January 2002 (Barraza *et al.* 2004). Environmental data collected during this period suggested that *Pyrodinium* blooms may be influenced by sea surface temperature, wind force and the effect of rain.

In the southwestern part of Mexico, Cortés-Altamirano *et al.* (1993) reported an outbreak of *Pyrodinium bahamense* var. *compressum* in December 1985 and in November 1996; Orellana *et al.* (1998) found a bloom in the same area. Several reports have been referred to on similar dates in Guatemala, Panama and Mexico (Sierra-Beltrán *et al.* 2004).

In the beginning of November 2005, Nicaragua reported water discolouration covering a large area in the Gulf of Fonseca. Subsequently, the Board of

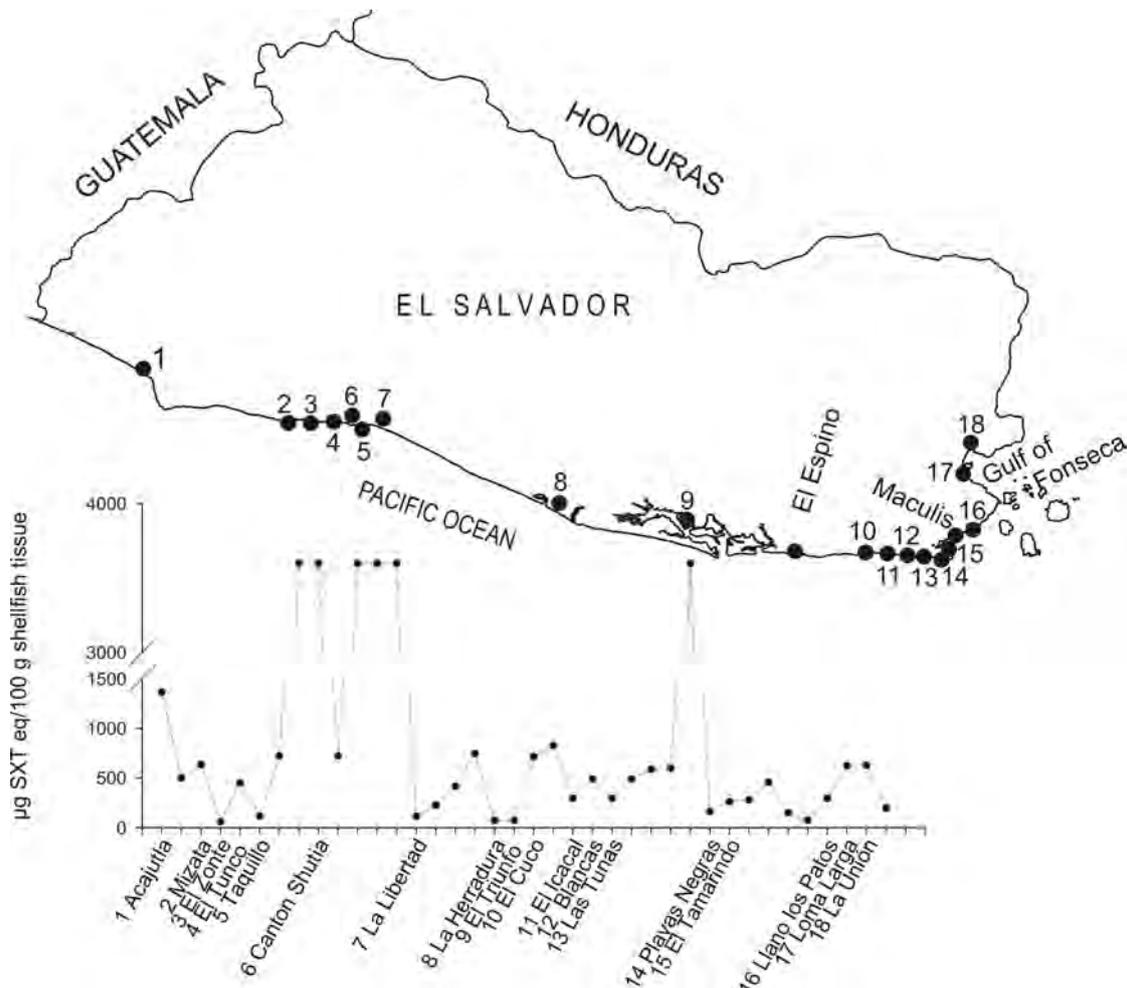
Health and Social Assistance (MSPAS), the Institute for Marine Sciences of El Salvador (ICMARES) and the National Committee for the Study of Red Tides (CONAMAR) initiated sampling along the coasts of El Salvador. *Pyrodinium bahamense* var. *compressum* was the species responsible for the discolouration. This study reports on the toxicity caused by an exceptional bloom of this species associated with oceanographic conditions.

### Material and Methods

To evaluate the progression of the *Pyrodinium* bloom a programme was established along the coast of El Salvador following the observed water discolouration. Samples for invertebrates were collected by hand from natural banks to analyze for saxitoxin (STX) by mouse bioassay and HPLC methods. The samples were frozen (-20 °C) until analysis. *Pyrodinium* monitoring was conducted at 24 sites along the Salvadoran coast (Fig. 1). Water samples were collected at each site for cell counting by the Utermöhl method (Hasle 1978). The Scanning Electron Microscope was used to positively identify the species.

### HPLC

For each turtle tissue and shellfish, about 200 mg were homogenized for toxin extraction. In the case of



**Figure 1.** Monitoring sites along the El Salvador coast. The graph shows saxitoxin levels found at monitoring sites along the El Salvador coast from 16 November to the end of December 2005. At Las Tunas, the maximum value was found in *Protothaca asperrima*, at Taquillo in *Crassostrea iridescens*, and at Shutia in *C. iridescens* and *C. corteziensis*.

jellyfish, three samples with 4-5 organisms were obtained. Jellyfish from each zone were put together and homogenized. Extraction of PSP-toxins was carried out by adding 4 ml of acetic acid (0.03N) to each sample; sonicating (35 KHz) each sample for 5 minutes in an ice bath and then clarifying each by centrifugation (3000 rpm for 5 min). The resulting supernatant was filtered with a single-use syringe filter (0.45 µm). An aliquot (150 µl) of the clarified extract was hydrolysed with HCl (1M). Ten µl of hydrolysed and unhydrolysed extracts were injected into the HPLC system (HP 1100). Chromatography was performed as indicated by Hummert *et al.* (1997) and Yu *et al.* (1998). Identification of PSP toxins was carried out by comparing chromatograms obtained from sample extracts with those resulting after the injection of standard solutions (National Research Council Canada). Toxicity factors to transform the analogs to saxitoxin equivalents were those given by Oshima (1995). Results are given in Table 1.

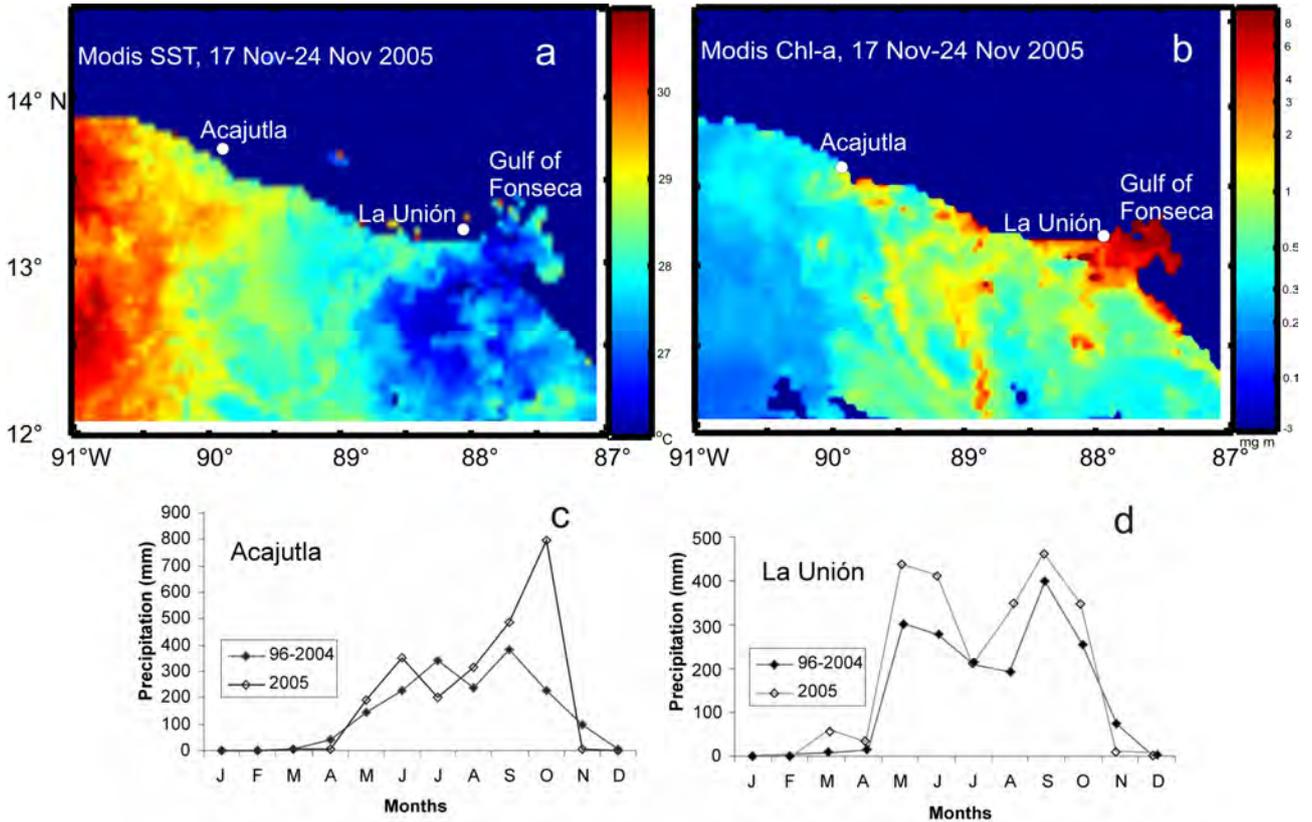
**Table 1.** Saxitoxin concentrations in the turtle *Lepidochelys olivacea* and the jellyfish *Stomolophus meleagris* collected at different sites.

Site	Date	µg STXeq / 100g wet tissue	Type of tissue
El Cuco	01/12/06	11.71	Jellyfish
El Espino	01/08/06	19.14	Turtle muscle fin
	01/09/06	12.15	Jellyfish
	01/10/06	0.41	Turtle muscle fin
El Tamarindo	01/16/06	433.96	Turtle brain
	01/16/06	27.99	Turtle liver
	01/16/06	63.03	Turtle rectum
	01/06/06	1.52	Turtle muscle fin

**Results and Discussion**

The mouse bioassay results demonstrated PSP toxin concentrations in many of the shellfish samples collected in El Salvador were higher than the maximum permissible level established by USFDA for human consumption [80 µm STX/100 g of shellfish wet weight]. The ranges found were from 60.4 µg SXT/100 g wet tissue in El Zonte on 14 December 2005 to >4000 µg SXT/100 wet tissue at Las Tunas in the clam *Protothaca asperrima*, at Taquillo Beach in

the rock oyster *Crassostrea iridescens* and at Shutia in the oyster *Crassostrea corteziensis* at the end of December (Fig. 1). Concentrations from January to March 2006 ranged from 72.5 in Las Tunas to 352.6 µg SXT/100 g wet tissue in the bivalves *Anadara similis* and *A. corteziensis* (data not shown). Concentrations of saxitoxins in the Pacific southwestern part of Mexico obtained by mouse bioassay monitoring were 58-204 µm STX eq/100 g wet tissue in *Crassostrea iridescens* (Rodríguez, personal communication).



**Figure 2.** MODIS SST data (upper left) and MODIS Chl-*a* data (upper right) for the period 17-24 November 2005. Lower panel shows decadal average precipitation (1996-2004) obtained from two meteorological stations. Note that the year 2005 had the maximum precipitation.

**Table 2.** Average relative abundance of *P. bahamense* var. *compressum* (cells/Liter) obtained from water samples taken at 1.5 m depth along the El Salvador coast from November 2005 to March 2006. (nd= not done).

Sites of collection	Year 2005		Year 2006				Total number of samples analyzed
	November	December	January	February	March	April	
Gulf of Fonseca	36,600,000	43,000,000	15,000,000	1,850,000	15,000	nd	66
Maculis	29,000,000	48,900,000	741,000	940,000	nd	nd	16
Playas Negras	18,300,000	9,355,000	870,000	1,730,000	nd	nd	16
Barra de Santiago	1,000	1,900,000	2,529,000	3,200	nd	nd	10
El Espino	nd	nd	13,300,000	21,000	nd	nd	8
El Zonte-Taquillo	not done	19,830,000	3,200,000	1,054,000	160,000	92,000	20
Los Cobanos	371,000	27,979,000	12,289,000	5,102,000	458,000	7,000	76

Table 2 gives an average of the relative abundance of *P. bahamense* var. *compressum*, showing that cell concentrations decreased as of January 2006 and disappeared after April. According to our results and other available data the bloom was considerably denser compared to earlier reports from the area. It is clear that an intensive monitoring programme is required to address human health and wildlife impacts.

Saxitoxin concentrations in brain tissue reached 443.96 µg STX per 100 g wet tissue of the green turtle *Lepidochelis olivacea* (Table 1). Although there are no reports on lethal doses for turtles, it is plausible that saxitoxin was the causative agent for turtle mortality. It is known that jellyfish are included in the diets of some sea turtles (Mortimer 1982). The presence of saxitoxins in the analyzed jellyfish points to these organisms as a vector in the saxitoxin food chain: *P. bahamense* var. *compressum*-jellyfish-turtle. The high concentration of saxitoxins in both jellyfish and turtle tissues gives support to this hypothesis. If so, this is the first report of the saxitoxin and turtle mortality link as well as the jellyfish being a vector in toxin transfer in this region.

### Oceanographic Conditions in the Area

Monthly MODIS SST data for 2002-2006 show that the waters off the Gulf of Fonseca in November 2005 were colder than usual. Weekly MODIS data show the lowest SST values during the week of 17-24 November 2005, as a result of a cyclonic eddy present in this area (Fig. 2A), which is visible in altimetry-derived SSH anomalies (not shown). Cyclonic eddies often develop a high-chlorophyll core, which is associated with upwelled nutrients. Consistently, the corresponding MODIS Chl-*a* data reveals a complex pattern, with high chlorophyll concentration values observed around the periphery of the cyclonic eddy (Fig. 2B). The advection of warm water was clearly observed during subsequent days (not shown). Note that the high precipitation observed during October 2005 (Figs 2C-D) is an additional mechanism of fertilization of coastal waters. Thus, it seems plausible that high levels of nutrients, supplied by both the cyclonic circulation located off the Gulf of Fonseca and river runoff are related to the formation and maintenance of the bloom observed in November/December 2005.

From the observed data and the literature (Sierra Beltrán *et al.* 2004), it appears that blooms of *P. bahamense* var. *compressum* are preceded by a period of cold water.

### Acknowledgements

We thank the institutions of El Salvador, particularly MSPAS for financial support and assistance during field collection and bioassay. Special thanks to F. Chicas Head of ICMARES and their students, to R. Luna and M.E. Zamudio for technical assistance, and to C. Ramirez and R. Rodríguez for providing unpublished technical reports.

### References

- Barraza, J.E., Armero, J.A. & Valencia de Toledo, Z.M. (2004). *Revista Biología Tropical* 52: 1-4.
- Cortés-Altamirano, R., L. Muñoz & Sotomayor, O. (1993). *An. Inst. Cienc. Mar y Limnol. UNAM* 20: 43-50.
- Hasle, G.R. (1978). In: *Phytoplankton Manual*, Sourin, A. (ed.), UNESCO, Paris, pp. 191-196.
- Hummert, C., Ritscher, M., Reinhardt K. & Luckas, B. (1997). *Chromatographia* 45: 312--316.
- Mata, L., Abarca, G., Marranghello, L. & Viquez, R., (1990). *Revista Biología Tropical* 38: 129-136.
- Mortimer, J.A. (1982). In: *Biology and Conservation of Sea Turtles*, Bjornal, K.A. (ed.), Smithsonian Inst. Press, pp. 103-109.
- Sierra-Beltrán, A.P., Lluch-Cota, S.E., Cortés-Altamirano, R., Cortés-Lara, M.C., Castillo-Chávez, Carrillo, L., Pacas, L., Viquez, R. & García-Hansen, I. (2004) *Rev. Biol. Trop.* (suppl. 1): 99-107.
- Orellana, E., Martínez, E., Muñoz, L., López, P., Cabrera, E. & Ramírez, C. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T. (eds), UNESCO, Paris, p. 60.
- Oshima, Y. (1995). In: *Manual of Harmful Marine Microalgae*, Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D. (eds), IOC Manuals and Guides 33, pp. 81-94.
- Rosales-Loessener, F. Porras, E. & Dix, M.W. (1989). In: *Red Tides : Biology, Environmental Science and Toxicology*, Okaichi, T., Anderson, D.M. & Nemoto, T., (eds), Elsevier, New York, pp. 113-116.
- Vargas-Montero, M. & Freer, E. (2003). In: *Molluscan Shellfish Safety*, Villalba, A., Reguera, B., Romalde, J.L. & Beiras, R. (eds), UNESCO, Paris, pp. 211-217.
- Yu R.C., Hummert, C., Luckas, B., Qian, P.Y. & Zhou, M.J.. (1998). *Chromatographia* 48: 671-676.
- Zhou, M.J.. (1998). *Chromatographia* 48: 671-676.

## Identifying and detecting harmful algal bloom species using a colour imaging flow cytometer (FlowCAM®)

N. Poulton<sup>1</sup>, H. Nelson<sup>2</sup> and C. Sieracki<sup>2</sup>

<sup>1</sup>Bigelow Laboratory for Ocean Sciences, 180 McKown Point, West Boothbay Harbor, ME 04575 USA, npoulton@bigelow.org <sup>2</sup>Fluid Imaging Technologies, 258 Cross Point Road, Edgecomb, ME 04556 USA

### Abstract

The ability to detect, identify and enumerate harmful algal species is a requirement in coastal ecosystems for monitoring programmes and early detection of harmful bloom events. To date, most monitoring programmes utilize microscopy for identifying bloom species in field samples, which can be laborious and time consuming. Recently, automated techniques for monitoring and detecting target species have been tested in phytoplankton monitoring programmes. These methods include instruments that utilize optical flow through systems. FlowCAM® is an imaging-flow-cytometer that combines the capabilities of a flow cytometer with a digital-imaging microscope, and automates phytoplankton detection and enumeration. Previously, FlowCAM® has been shown to successfully detect and enumerate harmful algal bloom species (*Alexandrium fundyense* and *Karenia brevis*) from laboratory and field samples. Here we present data from a new “colour” FlowCAM®. The use of colour provides additional criteria for distinguishing between closely related harmful algal species. In this study, the detection of algal species was enhanced by 5-7 % when using a colour FlowCAM when compared to a traditional black and white FlowCAM. Overall, the key benefits of this technology are the ability to analyze phytoplankton continuously, determine the size, and most importantly the collection of colour digital images for further analysis.

### Introduction

Early detection, identification and enumeration of harmful algal bloom species is a continuous challenge for scientists and monitoring groups to be able to mitigate the effects of blooms on the human population. Monitoring programmes currently use microscopic methods that sometimes combine molecular approaches and remote sensing techniques to determine the presence or absence of a particular species in coastal waters. Many of the manual methods used are time-consuming and laborious; more automated methods are slowly being implemented. One instrument, the Flow-CAM® (Fluid Imaging Technologies, Inc.) is a flow-cytometer designed to image, count and characterize particles that are in the micro-size range (5-800 µm) (Sieracki *et al.* 1998). Microplankton are imaged by the FlowCAM as a water sample passes through a rectangular glass tube (2.0 mm x 0.1 mm cross section). The FlowCAM acquisition and analysis software allows the user to determine cell abundance and to define image libraries which are used for image recognition, identification, filtering and sorting from mixed cultures or field samples (See *et al.* 2005; Buskey and Hyatt 2006).

In this study, we compare the ability of a FlowCAM to sort and identify target species using images generated by black and white (B&W) and colour

cameras. Using user-defined image libraries of target algal species we test the ability of the recognition software to discriminate different algal species from a pre-mixed field sample.

### Materials and Methods

Using a combination of cultures and field samples, samples for analysis using both a B&W and colour FlowCAM were prepared and processed simultaneously. Three dinoflagellate cultures were used: *Alexandrium fundyense*, *Karenia brevis* (strain CCMP 2228) and *Scrippsiella* sp. (strain CCMP 772). The *A. fundyense* strain, CB-301, was clonally isolated from a cyst from Casco Bay, Maine. The other cultures were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP). *K. brevis* was cultured in 25-ml glass tube using L1-Si medium (Guillard and Hargraves 1993) with 0.2-µm-filtered coastal seawater collected in Boothbay Harbor, ME. Both *A. fundyense* and *Scrippsiella* were cultured in f/2-Si media (Guillard 1975). All three cultures were incubated and grown at 20 °C using a 14:10 L:D cycle.

A summer field sample was obtained from Boothbay Harbor, ME and concentrated 8x using a 20-µm nylon mesh. The field sample concentrate was pre-filtered using 100-µm nylon mesh to remove larger

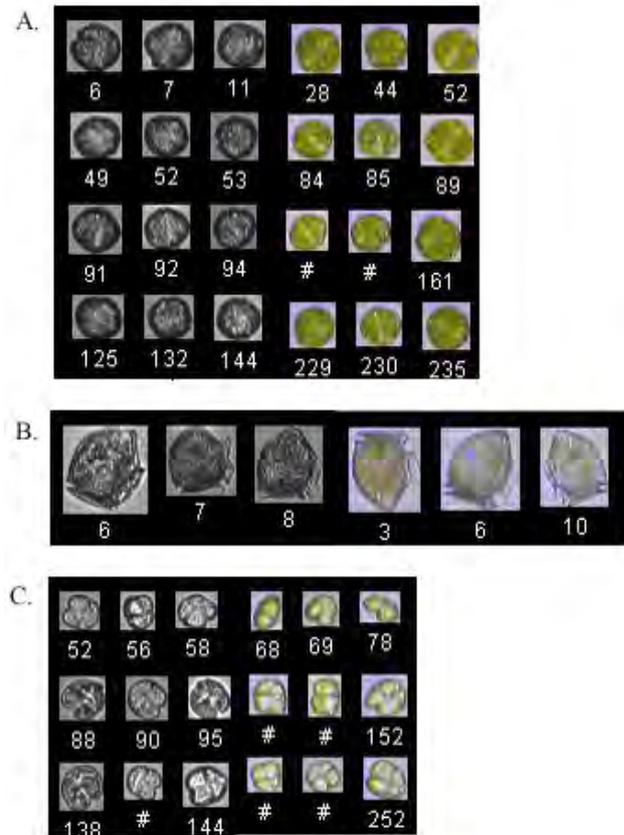
organisms that could potentially block the FlowCAM flow cell. Samples were run without chemical fixatives in order to better preserve cellular fluorescence. Each FlowCAM was equipped with a 10x objective and a 100- $\mu\text{m}$  depth flow cell. For analysis a 50-ml sample was prepared using a 5-ml mixture of the three cultured dinoflagellates and 45 ml of field sample concentrate. Two 10-ml sub-samples of the culture + field mixture were processed and counted using fluorescence mode. In the fluorescence mode, a cell is detected using a green laser (532 nm) for excitation and counted based on a red fluorescence signal (emission > 650 nm) that is within a user-defined threshold. In this study, one FlowCAM was equipped with a Sony B&W high resolution camera (Sony 1224 x 768) and the other with a colour camera (Sony 1224 x 768).

Prior to each run on the FlowCAM, the pump and flow cell were primed with filtered seawater (FSW). Each dinoflagellate culture was run individually as a culture control to collect one hundred images of each species from both B&W and colour FlowCAMs to be used as separate user-defined image libraries. All samples were processed through each FlowCAM at approximately  $0.35 \text{ ml min}^{-1}$ .

Using the images collected during the culture control runs and the Boothbay Harbor field sample, image libraries were created for five different dinoflagellate species. Two additional dinoflagellate species, *Dinophysis* sp. and *Prorocentrum* sp. were identified in the field sample using the FlowCAM VisualSpreadsheet (VSp) analysis software. These images were identified and saved separately in additional image libraries (examples of library images are depicted in Figure 1).

Using the data and images obtained from the culture+field mixture, each run was analyzed by filtering and sorting all of the images in a run using the appropriate algal libraries. The filtering process compares all the parameter values from the images in each library to the parameter values from images in the field mixtures. Each image from the culture+field mixture is given a filter score depending on the image's similarity to the user-defined library. The number of parameters measured for each cell varies and depends on which FlowCAM was used. The B&W FlowCAM has 18 different parameters (e.g. length, width, equivalent spherical diameter (ESD), roughness, aspect ratio) and the colour FlowCAM has 22 parameters, with 6 additional parameters that describe 6 colour optical properties of each image (e.g. average red, average green, and ratios of the average colour values to each other). After each run the data and images are filtered and sorted using the image libraries. The number of

positively identified cells are counted and compared to the total number within the sample (user identified – a tolerance of 5 false positives was utilized). The percentage sorting efficiency (% positively identified) is determined for each harmful algal species and the results of the two FlowCAMs are compared.



**Figure 1.** Harmful algal species images generated by a both a B&W and a colour FlowCAM. The images are subsets of user-defined libraries used for pattern recognition: A. *A. fundyense*, B. *Dinophysis* sp., C. *K. brevis*.

In order to understand how the different parameters measured by the a B&W or colour camera FlowCAM interact, a principle components analysis (PCA) using XLSTAT (Addinsoft, Inc.) was performed to determine the benefit of adding 6 colour parameters (variables) of the colour FlowCAM. Using the library images generated for the cultured cells (*A. fundyense*, *K. brevis* and *Scrippsiella* sp.) and library images generated from field samples (*Dinophysis* sp. and *Prorocentrum* sp.) a PCA analysis was performed on all the parameters (variables) measured by each FlowCAM.

## Results and Discussion

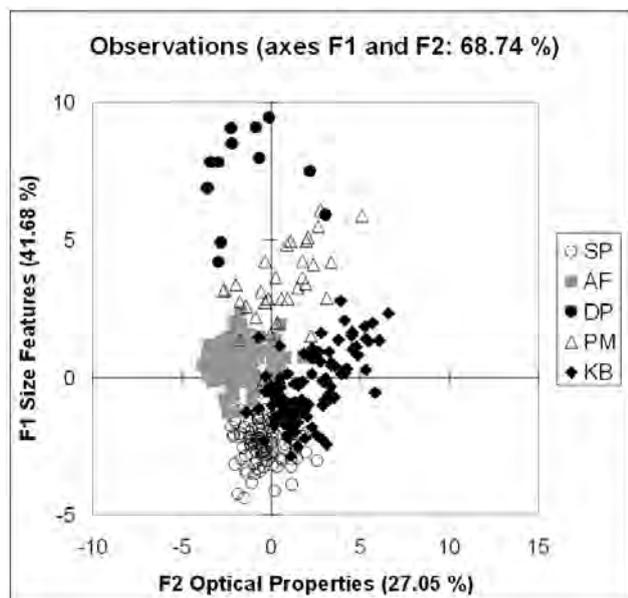
### Pattern recognition

Using the user-defined image libraries of *A. fundyense*, *K. brevis* and *Dinophysis* sp. the FlowCAM VSp software was able to positively sort and identify 67

% *A. fundyense*, 84 % *K. brevis*, and 87 % *Dinophysis* using a B&W FlowCAM and 75 % *A. fundyense*, 89 % *K. brevis*, and 92 % *Dinophysis* using a colour FlowCAM. Overall, the colour FlowCAM provided a 5-7 % increase in positive identification using colour image libraries.

#### Principle components analysis

From the PCA of the parameters determined by both B&W and colour FlowCAMs, two principle components (factors) were identified comprising 64-69 % of the variability. The first factor was associated with the size features of the particles/cells (e.g. length, width, ESD, and area).



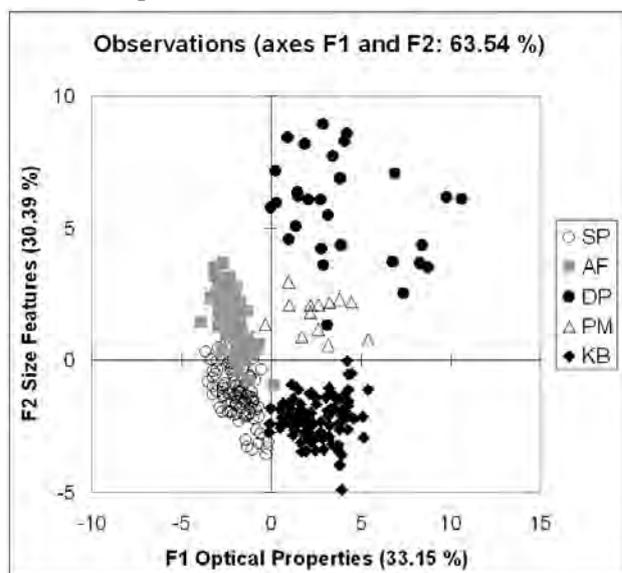
**Figure 2.** Representation of the B&W FlowCAM parameters (variables) as a function of the first and second principle components (F1 and F2) that comprise 69 % of the variability. SP = *Scrippsiella*, AF = *A. fundyense*, DP = *Dinophysis* sp., PM = *Prorocentrum*, KB = *K. brevis*.

The second factor was associated with the optical properties of the image (e.g. intensity, transparency, aspect ratio, and colour properties). Clear separation of species was not possible using the B&W FlowCAM (Fig 2. SP, AF and KB). In contrast, the colour FlowCAM yielded better resolution among the dinoflagellates. This was due to the inclusion of six additional colour parameters that enhanced the optical properties of the cells (Fig 3. SP, AF, KB).

Overall, there is an added benefit to using a colour FlowCAM for pattern recognition and identification of harmful algal species from mixed cultures or field assemblages. At present only a few parameters exist (max. 22), as the number of parameters and factors

used to identify and differentiate species (or genera) increases, the ability to identify additional harmful taxa will continue to improve. However, the actual number of parameters required to identify specific species will vary depending on the algal composition of the sample and number of parameters available to the user.

Overall, the use of FlowCAM to identify harmful algal bloom species has been quite successful. There are limitations, as certain species are morphologically and optically similar to one another, such as *Alexandrium* spp. and other thecate dinoflagellates (eg. *Scrippsiella* spp.). Whereas, other species such as *Dinophysis* and *Karenia* are more distinctive in nature from other plankton.



**Figure 3.** Representation of colour FlowCAM parameters (variables) as a function of the first and second principle components (F1 and F2) that comprise 64 % of the variability. See Fig. 2 labels.

#### References

- Buskey, E.J. & Hyatt C.J. (2006). Harmful Algae 5: 685-692.
- Guillard, R.R.L & Hargraves, P.E. (1993). Phycologia 32: 234-236.
- Guillard, R.R.L. (1975). In: Smith, W.L., Chanley, M.H. (eds), Culture of Marine Invertebrate Animals, Plenum Publishing Corp., New York, pp. 29-60.
- See, J.H., Campbell, L., T.L. Richardson, Pinckney, J.L. Shen R. & Guinasso Jr., N.L. (2005). J. Phycol. 41: 305-310.
- Sieracki, C.K., Sieracki, M.E. & Yentsch, C.S. (1998). Mar. Ecol. Prog. Series 168: 285-296.

## Retrospective GIS analyses of the Florida red tide database

K. A. Steidinger<sup>1,2</sup>, J. A. Tustison<sup>2</sup>, R. H. Weisberg<sup>3</sup>, A. Barth<sup>3</sup> and C. A. Heil<sup>2</sup>

<sup>1</sup> University of South Florida, Florida Institute of Oceanography, 830 1st Street So., St. Petersburg, FL 33701 USA; <sup>2</sup> Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 8th Ave. SE, St. Petersburg, FL 33701, USA, jacob.tustison@myFWC.com, cindy.heil@myFWC.com;

<sup>3</sup> University of South Florida, College of Marine Science, St. Petersburg, FL 33701, USA, weisberg@marine.usf.edu, abarth@marine.usf.edu

### Abstract

The use of GIS technology to visualize point, line, and polygon data in large datasets, either in a 2-D or 3-D format, provides another valuable tool for graphing and analyzing datasets. This tool can even be adapted for forecast modelling. The technology has been applied to Florida red tides using a large historical database of *Karenia brevis* counts to show movement of blooms longitudinally and latitudinally, initiation of blooms > 18 km offshore, the influence of currents on movement of *K. brevis*, and the influence of hurricanes on distribution and intensity of blooms. Present-day count data and circulation data are being used to develop a HAB bulletin to forecast movement of blooms.

### Background

*Karenia brevis* is a toxic, unarmoured dinoflagellate that can cause neurotoxic shellfish poisoning, animal mortalities, and respiratory distress. It is distributed throughout the Gulf of Mexico but blooms are most frequent in coastal waters off west coast Florida in late summer – fall. Occasionally, it is transported out of the Gulf into the North Atlantic up to the Carolinas, carried by the Gulf Stream. *Karenia brevis* has only been recorded once outside of this region in Trinidad (Lackey 1956). The State of Florida has maintained a red tide database since 1954 using data from local, state and federal agencies, universities, private laboratories, and other sources including volunteers. The data itself is principally *K. brevis* cell counts, geographic coordinates, depth, and date. Additional data, e.g., salinity, temperature, light, dissolved oxygen, nutrients and other variables are available for some monitoring or research cruises or long term Tampa Bay water quality monitoring but not for most event response samples. More recently, other *Karenia* species have been recorded. Two of these, *K. mikimotoi* and *K. papilionacea* have been recorded back to the 1960s (Heil *et al.*, these proceedings). An average of 867 (from 1954 to 1994) and 2929 (1995-2003) samples were collected each year, thus sampling effort was increased 3.3 fold from 1995. The database is further biased by 80 % inshore vs only 20 % offshore and 80 % surface vs only 20 % deeper water because the majority of samples are event response. These sampling biases were due to the need for rapid assessment of where blooms were and their concentrations (need to close shellfish harvesting areas inshore). Only re-

search cruises had designated sampling plans and some were conducted along transects from inshore to offshore on a routine basis. Sampling effort, which depends on programme needs, can bias the database (see [http://research.myfwc.com/features/view\\_article.asp?id=25871](http://research.myfwc.com/features/view_article.asp?id=25871)) and the data cannot be used to assess increase in bloom frequency or intensity. Although there are difficulties with statistical analyses because assumptions are not met, and sampling biases, the database is valuable and can be used to 1) map red tide movement cross shelf and along shore, 2) assess the offshore origin of red tide build-up on the shelf, 3) integrate circulation model trajectories and cell count point data to forecast movement on the shelf, and 4) assess the influence of hurricanes on intensity and distribution of red tides.

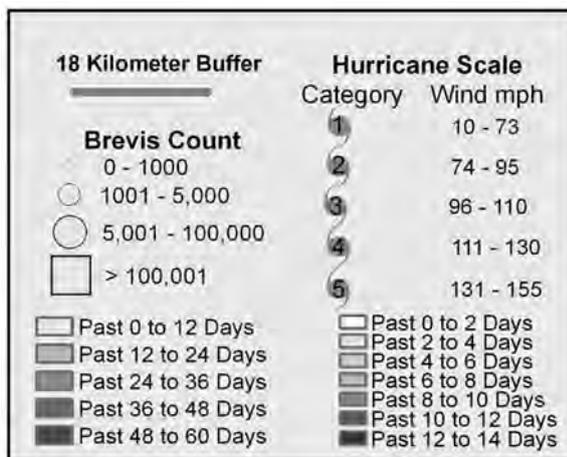
Today, advanced technology in the form of satellite images, continuously recorded data from other sensors as well as new approaches continues to increase the size and value of the Geographic Information Systems (GIS) database. GIS software and applications are able to handle large, long-term datasets and seamlessly integrate multiple datasets, e.g., cell counts, salinity, temperature, current speed and direction, winds, etc. This tool has the capability to integrate point, line, and polygon data.

### Results and Discussion

Tester and Steidinger (1997) graphed the spatial and temporal distribution for the 1976, 1979, and 1985 blooms to demonstrate initiation of red tides on the west Florida shelf > 18 km from shore. In comparison, when locations are plotted in GIS format and

*K. brevis* counts  $L^{-1}$  are coded for abundance (shape and size of symbol) and recent or older sample dates (colour) (see Fig. 1 key), sequential daily, weekly or other time period maps can be looped to visualize the movement of red tide over time cross shelf and alongshore. (Figs 1-4, see <http://ocean.floridamarine.org/12thHABconfms>) for an animation of representative sequential maps. Blooms follow a sequence in development: initiation, growth, maintenance/concentration, and termination/dissipation (Steidinger 1975). *Karenia brevis* blooms start at low population concentrations of  $10^4$  cells  $L^{-1}$  18-76 km from shore, and grow slowly. *In situ* growth is typically 0.2-0.3 divisions per day (Steidinger *et al.* 1997). Populations can then be transported onto the inner shelf to near-shore waters. In 1976, two monthly transects out to 70 km, one off Tampa Bay and the other off of Venice, did not reveal the presence of *K. brevis* until 22 September at 19 km at 20,000 cells  $L^{-1}$ . The bloom developed and moved inshore and south, then terminated in January 1977. In fall of 1979, a *K. brevis* bloom in the Big Bend area of the Florida shelf originated 80-130 km offshore and proceeded to grow and move inshore and south. Another example is the fall 1985 bloom where cell counts of 100,000 cells  $L^{-1}$  or greater were found 20-27 km offshore. The bloom was gradually transported onshore and south.

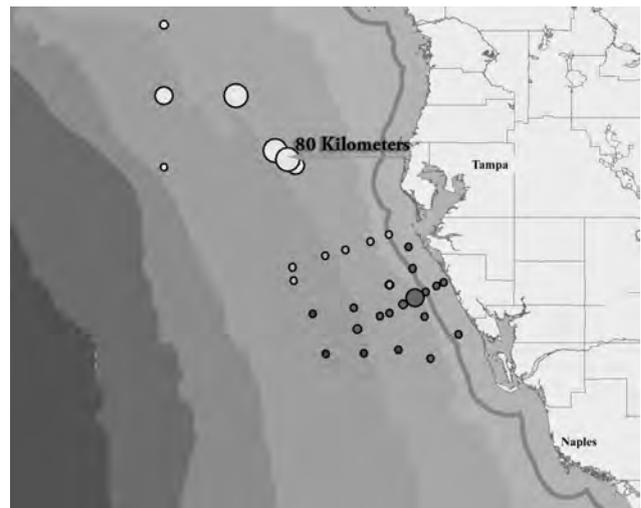
Movement of *K. brevis* blooms is effected by forcing factors such as Eckman layer currents transporting waters from the upwelled region on the northwest Florida shelf. This water is moved and concentrated into nearshore environs from Tampa Bay to Charlotte Harbor as documented by Weisberg and He (2003) for 2001. Since then Weisberg and colleagues (unpublished) have developed a bottom circulation



**Figure 1.** Key to the symbols and colours representing *Karenia brevis* counts and the wind speed associated with a hurricane. Colour indicates the age of the data, e.g., was it within the last 12 days or the last 60 days.



**Figure 2.** Initiation of the 1976 red tide off Florida, data for 22 September 1976.

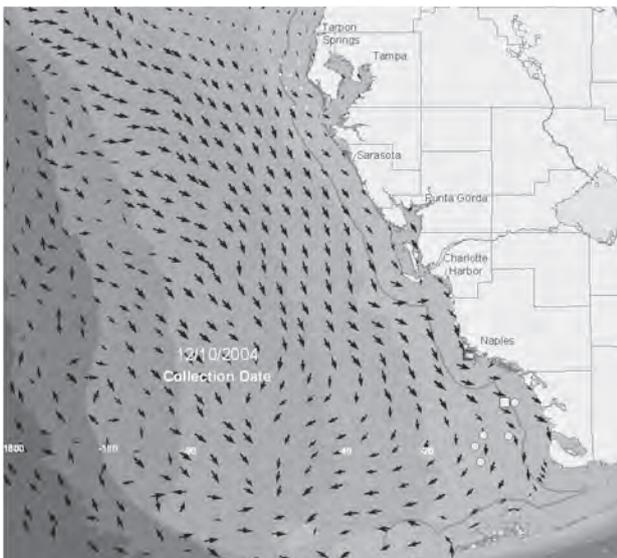


**Figure 3.** Initiation of the 1979 red tide off Florida, data for 27 November 1979.

model that when integrated with *K. brevis* counts can be mapped to show the influence of currents on the alongshore movement of blooms (Fig. 5, see <http://ocean.floridamarine.org/12thHABconfms> for the sequence of animated movement). This layered data approach (bottom and surface currents and cell count data) can be integrated to forecast the movement of shelf blooms and is in the preliminary stages of development. Based on a coastal observing system network, current models can be initialized and run to show the movement of particles such as *K. brevis* (see [http://ocgmod1.marine.usf.edu/WFS/plot\\_hab.html](http://ocgmod1.marine.usf.edu/WFS/plot_hab.html) for the USF-FWC HAB Bulletin). This is currently



**Figure 4.** Initiation of the 1985 red tide off Florida, data for 10 September 1985.

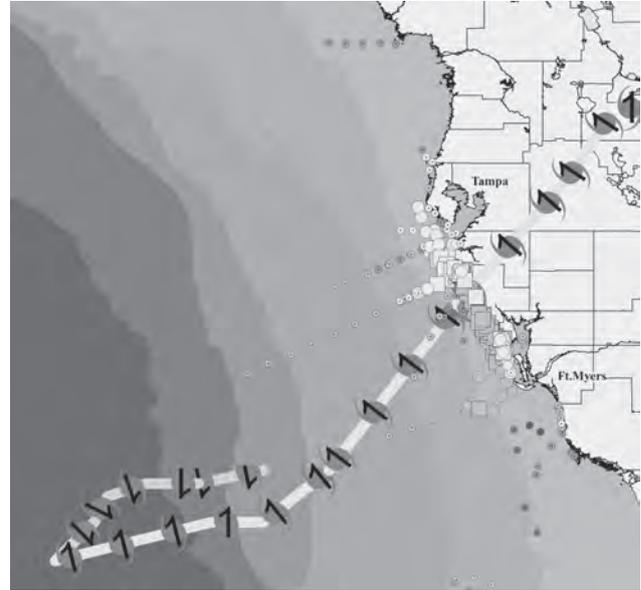


**Figure 5.** Representative integration of bottom current data with *K. brevis* count data to show influence of currents on southerly movement of bloom.

done twice weekly, but can eventually be done daily during events.

Data visualization of historic and current *K. brevis* point counts has proven valuable in analyzing movement of blooms and has much potential as a forecast tool. In addition to these applications, other layers such as hurricanes can be visualized and assessed. One question being, do hurricanes terminate Florida red tides. Tracking Hurricane “Gabrielle” from the 2001 season demonstrates the value of the GIS approach. Gabrielle crossed the Florida peninsula on 16 September 2001 from west to east as a Cat-

egory 1 hurricane. At the same time there was a red tide inshore from Clearwater down to Charlotte Harbor (Fig. 6 and see animation). After passage of the hurricane, it is clear that the bloom was spread further offshore. In this case, the hurricane did not dissipate the bloom. Other hurricanes will be analyzed in the same manner.



**Figure 6.** The passage of Hurricane Gabrielle across Florida right over a coastal red tide.

See <http://ocean.floridamarine.org/12thHABconfirms> for full key for symbols and animation of sequential GIS maps for movement of blooms.

### Acknowledgements

We would like to thank K.O’Keife and H. Norris, FWRI for their support and assistance. Additionally we thank A. Azcarate Alvera of USF for her assistance with data and the anonymous reviewers.

### References

- Lackey, J.B. (1956). *Quart. J. Florida Acad. Sci.* 19: 71.
- Steidinger, K.A. (1975). In *Proceedings of the 1st International Conference on Toxic Dinoflagellate Blooms*, LoCicero, V. (ed.), Massachusetts Science and Technology Foundation, pp. 153-162.
- Steidinger, K.A., Vargo, G.A., Tester, P.A. & Tomas, C.R. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (eds), Springer-Verlag, pp. 133-153.
- Tester, P.A. & Steidinger, K.A. (1997). *Limnol. Oceanogr.* 42: 1039-1051.
- Weisberg, R.H. & He, R. (2003). *J. Geophys. Res.* 108 (C6), 3,184, doi:10.1029/2002/C001407.

## Developing operational capabilities for nowcasts and forecasts of harmful algal blooms

R.P. Stumpf

NOAA National Ocean Service, 1305 East-West Highway, Silver Spring, MD 20910 US Richard.  
stumpf@noaa.gov

### Abstract

Managers and communities need forecasting systems that address “nowcasts”—where a harmful algal bloom (HAB) is today; and “forecasts”—where it will be in the near future. While characteristics of HABs differ between regions, forecasting systems have key commonalities. Forecasting systems have three basic requirements: data on HAB locations; transport models; and analysis of the location data and models in a way that makes sense to managers. To determine HAB locations, the suite of observations may include samples, manual or automated detectors, and remote observations. To determine transport, the options may include heuristic models or single- or multi-dimensional transport models. Effective integration of observations and models requires an analyst, much as an analyst is needed for a weather forecast. In contrast, “predictions”, predicting the initiation and dissipation of HABs, will use different tools: ecological measurements and models. These will tend to involve a greater diversity of physical and ecological observations. While an initiation model can aid in directing sampling to find a new HAB, the ecological data requirements of that model should not be confused with the basic “forecast” requirements of location, transport, and analysis.

### Introduction

Problems associated with blooms of phytoplankton are global and appear to be increasing in severity and extent (Glibert *et al.* 2005). Monitoring for toxic HABs is critical for protecting public health, wild and farmed fish and shellfish, and endangered species (such as marine mammals). The cost and logistics of sampling and the economic impact of delays in warning (while samples are processed) presents the need for effective forecasts of HABs.

Three types of forecasts are needed for managers:

*Nowcasts* of today’s HAB locations, which would improve the value of sampling programs by providing more targeted response;

*Forecasts* of HAB location in the next few days, which would allow strategic planning of sampling and advisories;

*Predictions* of HAB characteristics (including when a HAB may start, whether it will persist, when it will dissipate, what will be its potential severity), which are needed for strategic planning.

Nowcasts and Forecasts fundamentally differ from Predictions—the former emphasizing location, the latter emphasizing ecological characteristics. As a result, implementing the two groups (Nowcast/ Forecasts vis-à-vis Predictions) will differ in both scientific and technical perspectives.

Because of the critical economic and public health issues of HABs, these forecasts (in the general sense) need to be implemented operationally in order to have long-term value. The term “operational” is in-

terpreted differently by different groups. A “forecast” should meet several requirements in order to assure that it is operational:

*Scheduled*: the forecast is produced on a schedule consistent with management needs;

*Reliable*: the forecast system has the necessary redundancy, including backups of facilities and staff, and operates during holidays, etc.;

*Institutional*: the system is supported by an institution, rather than by an individual. This involves training, operations manuals, etc. that will assure continuity even with personnel changes.

The classic example of an operational forecast is the weather forecast. Forecast offices have schedules; their staff, analytical, and computational facilities have backups; and the forecasts are managed by national weather bureaus or comparable entities. In contrast, a model that is run daily on a research investigator’s computer is not operational.

This paper will discuss components relevant to these topics and provide examples of how they are being explored in forecast systems.

### Methods Nowcasts/Forecasts

The critical information for a nowcast is the location of a HAB. While this is the simplest objective in theory, it is complex in practice. A comprehensive nowcast requires real-time observations that identify presence or absence of HABs everywhere the information is needed. Such a requirement points to the need for a hindcast of transport.

Forecasts have essentially the same requirements as nowcasts. Real-time observations of locations are required. Transport depends on the ability to have a nowcast and forecast of currents.

In both cases, the incomplete HAB location data means that most locations need to be modelled. However, in most cases, few other models are needed, as grazing, growth and other factors are not important over the time scales in question (toxic HABs result from relatively slow growing algae; Smayda, 1997). It could be argued that swimming or buoyancy behaviour of flagellates would be important, but if a bloom is in a certain water feature yesterday, it can be reasonably assumed that it will be in it today.

#### *Data and models*

The determination of location involves integration of data from a variety of sources and ages. Unlike physical models, where assimilation schemes can be straightforward and the requisite data sets (like water level) have coherency and predictability, HAB nowcast “models” must use highly variable HAB distributions captured by sketchy and inconsistent data sets. These might include water samples taken over a week, buoys partly sampling patchy blooms, satellite data limited by clouds, and modelled patterns.

Location data is critical for nowcasts and forecasts. The primary tool for most HABs is cell concentration. Toxin measurements in water and in shellfish are also used. A variety of new capabilities are appearing that will provide HAB locations. Autonomous underwater vehicles (AUVs) with HAB specific sensors would allow flexible monitoring. An example is the Brevebuster sensor on the Slocum glider, developed by Kirkpatrick *et al.* (2000). Moored sensors in critical or sentinel areas (the latter including months of bays) are obvious. Handheld units, such as the Jellet sensor are suitable, such as used in the Olympic Region HAB (ORHAB) operational monitoring effort. Similarly, if a HAB has a characteristic that is obvious to a person (strongly discoloured water, respiratory irritation), a volunteer network can be established. For HABs that can be identified by remote sensing, there are satellites or aircraft.

Transport models can involve any type of model, these include heuristic, one-dimensional (1-D), 2-D, and 3-D. Most coupled ecological/hydrodynamic models run the transport from a 3-D model. While three-dimensional (3-D) numerical models are presumed as the primary tool, the other types of models can be useful. A 1-D model accurately hindcast the spread of *Karenia brevis* (*Gymnodinium breve* at the time) along the US southeast coast in 1987-1988 (Test-

er *et al.* 1991), and is used effectively for along-coast transport on the Florida Gulf coast today (Stumpf *et al.* 2003). Heuristic models, particularly on transport caused by changes from upwelling to downwelling appear to have value on the Iberian peninsula. Several systems have 3-D models in place, including Norway, Denmark, and Finland, that are used as indicators of potential transport of potential HABs (some details are found below).

#### *Methods: predictions*

A prediction of initiation or demise of a HAB is the primary goal of most research into HABs. Prediction involves a complex combination of topics, for example: geography (why does a HAB form here rather than there) which is a part of the GEOHAB (Global Ecology and Oceanography of HABs) program, climatology (when do HABs develop), and ecology and oceanography (how does the organism interact with the environment and when does the environment favour a HAB).

#### *Data and models*

Predictions have much more complex requirements. A prediction requires an ecological model. While this model could be numerical or a heuristic, rule-based model, it can be expected to need a variety of inputs. However, these do not need to include the location of the actual HAB. Types of data could be nutrients, winds, upwelling, temperature, locations of cyst beds, and other factors that could affect the development and growth of a bloom. Even for demise, location is only a small part of the problem, the significant question is what ecological factors will cause the bloom to end.

#### *Examples*

There are examples of forecast systems in different countries. The capabilities differ from research to demonstration to operational. The systems are not necessarily documented in the standard literature; web-sites are often the key source for information.

#### *US Gulf of Mexico*

The US National Oceanic and Atmospheric Administration (NOAA) has an operational forecast system for *Karenia brevis* in the eastern Gulf of Mexico and a parallel demonstration system for the western Gulf ([csc.noaa.gov/crs/habf](http://csc.noaa.gov/crs/habf)). The system provides nowcasts and 1-3 day forecasts of the location and potential impact of *Karenia* HABs on the coast. The capability uses a combination of analyzed satellite data, state monitoring data, environmental data and models for transport and human respiratory irritation (Stumpf *et al.* 2003; [csc.noaa.gov/crs/habf/](http://csc.noaa.gov/crs/habf/)).

### Sweden

Two capabilities exist for cyanobacterial blooms: a monitoring system through the Swedish Meteorological and Hydrological Institute ([www.smhi.se](http://www.smhi.se)), and an experimental effort by the University of Kalmar for Öland ([www.hik.se/alg](http://www.hik.se/alg)). The SMHI system is based on field and satellite data. The Kalmar program, "Miss Alga", is based on daily sampling and reporting by volunteers, which is analyzed by the university and assessed for risk to swimming.

### Finland, Baltic Sea

The Finnish Institute of Marine Research set up a nowcast system for cyanobacterial blooms using data collected from a variety of sources, including fluorescence sensors on ferries ([www.fimr.fi/en/itamerikanta.html](http://www.fimr.fi/en/itamerikanta.html)).

### Norway

Two efforts are ongoing in Norway. The Norwegian Institute of Marine Resources has a monitoring and nowcasting effort ([algeinfo.imr.no](http://algeinfo.imr.no)). The Norwegian Meteorological Institute includes flagellates as a demonstration in the MONCOZE modelling effort in order to support algal monitoring ([moncoze.met.no](http://moncoze.met.no)). MONCOZE uses a combination of data types and ecological models to produce the HAB position. However, they note that the blooms with greatest impact (*Alexandrium* and *Gymnodinium*) cannot be accurately modelled in this system with the available information.

### US Gulf of Maine

A research forecast is based on a transport and population dynamics model to determine start (Prediction) and transport (Forecast) of *Alexandrium* blooms (Anderson *et al.* 2005). This method provided support to managers during a severe bloom in 2005 and a moderate one in 2006.

### Korea

Korea has primarily a monitoring programme, but has included temperature indicators for predictions of blooms reaching bays of concern. Because colour can be an indicator, additional work has included colour analyses (Kim *et al.* 2003).

### Japan

Comprehensive monitoring programmes are ongoing in Japan. Some prediction capabilities are resulting from analyses of environmental conditions that lead to blooms (Ishizaki *et al.* 2006).

### Portugal/Spain

Downwelling conditions favour introduction of HABs in the estuaries along the coast. This is being used as a prediction tool (Moita *et al.* 2003).

### HABES programme

The Harmful Algal Bloom Environment Sensing (HABES) programme funded by the European Union ([www.habes.net](http://www.habes.net)), has started some heuristic capabilities for prediction through fuzzy logic that allow for predicting onset of HABs. The studies cross several European countries (Ireland, UK, Spain, Sweden, Finland, Netherlands).

### Other

Capabilities for nowcasts are being developed in Denmark ([www.waterforecast.com](http://www.waterforecast.com)) and the Netherlands ([www.wldelft.nl](http://www.wldelft.nl)).

### Conclusions

While significant progress has occurred, several areas require investigation. Of these, the most important are data integration capabilities, and accuracy assessment. Methods for accuracy assessment of many models are sketchy and inconsistent. Determining the accuracy of modelled distributions will be a particular research challenge. Continued development of data integration techniques will be critical to improved forecasting. Ecological prediction of the likelihood of a bloom does not replace the key management need of identifying the current and forecast location.

### References

- Anderson, D.M., Keafer, B.A., McGillicuddy, D.J. Mickelson, M.J., Keay, K.E. Libby, P.S., Manning, J.P., Mayo, C.A., Whittaker, D.K., Hickey, J.M., He, R., Lynch, D.R. & Smith, K.W. (2005). Deep Sea Research II, 52: 2856-2876.
- Glibert, P.M., Anderson, D.M., Gentien, P., Granéli, E. & Sellner, K.G. (2005). Oceanography 18: 132-141.
- Ishizaka, J. Y., Kitaura, Y., Touke, Y., Sasaki, H., Tanaka, A., Murakami, H., Suzuki, T., Matsuoka, K. & Nakata, H., (2006). J. Oceanogr. 62: 37-45.
- Kim, H.G., Lee, S.G., Lee C.K. & Lim, W.A. (2003). Workshop on Red Tide Monitoring, [fol.fs.a.u-tokyo.ac.jp/rtw](http://fol.fs.a.u-tokyo.ac.jp/rtw).
- Kirkpatrick, G.J., Schofield, O.M., Millie, D.F. & Moline, M. (2000). Limnol. Oceanogr. 45: 467-471.
- Moita, M.T., Oliverira, P.B., Mendes, J.C. & Palma, A.S. (2003). Acta Oecol. 24 (suppl.1): 125-132.
- Smayda, T. (1997). Limnol. Oceanogr. 42: 1137-1153.
- Stumpf, R.P., Culver, M.E., Tester, P.A., Tomlinson, M., Kirkpatrick, G.J., Pederson, B.A., Truby, E., Ransibrahmanukul, V. & Soracco, M. (2003). Harmful Algae 2: 147-160.

## Using remote sensing to aid in the detection and monitoring of *Microcystis aeruginosa* in western Lake Erie and Saginaw Bay, USA

M.C. Tomlinson<sup>1</sup>, T.T. Wynne<sup>1</sup>, R.P. Stumpf<sup>1</sup>, J. Dyble<sup>2</sup>, G.L. Fahnenstiel<sup>2</sup> and P.A. Tester<sup>3</sup>

<sup>1</sup>NOAA, National Ocean Service, Center for Coastal Monitoring and Assessment, 1305 East-West Hwy, N/SCI1, Silver Spring, MD 20910 USA, Michelle.Tomlinson@noaa.gov; <sup>2</sup>NOAA, Great Lakes Environmental Research Laboratory, 2205 Commonwealth Blvd., Ann Arbor, MI 48105, USA; <sup>3</sup>NOAA, National Ocean Service, Center for Coastal Fisheries and Habitat Research, Beaufort, NC 28516 USA

### Abstract

*Microcystis aeruginosa* has become a dominant component of the summer phytoplankton population in Saginaw Bay and western Lake Erie, USA. Expansive blooms of *Microcystis* have caused considerable concern to the Great Lakes region because of the capability of these blooms to produce the hepatotoxin microcystin, and simultaneous use of the waters for recreation and drinking water supplies. Microcystin concentrations above the recommended treated drinking water limit of  $1 \mu\text{g L}^{-1}$  have been measured in both regions and could pose a threat to human health. Some cyanobacteria blooms, such as those caused by *Microcystis*, have unique scattering and absorption properties, due to the production of surface scums and the dominant accessory pigment, phycocyanin. In an effort to better detect and monitor these blooms, satellite-derived products from SeaWiFS were compared with *in situ* measurements of *Microcystis* to determine its usefulness for detection. Phycocyanin has an absorption peak centered at 620 nm, which coincides with a spectral band available on MERIS. Therefore, a phycocyanin algorithm was applied to a MERIS image from August 2004, to determine its usability in monitoring *Microcystis* in western Lake Erie.

### *Microcystis* Detection

Blooms of toxic cyanobacteria are of increasing concern worldwide. In the Great Lakes (GL) region, the dominant bloom-forming cyanobacterium, *Microcystis aeruginosa*, has increased in abundance since the mid-1990s in Saginaw Bay and western Lake Erie, USA (Vanderploeg *et al.* 2001). Because microcystin is a hepatotoxin, the recent resurgence of *Microcystis* is of particular concern since GL waters are used for recreational activities and are the primary source of drinking water.

Surface blooms such as those formed by *Microcystis* spp. are highly reflective due to the presence of gas vacuoles, and in high biomass strongly discolour water (Budd *et al.* 2001). These blooms are distinctively green in the GL which distinguishes them from diatom or dinoflagellate blooms (Fig. 1). However, chlorophyll *a* (chl *a*) imagery alone cannot accurately distinguish cyanobacteria since these blooms can occur in mixed assemblages with other phytoplankton.

The presence of gas vacuoles in *Microcystis* and the subsequent tendency to produce surface scums results in a high relative reflectance at near-infrared wavelengths. As a result, *Microcystis* should be visible using satellite remote sensing (Stumpf and Tomlinson 2005). *Microcystis* blooms may be directly detected from the red reflectance bands of Sea-viewing

Wide Field-of-view Sensor (SeaWiFS) (670 nm) and/or Moderate Resolution Imaging Spectroradiometer (MODIS) (667 nm) when present at concentrations necessary to produce surface scum. It was found that the AVHRR (Advanced Very High Resolution Radiometer) visible and near infrared bands (NIR) can detect the high relative reflectance in the red and near infrared wavelengths that is produced by concentrated blooms (Budd *et al.* 2001). Due to these unique char-



**Figure 1.** “True colour” Landsat image on 18 August 2003 from Ohiolink.edu. Bright green indicates the location of a *Microcystis* bloom.

acteristics, surfacing blooms have been seen in single-band imagery. The most dramatic example occurred in the Baltic Sea where single-band AVHRR imagery characterized the extent of *Nodularia* blooms over several years (Kahru *et al.* 2000). However, suspended sediment also causes high red remote sensing reflectance and may need to be corrected for, especially at river mouths or during resuspension events.

The dominant photosynthetic accessory pigment for *Microcystis* is phycocyanin, which has an absorption peak at approximately 615 nm (Bryant 1994; Simis *et al.* 2005). The most significant change in the absorption spectra for cyanobacteria occurs between 620 and 650 nm (Kutser *et al.* 2006). Current sensors available for monitoring the Great Lakes region (SeaWiFS and MODIS) do not have sufficient resolution in this portion of the spectrum. A phycocyanin algorithm has been developed from the Medium Resolution Imaging Spectrometer (MERIS) imagery, which contains a spectral band that overlaps with this phycocyanin absorption peak (Simis *et al.* 2005). Due to the 2-3 day repeat of MERIS scenes and their spectral resolution, these products seem most suitable for monitoring cyanobacterial blooms such as *Microcystis* in the GL (Kutser *et al.* 2006).

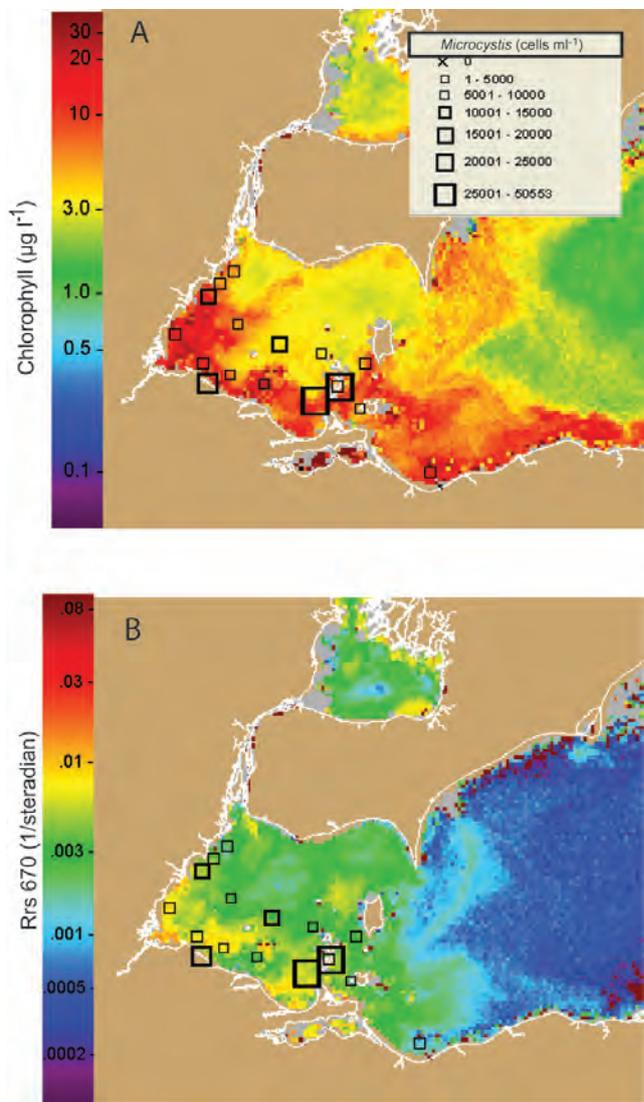
#### Methods: Chlorophyll and Red Reflectance

Chl *a* using the standard OC4 algorithm (which is based on a ratio of blue to green) and the red remote sensing reflectance (Rrs670 nm) band from SeaWiFS were examined to determine whether they could accurately identify a bloom of *Microcystis* that occurred in western Lake Erie in 2004. Basin-wide sampling for *Microcystis* abundance and microcystin concentrations was conducted in western Lake Erie 28-30 August 2004. Samples were collected from just below the surface at 18 stations and filtered onto GF/F filters for microcystin analysis, or preserved in Lugol's medium for counts of *Microcystis* colony abundance. Samples for microcystin analysis were lyophilized under vacuum, extracted in 75 % MeOH with sonication and measured by HPLC (modification of Lawton *et al.* 1994). All *Microcystis* species were included in counts of colony abundance. These field estimates of *Microcystis* abundance and microcystin concentration were compared with SeaWiFS-derived chl *a* and Rrs670 products on 31 August 2004.

Phycocyanin is the dominant accessory pigment in *Microcystis*. Simis *et al.* (2005) developed an algorithm for MERIS used to detect phycocyanin concentration ([PC]) as:

$$[PC] = a_{pc}(620)/a_{pc}^*(620)$$

where  $a_{pc}^*$  is the specific absorption coefficient of phycocyanin, estimated to be  $0.007 \text{ m}^2 \text{ mg}^{-1}$ , and  $a_{pc}(620)$  is the absorption of phycocyanin at 620 nm, expressed in equation 4 in Simis *et al.* (2006). The equation for phycocyanin concentration assumes that coloured dissolved organic material (CDOM) makes up <10 % of the total absorption. At higher wavelengths (620 nm), where CDOM absorption is low, this is a valid assumption. The algorithm is valid in turbid waters where cyanobacteria blooms are present in relatively high concentrations, otherwise the algorithm will yield a negative result in which case the phycocyanin concentration may be assumed to be negligible. The phycocyanin algorithm was applied to a 30 August



**Figure 2.** Comparison of *Microcystis* cell counts (purple squares) from 28-30 August 2004 with SeaWiFS-derived products for 31 August 2004: (A) chl *a* with cell counts and (B) Rrs 670 with cell counts.

2004 image and compared with field estimates of *Microcystis* abundance.

## Results

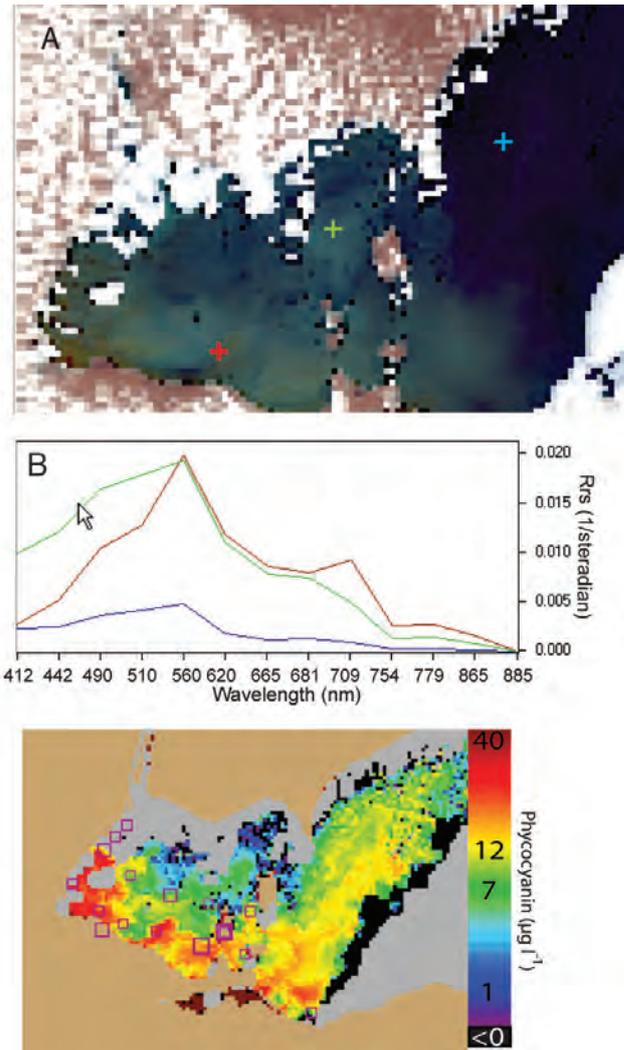
A direct correlation between field measurements of *Microcystis* abundance and chl *a* was not evident (Fig. 2A). In addition, cell abundance was uncorrelated with Rrs670 (Fig. 2B).

A false colour image produced from MERIS, where the RGB was shifted towards the NIR (R=865 nm, G=670 nm, B=555 nm), was created to demonstrate the variation in water colour and spectral absorption spectra within the image. In Fig. 3A, areas within the *Microcystis* bloom appear brown (red +), whereas areas outside the bloom appear turquoise (green +) and dark blue (blue +). Spectra extracted from the imagery, in the area of the *Microcystis* bloom, show a peak in the red to near-infrared (709 nm) bands relative to the other water types (Fig. 3B). In addition, absorption in the blue portion of the spectrum may indicate the presence of chlorophyllous and/or carotenoid pigments, CDOM, and tripton within the *Microcystis* bloom, in addition to high chl *a*.

Initial comparisons between the phycocyanin image product developed from MERIS and cell abundance data collected within 3 days of the image indicate relatively high phycocyanin concentrations in the location of the *Microcystis* bloom (Fig. 3). The accuracy of the algorithm to quantitatively determine phycocyanin concentration was not examined within this study, as *in situ* phycocyanin concentration was not measured.

## Discussion

Although further investigation is necessary for determining the use of MERIS imagery in the detection and monitoring of *Microcystis* blooms in western Lake Erie, initial results indicate that the application of the phycocyanin algorithm has a stronger visual correlation with the *Microcystis* bloom in 2004, than does Rrs670 or chl *a*. Rrs670 may be useful in identifying massive blooms in which scum is produced. However, this is not a reliable detection method as blooms often occur below cell densities that cause scums. In addition, sediment is highly reflective in the red portion of the spectrum and will falsely predict the presence of *Microcystis* at the mouths of rivers (such as the Detroit River) or in areas of resuspension. Although the PC-algorithm's ability to accurately quantify the phycocyanin concentration was not investigated here, this is an area for further study. The phycocyanin al-



**Figure 3.** Results from MERIS image for 30 August 2004: (A) False colour image, brownish water seems to indicate area of *Microcystis* bloom; (B) Rrs spectra from 3 water types where the line colour corresponds to the coloured (+) shown in (A), indicating the location of the spectra; (C) phycocyanin as derived by Simis *et al.* (2006) algorithm.

gorithm was developed from *in situ* measurements collected in two Dutch lakes, and therefore may need to be adjusted for the Great Lakes region (Simis *et al.* 2005).

Reflectance spectra indicate that other pigmented material may be present in the location of the *Microcystis* bloom (Fig. 3B). Unlike the chl *a* algorithm used in this study, which is based on a ratio of the blue to green bands, the phycocyanin algorithm was developed from the red to near-infrared (709 nm) and orange (620 nm) bands and therefore does not rely on the blue portion of the spectra in which the absorption due to CDOM is high. Therefore, this algorithm is applicable to turbid eutrophic lakes (Simis *et al.*

2005). Others have also developed chl *a* algorithms for MERIS using the red portion of the spectra which may be more suitable than the OC4 algorithm used in this study (Gons *et al.* 2005).

Phycocyanin is a phycobiliprotein produced by many cyanobacterial species. Therefore, the concentration of phycocyanin will not distinguish *Microcystis* spp. from other cyanobacteria in the Great Lakes (e.g., *Synechococcus* spp., *Aphanizomenon* spp.). If proven applicable, this algorithm may be useful in the detection and monitoring of cyanobacterial blooms, but field validation will be necessary to determine species abundance. In addition, it is impossible to determine toxicity, or microcystin concentration from satellite. Therefore, the use of MERIS products in an early warning system for toxic *Microcystis* blooms will only aid in targeting sampling efforts during the typical bloom season in which *Microcystis* is dominant. Field validation is essential and incorporation of other real-time data streams will be necessary to accurately monitor and forecast toxic events due to *Microcystis*.

#### Acknowledgements

MERIS data was kindly provided by the European Space Agency. We would like to thank our reviewers, especially Dr. Stefan Simis for his critical review and thought provoking comments.

#### References

- Bryant, D.A. (1994). *The Molecular Biology of Cyanobacteria*. Dordrecht: Kluwer Academic Publishers.
- Budd, J.W., Beeton, A.M., Stumpf, R.P., Culver, D.A. & Kerfoot, W.C. (2001). *Verh. Internat. Verein. Limnol.* 27: 3787-3793.
- Gons, H.J., Rijkeboer, M. & Ruddick, K.G. (2005). *J. Plankton Res.* 27: 125-126.
- Kahru, M., Leppänen, J.M., Rud, O. & Savchuk, O.P. (2000). *Mar. Ecol. Progr. Ser.* 207: 13-18.
- Kutser, T.L., Metsamaa, N., Strömbeck, E. & Vahtmäe (2006). *Estuar., Coast. Shelf Sci.* 67: 303-312.
- Lawton, L.A., Edwards C. & Codd, G.A. (1994). *Analyst* 119: 1525-1530
- Simis, G.H., Peters, S.W.M. & Gons, H.J. (2005). *Limnol. Oceanogr.* 50: 237-245.
- Simis, G.H., Ruiz-Verdú, A., Domínguez-Gómez, J.A., Peña-Martinez, R., Peters, S.W.M. & Gons, H.J. (2007). *Remote Sensing of Environment* 106: 414-427.
- Stumpf, R.P. & Tomlinson, M.C. (2005). In: *Remote Sensing of Aquatic Environments*, Miller, R.L., Del Castillo, C.E. & McKee, B.A. (eds), Springer, Dordrecht, The Netherlands, pp. 277-296.
- Vanderploeg, H.A., Leibig, J.R., Carmichael, W.W., Agy, M.A., Johengen, T.H., Fahnenstiel, G.L. & Nalepa, T.F. (2001). *Can. J. Aquat. Sci.* 58: 1208-1221.

## Moving towards an operational harmful algal bloom forecasting system in Texas (USA)

Timothy T. Wynne<sup>1</sup>, Richard P. Stumpf<sup>1</sup>, Michelle C. Tomlinson<sup>1</sup>, Tracy A. Villareal<sup>2</sup>, Kirk Wiles<sup>3</sup>, Gary Heidem<sup>3</sup>, Meridith Byrd<sup>4</sup>, Dave Buzan<sup>4</sup>, and Lisa Campbell<sup>5</sup>

<sup>1</sup>NOAA/NOS Center for Coastal Monitoring and Assessment, 1305 East-West Highway, Silver Spring, MD 20910, USA, timothy.wynne@noaa.gov; <sup>2</sup>Marine Science Institute, The University of Texas at Austin, 750 Channel View Drive, Port Aransas, TX 78373, USA; <sup>3</sup>Texas Department of State Health Services, 1100 West 49th Street, Austin, TX 78756, USA; <sup>4</sup>Texas Parks and Wildlife Department, 3000 South-IH-35, Austin, TX 78068, USA; <sup>5</sup>Department of Oceanography, Texas A&M University, 3146 TAMU Texas A&M University, College Station, TX 77843, USA

### Abstract

Blooms of the toxic harmful alga *Karenia brevis* have been persistent and problematic in the Gulf of Mexico (USA) for decades. A heuristic ecological model based on satellite imagery has been shown to be effective in identifying likely *Karenia* blooms in the eastern Gulf of Mexico (Florida). “New” blooms are identified by satellite, and blooms that meet certain criteria are identified as likely *Karenia*. The method is core to the detection component of the operational Harmful Algal Bloom (HAB) forecast deployed by the National Oceanic and Atmospheric Administration (NOAA), in October 2004. The Texas coast commonly has false positives resulting from frequent resuspension events. The method was modified to compensate for these events. However, because of the infrequency of *Karenia* HABs along the Texas coast, only one major bloom event (2000) coincided with available SeaWiFS imagery. This event was used to validate the methods presented by Wynne *et al.* (2005). Since the publication of this paper the Texas coastline experienced a major bloom in 2005. The present study will demonstrate the efficacy of the algorithm using *in situ cell* count data collected from this event. The algorithm will then be used to expand NOAA’s HAB forecast system to include Texas.

### Introduction

*Karenia brevis*, a toxic dinoflagellate, has been responsible for large Harmful Algal Blooms (HABs) in the Gulf of Mexico for decades. *K. brevis* has the potential to cause massive fish kills, marine mammal mortalities, and respiratory irritation in humans. *K. brevis* can also cause Neurotoxic Shellfish Poisoning in various types of shellfish (Tomlinson *et al.* 2004). While *K. brevis* may cause large problematic blooms in Texas, such blooms occur far less frequently than in Florida, where they are an annual occurrence.

Tomlinson *et al.* (2004) developed a method to detect *Karenia* blooms using imagery from the Sea-viewing Wide Field-of-view Sensor (SeaWiFS). This method proved to be 80 % effective in identifying blooms in Florida. The technique finds new blooms based on an anomaly of chlorophyll against a 60-day running mean stopping two weeks prior to the day of interest. The 60-day mean was chosen to capture preceding seasonal conditions. The two-week lag is needed to reduce the likelihood that a stationary, persistent bloom will skew the mean. Anomalies with an increase of chlorophyll greater than  $1 \mu\text{g L}^{-1}$  relative to the 60-day mean are flagged as potential new blooms.

Using wind and geographical constraints, analysis can classify how likely new blooms are to be *Karenia*.

The same techniques when applied to the Texas coastline produced many false positives, co-occurring with frequent resuspension events. Such events confuse currently available bio-optical algorithms by introducing benthic chlorophyll as well as sediments into the surface waters where they are interpreted as new blooms in satellite imagery. Wynne *et al.* (2005) developed a method to correct the chlorophyll anomalies. By using the reflectance from the red band as a surrogate for resuspension and subtracting this signal out from the chlorophyll anomalies, the number of false positives was dramatically reduced.

Other phytoplankton species are capable of producing blooms in the gulf. *Karenia* typically blooms in the late summer in both Florida and Texas. Using climatology it is possible to rule out many other blooms from potentially being *Karenia*. For example spring diatom blooms, an annual occurrence on the Texas shelf, will not be classified as *Karenia* despite the appearances of anomalously high chlorophyll features. For this reason analysis will only take place from 1 June to 31 December.

The newly developed technique was tested with cell count data collected during the 2000 *Karenia* bloom, which affected the entire Texas coastline. This was the only major bloom that coincided with the presence of SeaWiFS imagery, which became operational in September 1997. There was a smaller bloom in 1999 in Texas, however, it had a short duration, small impacts, and there was a relative lack of *in situ* cell counts. Tester *et al.* (1998) determined that the minimum detection limit for *Karenia* was a concentration of at least 50,000 cells L<sup>-1</sup>. These cell counts were the upper limit of reported cell concentrations associated with the 1999 bloom event.

*Karenia brevis* monitoring on a state level is done almost entirely within the back bays and estuaries, where the shellfishing industry is located, and only after a fish kill or the presence of *Karenia* has been reported. The utilized chlorophyll algorithm is ineffective in these estuarine environments due to the high concentrations of organic materials which can be misinterpreted as chlorophyll by the sensor.

In 2005 Texas experienced a major bloom of *Karenia* throughout the lower portion of the coastline. In this paper we will test the efficacy of the algorithm developed by Wynne *et al.* (2005) in determining the bloom conditions.

## Methods

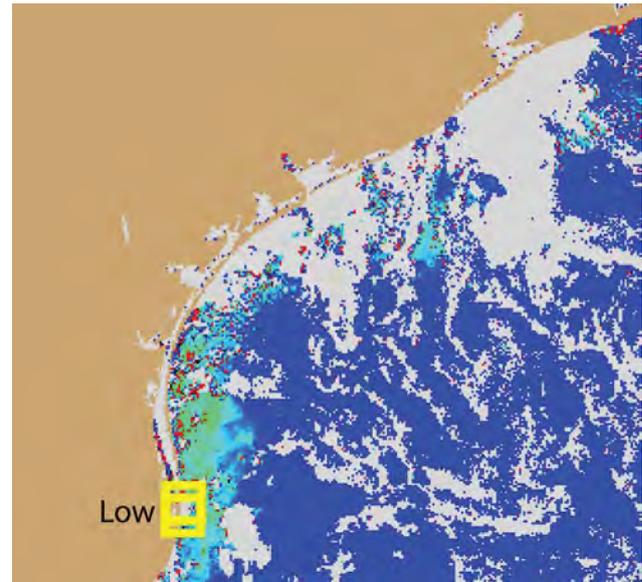
Cell count data were collected by both state and academic agencies (University of Texas, Texas A&M University, Texas Parks and Wildlife and Texas Department of State Health Services). We assembled any available data that were graciously made available for use in validating our algorithm. The SeaWiFS imagery was processed with the latest version of SeaDAS and was processed with a regional chlorophyll algorithm developed by Stumpf *et al.* (2000). The chlorophyll anomalies and red reflectance anomalies were calculated according to methods described by Tomlinson *et al.* (2004). The chlorophyll anomaly corrected for resuspension was then calculated according to the methods outlined by Wynne *et al.* (2005). Available cell counts were then overlaid onto the imagery to test the efficacy of the algorithm.

A cell count convention developed by the state of Florida was used to quantify bloom levels (Florida Marine Research Institute, 2001) (Table 1).

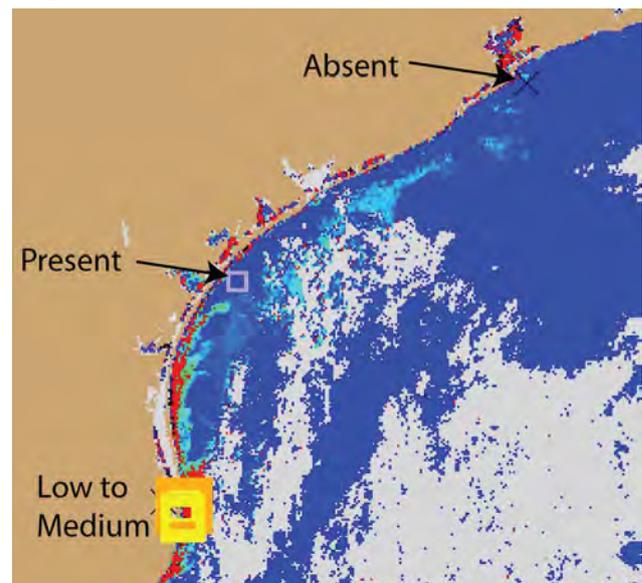
## Results

All available cell counts were overlaid onto the SeaWiFS imagery. The first positive cell counts that

were recorded were from 12 September (Fig. 1). These “Low” counts were still within the range of the satellite detection limit of *Karenia brevis* (50,000 cell L<sup>-1</sup>; Tester *et al.* 1998). 12 September was also when the *Karenia* bloom was first identified in the chlorophyll imagery. The imagery prior to 12 September was masked in clouds for a week making it impossible to observe the formation of the bloom from satellite. The bloom was persistent in the imagery from 12 September through mid November. The bloom most likely originated along the southern coast of Texas, near South Padre Island, and was then transported to the



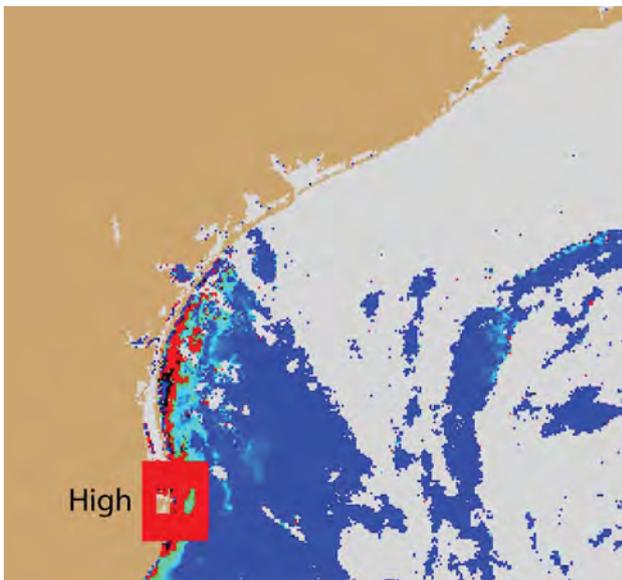
**Figure 1.** The SeaWiFS chlorophyll anomaly image from 12 September 2005, at the start of the bloom. Red indicates areas that have shown an increase in chlorophyll of at least 1  $\mu\text{g L}^{-1}$ , relative to a 60-day mean.



**Figure 2.** The anomaly image from 20 September. It illustrates how the bloom began expansion and intensification.

north. Fig. 2 shows the imagery from 20 September 2005 and illustrates the extension of the bloom further up the coast relative to Fig. 1. The bloom also appeared to intensify in the satellite imagery coinciding with cell counts in “Medium” levels. Fig. 3 shows the image from 30 September 2005, with “High” counts observed in the southern portion of the coastline as the intensification of the bloom continued.

This technique has been incorporated into the NOAA Harmful Algal Bloom Forecasting System (HAB-FS). The HAB-FS creates and sends bulletins to managers once a week (twice a week during a bloom) describing the conditions using data from: field cell counts, wind, and remote sensing (Stumpf *et al.* 2003).



**Figure 3.** The anomaly image from 30 September. Note that all of the *in situ* cell counts have reached “High” levels ( $> 1,000,000$  cells  $L^{-1}$ ).

### Discussion

Hurricanes and other major limatological forcing functions may cause the technique to fail, as the algorithm is unable to distinguish toxic algal blooms from non-toxic algal blooms. Not only will hurricanes cause short-term resuspension of benthic chlorophyll, but they also supply nutrients via run-off into the system which can fuel non-toxic algal blooms. In addition storms can create a destabilized water column, which is conducive to the formation of diatom blooms.

For these reasons, the imagery from the northern portion of the Texas coastline showed large algal blooms through much of the fall of 2005 following the passage of Hurricane Rita in late September.

**Table 1.**

Category	Cell Count	Symbol Used
Not Present	0	X
Present	1-999	□
Very Low a	1000 - 4999	■
Very Low b	5000 - 9999	■
Low	10,000 – 99,999	■
Medium	100,000 – 999,999	■
High	$> 1,000,000$	■

### References

- Florida Marine Research Institute. 2001. Red tides in Florida, 1954-1998: Harmful Algal Bloom Historical Database. CD-ROM, Version 1.0
- Stumpf, R.P., Arnone, R.A., Gould, R.W., Martinovich, P., Ransibrahmanakul, V., Tester, P.A., Steward, R.G., Subramaniam, A., Culver, M. & Penock, J.R. (2000). Sixth International Conference on Remote Sensing for Marine and Coastal Environments. Charleston SC. Veridian ERIM Intl. Ann Arbor MI, USA, p. 25\_27.
- Stumpf, R.P., Culver, M.E., Tester, P.A., Tomlinson, M., Kirkpatrick, G.J., Pederson, B.A., Truby, E., Ransibrahmanakul, V., & Soracco, M.. (2003). Harmful Algae 2: 147-160.
- Tester, P.A., Stumpf, R.P. & Steidinger, K.. (1998). Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO 2001.
- Tomlinson, M.C., Stumpf, R.P., Ransibrahmanakul, V., Truby, E.W., Kirkpatrick, G.J., Pederson, B.A. Vargo, G.A. & Heil, C.A. 2004. Remote Sensing of Environment 3-4: 293-303.
- Wynne, T.T., Stumpf, R.P., Tomlinson, M.C., Ransibrahmanakul, V. & Villareal, T.A. (2005). Harmful Algae 4: 992-1003.



## 5. POPULATION DYNAMICS



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Manger à trois : toxic and non-toxic *Karlodinium veneficum* strains with a predator, *Oxyrrhis marina*, and prey, *Storeatula major*

J.E. Adolf<sup>1</sup>, T.R. Bachvaroff<sup>2</sup> and A.R. Place<sup>3</sup>

UMBI Center of Marine Biotechnology, Columbus Center, Suite 236, 701 E. Pratt St., Baltimore, MD USA 21202, <sup>1</sup>adolf@umbi.umd.edu, <sup>2</sup>bachvaro@umbi.umd.edu, <sup>3</sup>place@umbi.umd.edu

### Abstract

The mixotrophic dinoflagellate *Karlodinium veneficum* (syn. *K. micrum*) is a common member of temperate, coastal phytoplankton assemblages that can occasionally form ichthyotoxic blooms. Relatively little is known of the mechanisms that allow *K. veneficum* bloom formation, but we do know that *K. veneficum* can be both predator and prey in natural assemblages. Here we present the results of an experiment wherein *K. veneficum* strains (toxic vs. non-toxic) were co-cultured with a predator (*Oxyrrhis marina*), and the cryptophyte *Storeatula major*, a shared prey item of *O. marina* and *K. veneficum*, to test the ability of karlotoxin to simultaneously inhibit a predator and increase prey capture efficiency. We hypothesized that *K. veneficum* would dominate the three-species mixture containing toxic *K. veneficum* strain CCMP 2064, and that *O. marina* would dominate the three-species mixture containing non-toxic *K. veneficum* strain MD5. We observed that toxic *K. veneficum* out-competed *O. marina* for cryptophytes while the non-toxic strain did not. Our results suggest that karlotoxin production gives *K. veneficum* a growth advantage by simultaneously inhibiting predator grazing and improving prey ingestion efficiency.

### Introduction

Harmful algal blooms (HAB) are ecological phenomena, that is, they result from interactions of the HAB species with its abiotic environment as well as with co-occurring algae and heterotrophic predators. The production of anti-herbivory allelochemicals is likely one mechanism whereby harmful algae (HA) reduce losses due to predation (Tillmann 2004). Other studies have shown that allelochemicals produced by mixotrophic HA aid in prey capture (Tillmann 1998; Skovgaard and Hansen 2003; Adolf *et al.* 2006a). *K. veneficum* is a toxic dinoflagellate that co-occurs with *O. marina* and cryptophytes in Chesapeake Bay (Li *et al.* 2000; Johnson *et al.* 2003) and most likely in other environments. We have been examining interactions between these organisms, and how they are mediated by karlotoxin, in order to better understand bloom formation by *K. veneficum*.

Comparisons of *K. veneficum* strain CCMP 2064 (hereafter referred to as 'toxic') and *K. veneficum* strain MD5 (hereafter referred to as 'non-toxic') have suggested an anti-herbivory role (against *O. marina*) for karlotoxins (Adolf *et al.* 2006b). Likewise, treatment of a cryptophyte prey of *K. veneficum* (*Storeatula major*) with a sub-lethal dose of karlotoxin (50 ng ml<sup>-1</sup>) result in increased *K. veneficum* grazing rates suggesting that karlotoxin increases prey capture efficiency in *K. veneficum* (Adolf *et al.* 2006a). Both of these observations are consistent with find-

ings that karlotoxin action depends on the presence of des-methyl sterols in the target species (Deeds and Place 2006). Here we present the results of an experiment wherein *K. veneficum*, *O. marina*, and *S. major* were mixed in three-species culture to test the ability of karlotoxin to simultaneously inhibit a predator and increase prey capture efficiency. We hypothesized that toxic *K. veneficum* would dominate the three-species culture with *O. marina* and *S. major* while *O. marina* would dominate the three-species culture containing non-toxic *K. veneficum* and *S. major*.

### Methods

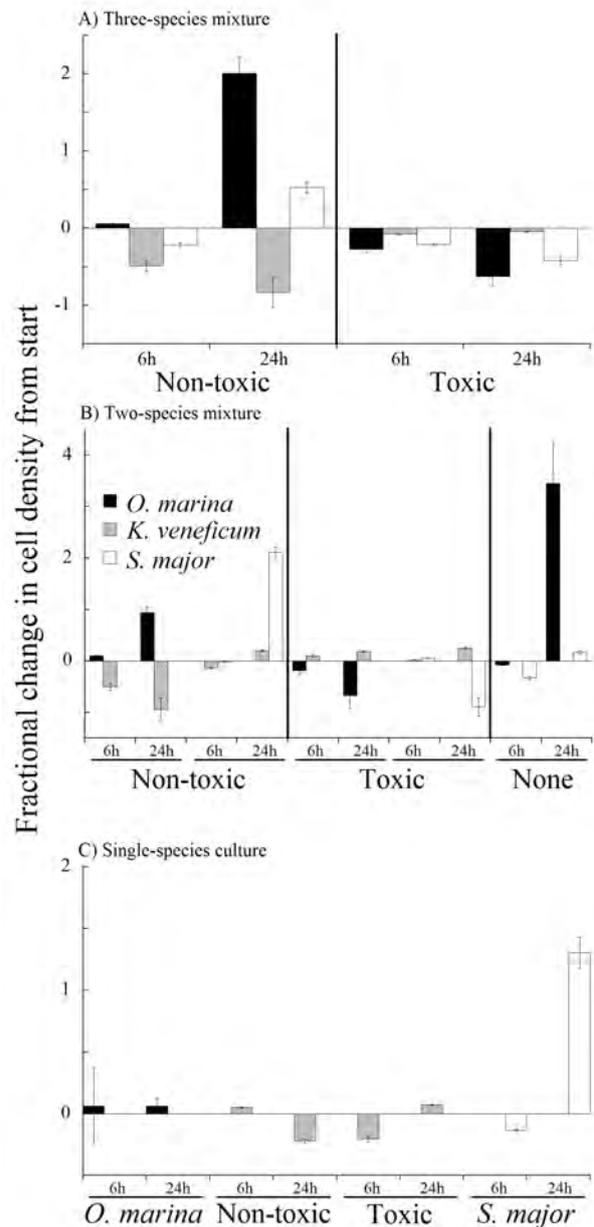
Algal cultures were maintained in F/2 medium at 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  PAR (12:12 L:D), 20 °C, salinity 15 ‰. Late-exponential to early-stationary phase cultures of toxic *K. veneficum* strain CCMP 2064 (toxic) and strain MD5 (non-toxic) were diluted to a density of 100,000 cells ml<sup>-1</sup>. A late exponential phase culture of the cryptophyte, *S. major*, was diluted to a density of 400,000 cells ml<sup>-1</sup>. A culture of *Oxyrrhis marina* that had been maintained on *S. major* prey, but last fed two-days preceding this experiment, was diluted to 10,000 cells ml<sup>-1</sup>. Microscopic and Coulter® counts verified the absence of food in the *Oxyrrhis* stock culture. The Coulter® Counter was also used to measure the Equivalent Spherical Volume (E.S.V.) of each species: *K. veneficum* MD5, 395 ( $\pm 41.7$ )  $\mu\text{m}^3$ ; *K. veneficum* CCMP 2064, 606 ( $\pm 74.0$ )  $\mu\text{m}^3$ ; *S. ma-*

*major*,  $190 (\pm 23.8) \mu\text{m}^3$ ; *O. marina*,  $3279 (\pm 341.5) \mu\text{m}^3$ . The experiment was set up, with each treatment run in triplicate, in polystyrene 24-well plates (Falcon) by pipeting from stock cultures to yield target cell densities of  $3700 O. marina \text{ ml}^{-1}$ ,  $44,000 K. veneficum \text{ ml}^{-1}$ , and  $74,000 S. major \text{ ml}^{-1}$ , in all mixtures. The order of addition was medium, *S. major*, *K. veneficum*, followed last by *O. marina*. Three species combinations, as well as two-species and single-species controls with a culture medium blank instead of the omitted species, were prepared. Data from a single species control of *O. marina* from a similar experiment (Adolf *et al.* 2007) was used as this control was not run in the present experiment. When all components had been added, the plates were wrapped in clear PVC film and placed back in the growth incubator. Cell samples were taken at 6 h (at which time ingested cells are clearly visible and not yet digested in both *K. veneficum* and *O. marina*) and 24 h by pipetting 1 ml from each well into a sterile Eppendorf tube containing 20  $\mu\text{l}$  of 50 % glutaraldehyde, to yield a final 1% glutaraldehyde solution. Preserved cell samples were stored under foil at  $4^\circ\text{C}$  before being prepared for examination by epifluorescence microscopy. To each sample,  $36 \mu\text{l}$  of 0.1 mg L-1 DAPI were added and allowed to incubate in the dark for 10-15 minutes. The entire contents of each tube was then vacuum filtered onto blackened  $0.22\text{-}\mu\text{m}$  pore size Nuclepore membrane filters and mounted on glass slides. These were stored frozen until examination. Each slide was examined using a Zeiss Axioplan 2 epifluorescence microscope at 250X with a Zeiss filter set No. 9 (ex. 450 – 490 nm; em. LP 550 nm) in order to count (1) *K. veneficum* (red fluorescent cells), (2) *S. major* (orange fluorescent cells), and (3) *S. major* ingested per *K. veneficum* (counted as orange fluorescent inclusions [O.F.I.], resulting from *S. major* phycoerythrin content, per *K. veneficum*). Each slide was also examined with a DAPI filter set in order to count (4) *O. marina*, and (5) *K. veneficum* ingested per *O. marina* (counted as dinoflagellate nuclei per *O. marina*). The last category was not quantitated for the 24 h samples as the dinoflagellate nuclei were not clearly visible inside *O. marina* at this point. ANOVA and t-tests were done on count data, where  $n = 9$  for each treatment (3 replicates, 3 counts each).

## Results and Discussion

### Mixtures with non-toxic *K. veneficum*

Figure 1 shows the fractional change (at 6 and 24 h) from starting cell density for the 3-species, 2-species



**Figure 1.** Fractional change from starting cell density for all treatments run. ‘Non-toxic’ and ‘Toxic’ refer to *K. veneficum* strain used. Starting densities were  $3700 \text{ ml}^{-1}$  (*O. marina*),  $44,000 \text{ ml}^{-1}$  (*K. veneficum*), and  $74,000 \text{ ml}^{-1}$  (*S. major*). Triplicate counts of each culture or mixture were made at 6 h and 24 h.

and 1-species experiments. At 6 h, *O. marina* density was similar in all 3-species and 2-species mixtures (ANOVA  $F_{4,40} = 0.69$   $p = 0.61$ ). At 24 h, *O. marina* densities in the 3-species and 2-species mixtures with non-toxic *K. veneficum* or with *S. major* indicated significant growth (ANOVA  $F_{4,40} = 14.6$   $p < 0.001$ ), equivalent to approximately 1 division per day. This increase in *O. marina* density was accompanied by a

significant decline in *K. veneficum* cell density, either when mixed with non-toxic *K. veneficum* alone (Fig. 1A) or in the presence of *S. major* (Fig. 1B). Non-toxic *K. veneficum* density was significantly reduced (24 h) in the 3-species and 2-species ('*O. marina* + non-toxic *K. veneficum*') mixtures compared to the single species control and non-toxic *K. veneficum* + *S. major* 2-species mixture (ANOVA  $F_{3,30} = 105.0$   $p < 0.001$ ). This contrasts with the single-species culture in which *O. marina* and non-toxic *K. veneficum* cell density changed little and counts at 6 h and 24 h showed no significant difference (t-test,  $p > 0.05$ ) (Fig. 1C). *S. major* density (24 h) was significantly higher in the single species culture and 2-species mixture containing non-toxic *K. veneficum* compared to other mixtures (ANOVA  $F_{5,48} = 51.9$   $p < 0.001$ ), but showed lower final (24h) density in 2-species culture with *O. marina* or in the 3-species non-toxic mixture. This pattern is consistent with the conclusion that grazing matched cryptophyte growth in mixtures containing *O. marina*, with or without non-toxic *K. veneficum*, and that non-toxic *K. veneficum* did not significantly graze *S. major*.

#### Mixtures with toxic *K. veneficum*

*Oxyrrhis marina* density declined significantly when mixed with toxic *K. veneficum* in the presence (Fig. 1A) or absence (Fig. 1B) of *S. major*. *O. marina* density (24 h) in mixtures containing toxic *K. veneficum* was significantly reduced (~64%) compared to densities at 6h (ANOVA  $F_{4,40} = 14.6$   $p < 0.001$ ). *O. marina* mortality was similar in the toxic *K. veneficum*-containing 3-species and 2-species ('*O. marina* + toxic *K. veneficum*') mixture (Fig. 1C), eliminating the possibility that the presence of *S. major* contributed to *O. marina* mortality in the 3-species mixture. Toxic *K. veneficum* density in the 3-species mixture (24 h) was unchanged from the single species toxic *K. veneficum* control and the 2-species mixtures containing toxic *K. veneficum* and *O. marina* or toxic *K. veneficum* + *S. major* (ANOVA  $F_{3,32} = 1.8$   $p = 0.18$ ). The change in the single species culture of toxic *K. veneficum* between 6 and 24 h (35% increase) was significant ( $p = 0.03$ ). *S. major* density declined significantly between 6 and 24 h in the toxic 3-species (Fig. 1A) and 2-species mixtures (Fig. 1B).

#### Predation

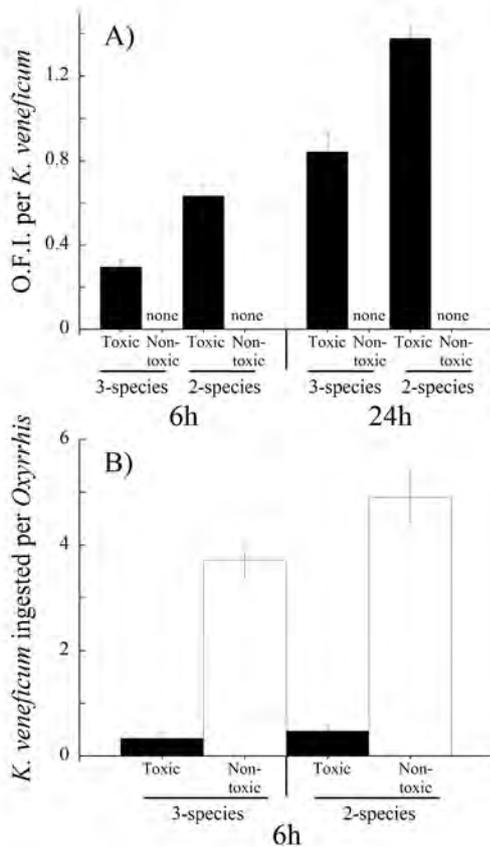
We observed predation of toxic *K. veneficum* on *S. major* and low grazing by *O. marina* on toxic *K. veneficum* in the 2-species and 3-species mixtures (Fig. 2). At both time points, grazing of toxic *K. veneficum*

on *S. major* was greater in the 2-species than the 3-species mixture (t-test,  $p < 0.001$ ,  $n=9$ ). By contrast, we did not observe ingested cryptophytes in non-toxic *K. veneficum* at either time point, but grazing of *O. marina* on non-toxic cryptophytes (6 h) was high in both the 2-species and 3-species mixtures (Fig. 2B). The grazing rates of *O. marina* on *K. veneficum* in the 2-species and 3-species mixtures (6 h) did not differ significantly for either strain (t-test  $p > 0.05$ ). The near depletion of non-toxic *K. veneficum* in the 3-species and 2-species mixture containing *O. marina* suggests continued grazing of *O. marina* on non-toxic *K. veneficum* although we could not directly count *K. veneficum* nuclei inside *O. marina* at this time point (see methods). We have never observed grazing of *K. veneficum* on *O. marina*.

#### Conclusions

Our results suggest that karlotoxin production gives *K. veneficum* a growth advantage by simultaneously inhibiting predator grazing and improving prey ingestion efficiency. Toxic *K. veneficum* out-competed *O. marina* in 3-species culture with cryptophytes while non-toxic *K. veneficum* did not. The success of toxic *K. veneficum* in this experiment was due to its grazing on cryptophytes and the mortality of *O. marina* exposed to toxic *K. veneficum*. That exposure to toxic *K. veneficum* was the source of *O. marina* mortality is supported by comparisons of *O. marina* densities (24h) in the different treatments we ran. If lack of food were the source of *O. marina* mortality then we would expect to see no mortality in the 3-species mixture containing *S. major* and toxic *K. veneficum* as potential prey for *O. marina*.

Increased grazing of toxic *K. veneficum* on *S. major* (Fig. 2A) in the absence of *O. marina* than in the presence of *O. marina* was an unexpected but intriguing result. There was no difference in *K. veneficum*  $\text{ml}^{-1}$  between these two treatments at the 6 or 24 h time points (t-tests,  $p=0.58$  and  $0.09$ , respectively) to explain this result. One obvious explanation would be that *O. marina* competed with *K. veneficum* for cryptophytes, but the following calculations suggest *O. marina* actually ingested fewer cryptophytes (6h) in the presence of toxic *K. veneficum*. Using the 74,000 *S. major*  $\text{ml}^{-1}$  as a starting density, and the observed 6h *S. major* density in the 2-species mixture of *O. marina* + *S. major* we can calculate that *O. marina* removed 24,000 *S. major* in 6 h in the absence of toxic *K. veneficum*. Using the same *S. major* starting density and the observed *S. major* density (6 h) in the toxic 3-spe-



**Figure 2.** (A) Grazing by *K. veneficum* strains on *S. major* (O.F.I. = orange fluorescent inclusions per *K. veneficum*). (B) Grazing by *O. marina* on *K. veneficum* strains. 24-h time point data were not available for this measurement due to digestion of prey inside *O. marina*.

cies mixture with toxic *K. veneficum* shows that, in total, ~16,000 *S. major* were removed. We know, based on the O.F.I.s in *K. veneficum* (6 h), that *K. veneficum* removed 12,000 *S. major* in the toxic 3-species mixture, leaving only 4,000 *S. major* unaccounted for and potentially grazed by *O. marina*. The suppression of feeding by *O. marina* on non-toxic prey by the presence of toxic *K. veneficum* is consistent with previous findings wherein *O. marina* was observed to feed less on non-toxic *K. veneficum* in the presence of toxic *K. veneficum* (Adolf *et al.* 2007), but excludes the possibility that reduced grazing of toxic *K. veneficum* on *S. major* in the toxic 3-species mixture was due to competition with *O. marina*. We suggest instead that *K. veneficum* lost some of its toxin to *O. marina* and thus had less to use for the purpose of prey immobilization (Adolf *et al.* 2006).

In *K. veneficum* we know that the presence or absence of karlotoxin (i.e. anti-grazing allelochemicals) is a matter of genetic differences between strains (Bachvaroff *et al.*, in prep.), not necessarily physi-

ological differences related to nutrient stress although nutrient stress will increase karlotoxin levels in toxic strains (Adolf *et al.*, in prep.). This contrasts with the model of HA competition stemming from three-species experiments reported by Mitra and Flynn (2006) wherein nutrient limitation is essential to induce grazing deterrents in HAB species. For *K. veneficum* we emphasize the importance of high biomass ( $10^4 - 10^5 \text{ ml}^{-1}$ ) and the presence of a toxic strain (Adolf *et al.* 2007). This experiment demonstrates that, without karlotoxin, *K. veneficum* is a good food source for *O. marina* and a poor grazer of *S. major*. Prey capture without the aid of karlotoxin is possible (pers. obs.) but apparently inefficient. Mixotrophic growth of toxic strains, aided by an abundance of cryptophyte prey in appropriate environments, is likely an important mechanism whereby *K. veneficum* achieves high biomass *in situ*.

### Acknowledgements

This work was funded in part by grants from National Oceanic and Atmospheric Administration Coastal Ocean Program under ECOHAB award #NA04-NOS4780276 to University of Maryland Biotechnology Institute, and Grant # U50/CCU 323376, Centers for Disease Control and Prevention and the Maryland Department of Health and Mental Hygiene. This is contribution # 07-157 from the UMBI Center of Marine Biotechnology, and # 210 from the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) programme.

### References

- Adolf, J. E., Bachvaroff, T.R., Krupatkina, D.N., Nonogaki, H., Brown, P.J.P., Lewitus, A.J., Harvey, H.R. & Place, A.R. (2006). In: Afr. J. Mar. Sci. Harmful Algae 2004, G. C. Pitcher, Probyn, T.A. & Verheye, H.M. (eds.), 28: 415-421.
- Adolf, J. E., Krupatkina, D., Bachvaroff, T. & Place, A.R. (2007). Harmful Algae 6: 400-412.
- Deeds, J.R. & Place, A.R. (2006). In: Afr. J. Mar. Sci. Harmful Algae 2004, G.C. Pitcher, Probyn, T.A. & Verheye, H.M. (eds), 28: 421-427.
- Li, A., Stoecker, D.K. & Coats, D.W. (2000). J. Plankton Res. 22: 2105-2124.
- Mitra, A. & Flynn, K.J. (2006). Biol. Lett. 2: 194-197.
- Skovgaard, A. & Hansen, P.J. (2003). Limnol. Oceanogr. 48: 1161-1166.
- Tillmann, U. (1998). Aquat. Microb. Ecol. 14: 155-160.
- Tillmann, U. (2004). J. Euk. Microbiol. 51: 156-168.

## A fuzzy logic model for *Alexandrium minutum* proliferations in harbours of the Catalan coast (NW Mediterranean)

M. Estrada<sup>1</sup>, L. Arin<sup>1</sup>, D. Blasco<sup>1</sup>, A. Blauw<sup>2</sup>, J. Camp<sup>1</sup>, E. Garcés<sup>1</sup>, N. Sampedro<sup>1</sup> and M. Vila<sup>1</sup>

<sup>1</sup>Institut de Ciències del Mar, CMIMA (CSIC), Pg. Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain, marta@icm.csic.es, <sup>2</sup>Delft Hydraulics, Rotterdamseweg, 185, 2629 HD Delft, The Netherlands, anouk.blauw@wldelft.nl

### Abstract

The dinoflagellate *Alexandrium minutum*, a paralytic shellfish toxin producer, forms recurrent blooms in many estuaries and semi-enclosed marine areas. We designed a fuzzy logic model, based on historical ecological information and routinely monitored meteorological variables, to predict the occurrence and intensity of *A. minutum* proliferations in two selected harbours (Olímpic Harbour of Barcelona and Arenys Harbour) of the Catalan coast (NW Mediterranean). The model showed the importance of long spells of calm weather in allowing the appearance of dense populations of *A. minutum* and successfully predicted 6 large blooms recorded in these harbours during the studied periods (1996-2002 for the Olímpic and 2000-2002 for Arenys). This result suggests that even with limited understanding of the processes affecting bloom dynamics, fuzzy models may be helpful in providing insight for management measures.

### Introduction

Deterministic models are useful tools in harmful algal bloom (HAB) research but they are difficult to apply in cases with poor knowledge of the ecological processes involved and lack of high quality data. The fuzzy logic approach aims to summarise complex processes in 'knowledge rules' combining qualitative and quantitative information and allows for a certain degree of uncertainty within the available data set (Blauw *et al.* 2006).

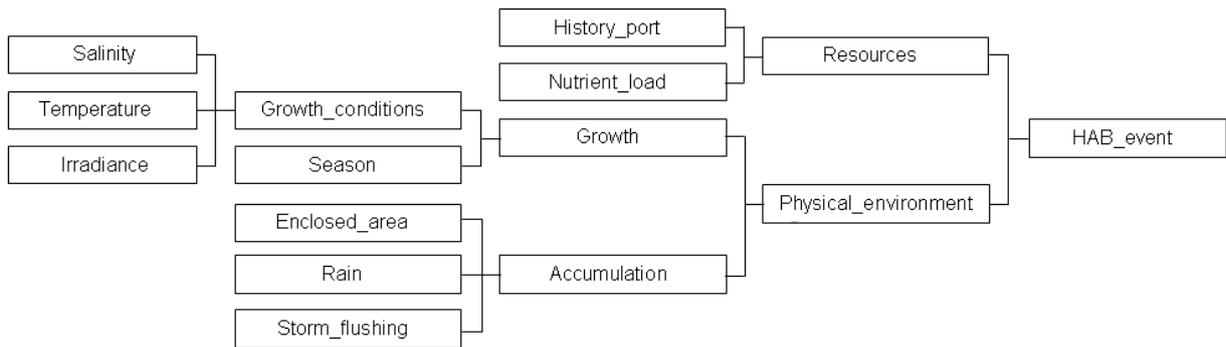
Observations in the Catalan coast (NW Mediterranean) suggest a conceptual model (Vila *et al.* 2001; Vila & Masó 2005) in which HAB events are linked to small-scale phenomena generated in areas of high water residence times due to natural (littoral fronts, semi-enclosed bays) or artificial (coastal infrastructures) factors. Within this framework, we hypothesised that rainfall followed by long spells of good weather would favour blooms, due to water column stabilization and nutrients associated with freshwater runoff. The aim of this work is to test these hypotheses and to present a fuzzy logic model for predicting the intensity of *Alexandrium minutum* blooms, based on monitoring data and historical series of meteorological variables. Given tourist interest for the Catalan coast, blooms of *A. minutum* (and other dinoflagellates) may cause problems not only due to toxicity but also for water discolouration. Understanding conditions favouring their appearance is important for management. The model was implemented and calibrated with a data set (1996-2002) from the Olímpic Harbour of Barcelona. Validation was carried out with data (2000-2002)

from the Arenys de Mar Harbour (40 km north of Barcelona), where the largest *A. minutum* proliferations of the region have been recorded.

### Model Implementation

We used the Ecofuzz software (available from [www.habes.net](http://www.habes.net) until at least 2008 and from WL | Delft Hydraulics thereafter). The first step in fuzzy model design is the development of a conceptual scheme that represents the relationships between input variables, intermediate variables and the final output parameter. The conceptual scheme developed for *A. minutum* (Fig. 1) is divided in several parts (see [www.habes.net](http://www.habes.net) for more details). The links leading to Growth\_conditions (intrinsic growth potential) reflect the general appropriateness of irradiance, temperature and salinity for *A. minutum* growth. In the Catalan coast, these variables are always within the required ranges. Season indicates the adequacy of the seasonal timing and together with Growth\_conditions determines environmental suitability for *A. minutum* growth.

The lower part of the scheme, leading to Accumulation, indicates the possibility of cell accumulation associated with low physical dispersion. Rain parameterizes the effect of precipitation and runoff, and Storm\_flushing represents the effect of wind. Enclosed\_area reflects the increase in water residence time due to harbour piers, but the model could be extended for application in open waters by parameterizing Enclosed\_area to account for the presence of littoral fronts of certain persistence and intensity in otherwise open littoral waters. In this case, runoff



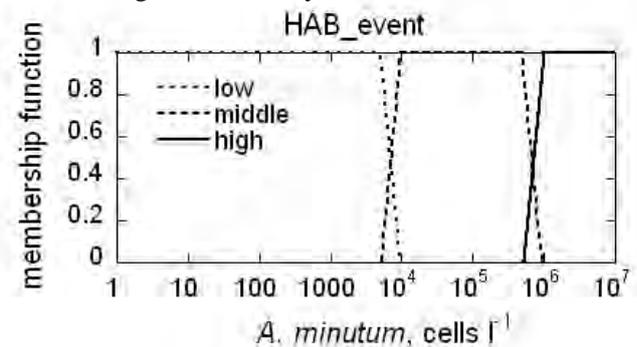
**Figure 1.** Conceptual scheme for *Alexandrium minutum* bloom development

and wind speed in the appropriate directions could be the main variables influencing the occurrence of these fronts.

Resources results from Nutrient\_load and History\_port that represent, respectively, estimates of historical information on nutrient availability (some harbours have higher overall nutrient inputs than others) and abundance of *A. minutum* (e.g., due to presence of cyst beds) in a particular area. They were included in the model to allow its use for different locations. Growth and Accumulation determine the suitability of the Physical\_environment, which combines with Resources to produce a prediction of the final output variable, *A. minutum* abundance or bloom intensity (HAB\_event).

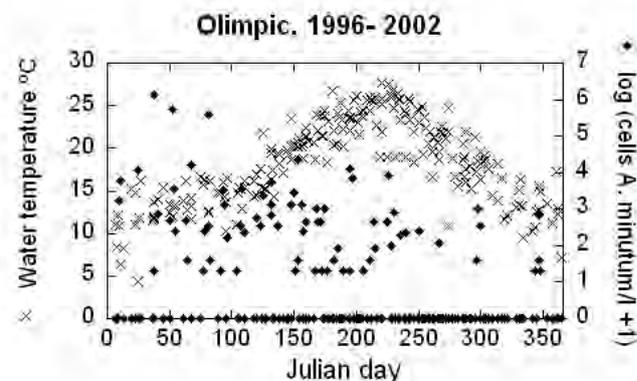
The following step is the implementation and adjustment of quantitative relationships and the definition of membership functions. Relationships between input and output variables are expressed in form of IF-THEN rules (see example in Table 1). Membership functions vary between 0 and 1 and express to what degree a value of a variable belongs to a class (such as low, middle or high in Fig. 2). Weekly to monthly *A. minutum* abundance data for the Olímpic and Arenys de Mar harbours were obtained as part of a monitoring plan ("Pla de vigilància de fitoplàncton nociu i tòxic a la costa catalana"), supported by the Agència Catalana de l'Aigua (Government of Catalonia). Some input variables such as Enclosed\_area, History\_port and Nutrient\_load were classified in terms of relative units ranging between 0 (low) and 1 (high), based on a semi-quantitative appreciation of historical data from the monitoring program. For both harbours, the value assigned to these three variables was 0.7 (indicating a strong degree of membership into the high categories for Enclosed\_area, History\_port and Nutrient\_load). Intermediate parameters such as Growth\_conditions, Resources, Physical\_environment, Growth and Accumulation were also recorded as relative values. The meteorological data were provided by the Olímpic

Harbour station of the Instituto Nacional de Meteorología and by the Vilassar de Mar station of the Servei Meteorològic de Catalunya.



**Figure 2.** Membership functions for *A. minutum* bloom intensity (HAB\_event in Fig. 1).

The monitoring data for the Olímpic Harbour indicated (Fig. 3) that the probability of strong *A. minutum* blooms is higher in winter (Julian days 30-100), middle in spring-summer (Julian days 130-250) and low outside these periods. These observations were used to parameterize the input variable, Season. No simple significant relationships were found between *A. minutum* abundance in the harbour and any of the meteorological (precipitation, wind speed and direction, air temperature, cloudiness) or hydrographical (T, S) variables routinely available.



**Figure 3.** Water temperatures and *A. minutum* concentrations in the monitoring samples taken from the Olímpic Harbour between 1996 and 2002.

However, some observation patterns, particularly concerning rainfall and wind speed (respectively, Rain and Storm\_flushing in Fig. 1), could be used to constrain bloom situations. In contrast to one of our initial hypotheses, examination of the Olímpic Harbour data set showed that rainfall above a certain intensity had a negative effect on *A. minutum* blooms, presumably due to flushing caused by runoff or by the winds associated with the storms. We observed also that high density winter blooms did not appear after strong winds. In the formulation of the knowledge rules, Rain was considered as high if it exceeded 20-25 mm in the previous 15 d. Storm\_flushing was high if there were one or more wind events with speeds  $>22 \text{ km h}^{-1}$  in the previous month. A partial example of the knowledge rules used in the model is given in Table 1.

**Table 1.** Example of knowledge rules between the degree of water confinement or Enclosed\_area (Encl.), Rain, Storm\_flushing (Storm.) and the output parameter Accumulation. The full table would include all the combinations between the different classes.

Input parameters			Output parameter
Encl.	Rain	Storm.	Accumulation
Low	Low	High	Low
High	Low	Low	High
Middle	Low	Low	Middle

## Results and Discussion

The model was run and calibrated with the Olímpic Harbour monitoring data and validated with an independent data set from the Arenys de Mar Harbour. The results are given in Table 2. The model performed fairly well for low concentrations and successfully predicted all observations exceeding  $10^6$  cells/L (high HAB\_event). Given the low number of observations and the limitations of available knowledge, the success with the high bloom intensities has to be taken with caution, but it does indicate the importance of prolonged periods of calm weather. Most errors occur between low and middle predicted abundance and tend to exaggerate observed population levels. This type of bias may be more desirable than the opposite, of under-predicting bloom intensity.

The results supported our initial hypothesis concerning the importance of high water residence times and improved our view of the relationships between rainfall and bloom development. Enhancing prediction ability would need a better understanding of *A.*

*minutum* population dynamics in relation to hydrographic and meteorological conditions. An additional difficulty is that, in many cases, *A. minutum* is not the dominant component of the phytoplankton community, although it can cause toxicity problems at relatively low concentrations. In spite of its simplicity, the fuzzy model presented here provided a conceptual framework for risk assessment and helped to identify a combination of environmental factors favouring bloom development.

**Table 2.** Comparison of observed and modelled *A. minutum* abundance (HAB\_event). Low, middle and high (Fig. 2) indicate the intensity of *A. minutum* blooms (HAB\_event in Fig. 1).

Olímpic (1996-2002)		Observed		
		Low	Middle	High
Modelled	Low	151	1	0
	Middle	36	2	0
	High	13	4	1
% correct		76	29	100

Arenys (2000-2002)		Observed		
		Low	Middle	High
Modelled	Low	61	6	0
	Middle	24	7	0
	High	6	1	5
% correct		76	50	100

## Acknowledgements

This work was supported by EU project HABES, (EVK2-CT-2000-00092). We thank the “Pla de vigilància de fitoplàncton nociu i tòxic a la costa catalana” (Agència Catalana de l’Aigua, Generalitat de Catalunya) for phytoplankton data, and the Instituto Nacional de Meteorología and the Servei Meteorològic de Catalunya for meteorological data. S. Anglès and J. Riba provided technical assistance.

## References

- Blauw, A.N., Anderson, P., Estrada, M., Johansen, M., Laanemets, J., Peperzak, L., Purdie, D., Raine, R. & Vahtera, E. (2006). *S. Afr. J. Mar. Sci.* 28: 365-469.
- Vila, M., Camp, J., Garcés, E., Masó, M. & Delgado, M. (2001). *J. Plankton Res.* 2: 497-514.
- Vila, M. & Masó, M. (2005). *Scientia Mar.* 69: 31-45.

## The Ebro Delta coastal embayments, a GEOHAB pilot site for the study of HAB population dynamics

M. Fernández-Tejedor<sup>1,6</sup>, L.M. Elandaloussi<sup>1,6</sup>, E. Mallat<sup>1,6</sup>, E. Cañete<sup>1,6</sup>, A. Caillaud<sup>1,6</sup>, P. Riobo<sup>2</sup>, B. Paz<sup>2</sup>, J. Franco<sup>2</sup>, D. Ibarra<sup>3</sup>, A. Cembella<sup>4</sup>, D. Blasco<sup>5</sup> and J. Diogène<sup>1,6</sup>

<sup>1</sup>IRTA, Apartado de Correos 200, Carretera del Poblenou km 5.5 E-43540 Sant Carles de la Ràpita, Spain, margarita.fernandez@irta.es, <sup>2</sup>IEO, Cabo Estai – Canido, 36200 Vigo, Spain, jose.franco@vi.ieo.es

<sup>3</sup>Department of Oceanography, Dalhousie University, 1355 Oxford Street, B3H 4J1, Halifax, NS, Canada, dibarra@dal.ca, <sup>4</sup>Alfred Wegener Institut, Am Handelshafen 12, D-27570 Bremerhaven, Germany, acembella@awi-bremerhaven.de, <sup>5</sup>Centre Mediterrani d'Investigacions Marines i Ambientals, CMIMA-CSIC Passeig Marítim de la Barceloneta, 37-49 E-08003 Barcelona, Spain, icmdir@icm.csic.es,

<sup>6</sup>Xarxa de referència d'R+D+I en Aqüicultura. Generalitat de Catalunya

### Abstract

Coastal embayments of the Ebro Delta in the NW Mediterranean include Fangar and Alfacs Bays. Both bays are active sites for the production of mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) and have been subjected to extensive studies of HABs. We present herein the first year results of a collaborative research project concerning HAB population dynamics in Alfacs Bay. In conjunction with the local monitoring programme, this project uses Alfacs Bay as a pilot site for comparative studies of HABs in coastal embayments within the GEOHAB programme. The distribution of harmful species was measured by direct counting along with chlorophyll-*a*, biooptical characteristics of the water column, and key environmental and physical parameters that could be crucial for understanding HAB dynamics. Toxicity was measured by analytical chromatography and by assessing cytotoxicity of phytoplankton recovered from net hauls. Evaluation of toxicity over a 52-week period showed that diarrhetic shellfish poisoning (DSP) episodes co-occurred with yessotoxins (YTX) in the shellfish from Alfacs Bay. The presence of DSP correlated with the abundance of *Dinophysis* species in only about half the incidences where DSP was measured. The source of the remaining DSP events is unknown and no environmental triggers for toxin production is yet evident. There was no evidence that the abundant *Pseudo-nitzschia* species were producing domoic acid.

### Introduction

#### *The Coastal Embayment*

Alfacs Bay has a surface area of ca 50 km<sup>2</sup>, and volume of 191x10<sup>6</sup> m<sup>3</sup>. Average and maximal depths are 4 m and 6 m, respectively, and there is a 3-km opening to the Mediterranean (Camp and Delgado 1987). Coastal currents, wind and freshwater inputs from agricultural irrigation runoff affect circulation patterns and the residence time of water. Harmful algal blooms have been responsible for recurrent DSP and ichthyotoxic events.

#### Project Description

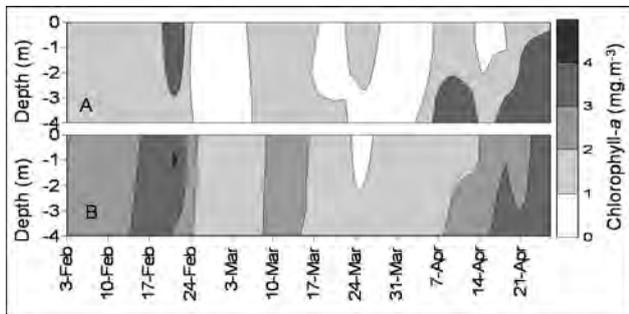
The overall goal of this project is to improve monitoring and prediction of harmful algal blooms in coastal embayments by comparative studies of two coastal embayment ecosystems: Ship Harbour in Nova Scotia, Canada and Alfacs Bay in Spain. This research is a component of the international programme on Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB) and part of the Core Project on HABs in

Fjords and Coastal Embayments. The experimental design includes measurements of the physical, chemical and biological characteristics of coastal embayments, including nutrient loading and the degree of stratification and mixing of the water column. These data are used to determine how residence time and water circulation patterns affect HAB population dynamics. Critical taxonomy was performed on the vegetative cells of potentially toxic algal species, and on benthic and epiphytic growth on aquaculture installations. Algal cultures were established after isolation from net hauls and benthic samples to identify the characteristics of HAB species. Advanced analytical methods were used to determine toxin cell quotas and toxin profile of isolates and natural populations by HPLC with fluorescence detection (HPLC-FLD) and by liquid chromatography coupled with mass spectrometry (LC-MS). Toxins were also determined in shellfish and in the dissolved phase in the water column. Dissolved phase toxins were accumulated on DIAION resin deployed in series in small Nitex bags (SPATT technique) within and adjacent to aquaculture instal-

lations. Two Tethered Attenuation Coefficient Chain Sensors (TACCS; Satlantic, Inc.) were deployed in the water column inside and outside the mussel farms during February, March and April 2005 to determine the effect of bivalve grazing on populations of HAB species and other phytoplankton. Multidimensional analyses were performed on data sets gathered during the specific field experiments, and previous monitoring data were integrated with information on growth and toxicity from culture experiments.

**Preliminary Results**

Seston depletion within the mussel farms during TACCS deployment was 16, 13, 7 and 6 % for 0-1, 1-2, 2-3, and 3-4 m depth intervals, respectively. Chlorophyll-*a* depletion was estimated to be 38, 45, 45 and 34 % for the same depth intervals (Fig. 1).

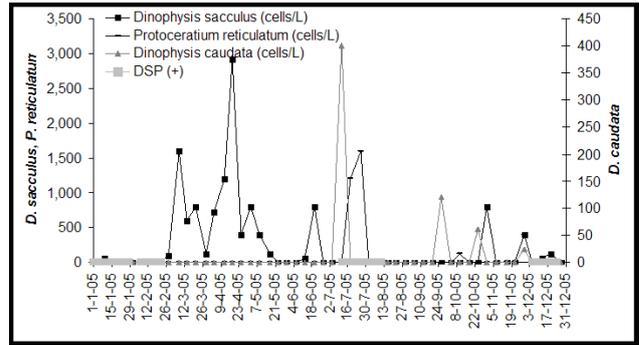


**Figure 1.** Chlorophyll-*a* concentration inside the mussel farm (A) and outside the farm (B) in February, March and April 2005.

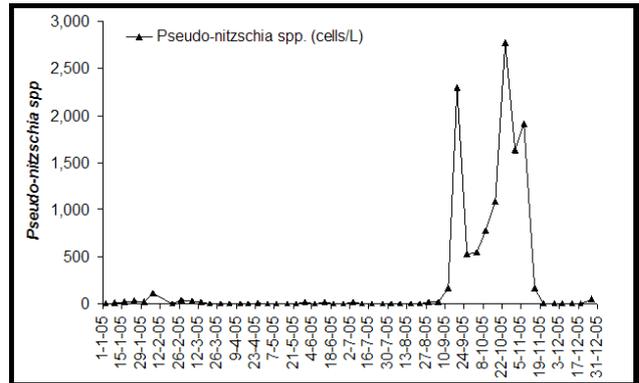
Major HAB events in Alfacs Bay during 2005 were caused by *Dinophysis sacculus*, *D. caudata*, *Prorocentrum reticulatum* and *Pseudo-nitzschia* spp. *Dinophysis sacculus* was abundant in March, April, May and December. Its maximal cell concentration was  $2.9 \times 10^3$  cells L<sup>-1</sup>. *Dinophysis caudata* was present in low cell numbers in July, September, October and November. *Prorocentrum reticulatum* attained maximal cell concentrations ( $1.6 \times 10^3$  cells L<sup>-1</sup>) in July; it was present in only low abundance in October and December. Four DSP toxin events (positive mouse bioassay for shellfish samples) were registered during 2005 accompanied by the presence of both yessotoxins and okadaic acid (Fig. 2).

*Pseudo-nitzschia* spp. attained maximal cell concentrations ( $2.7 \times 10^6$  cells L<sup>-1</sup>) in October (Fig. 3). The species present during the bloom were *P. calliantha*, *P. multistriata* and *P. delicatissima* (Quijano et al. 2006).

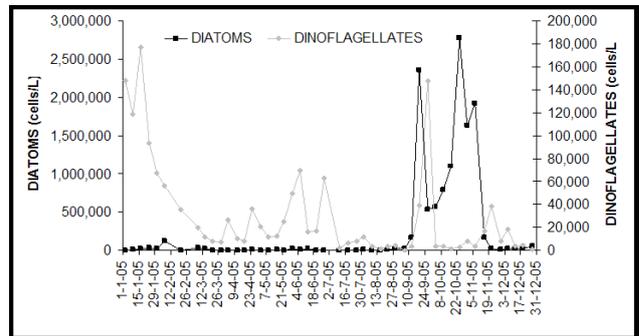
The decrease in dinoflagellate abundance was coincident with a minimum in the stratification index (Fig. 5). Diatoms were abundant during the low



**Figure 2.** *Dinophysis sacculus*, *D. caudata*, *Prorocentrum reticulatum* cell concentrations during 2005. Periods of DSP toxicity (+) in shellfish samples are represented in grey along the x-axis.



**Figure 3.** Cell concentrations of *Pseudo-nitzschia*.



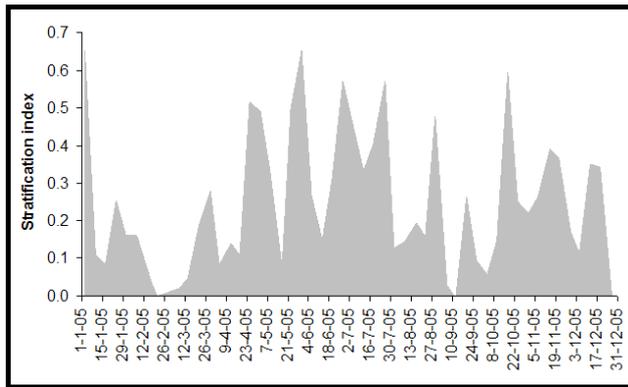
**Figure 4.** Total cell concentrations of diatoms versus dinoflagellates.

stratification period in September and October, and decreased in November (Fig. 4).

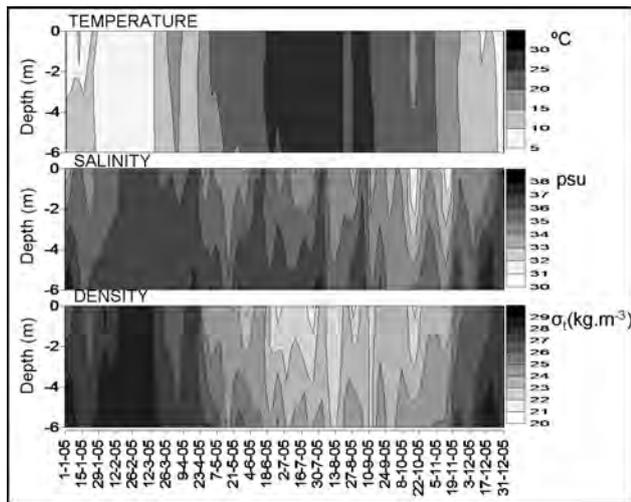
Dinoflagellates were most abundant in January when *Prorocentrum micans* was dominant, but during February and March dinoflagellate abundance decreased (Fig. 4).

The decrease in dinoflagellate abundance was coincident with a minimum in the stratification index (Fig. 5). Diatoms were abundant during the low stratification period in September and October, and decreased in November (Fig. 4).tv

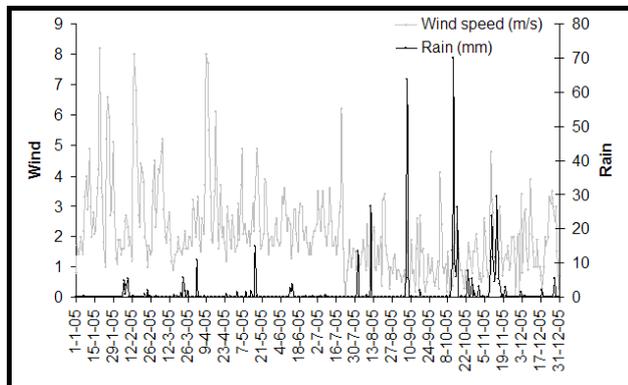
The water temperature ranged between 5.3 and 28.7 °C, and salinities between 29.9 and 37.7 psu. Low salinities (Fig. 6) were measured in October and



**Figure 5.** Stratification index based on the sigma-t values at the bottom and at 0.5 m depth.



**Figure 6.** Temperature ( $^{\circ}\text{C}$ ), salinity (psu) and sigma-t ( $\sigma_t$ ) ( $\text{kg m}^{-3}$ ).



**Figure 7.** Wind ( $\text{m s}^{-1}$ ) and rain (mm) in Alfacs Bay.

November after heavy rains (Fig. 7). The water column was well mixed in February and September.

The water temperature ranged between 5.3 and 28.7  $^{\circ}\text{C}$ , and salinities between 29.9 and 37.7 psu. Low salinities (Fig. 6) were measured in October and

November after heavy rains (Fig. 7). The water column was well mixed in February and September.

Strong wind events occurred in January, February and April (Fig. 7); they caused different degrees of water mixing (Fig. 6).

### Perspectives

Some of the DSP events during 2005 in Alfacs Bay cannot be explained by examination of cell abundance of *Dinophysis* spp., *Prorocentrum* spp. and/or *Protoceratium reticulatum*. The toxin content of the shellfish, phytoplankton and SPATT bags will allow a better understanding when the analysis is complete.

*Pseudo-nitzschia delicatissima* isolated from Alfacs Bay has been shown to produce domoic acid in culture (Elandaloussi *et al.*, this volume). Although high concentrations of this species were registered during 2005, shellfish samples did not contain domoic acid.

Integration of all the parameters by comparative studies in other GEOHAB pilot sites will increase our knowledge of HAB dynamics and allow to improve monitoring and prediction of HABs in coastal embayments.

### Acknowledgements

This project was funded by the NRC-SEPOCYT agreement between Canada and Spain, Ministry of Education and Science, Spanish Government DGPi-AM, Fisheries Department, Generalitat de Catalunya, IFOP Programme, and the European Union. We acknowledge support received by Karl Andree and technical staff from IRTA-Sant Carles de la Ràpita, the Alfred Wegener Institute (B. Krock) and Dalhousie University (S. Kirchhoff).

### References

- Camp, J. & Delgado, M. (1987). *Inv. Pesq.* 51: 351-369.
- Elandaloussi, L., Venail, R., Fernández-Tejedor, M., Diogène, J., Quijano, S., Garcés, E., Camp, J. & Andree, K. (this volume).
- Quijano-Scheggia, S., Garcés, E., Sampedro, N., Fortuño, J.M., van Lenning, K. & Camp, J. (2006). Book of Abstracts, 12th International Conference on Harmful Algae.

## Bottom cell clusters as inocula for bloom initiation of *Alexandrium catenella* in a shallow lagoon (Thau, Southern France)

B. Genovesi-Giunti<sup>1</sup>, A. Vaquer<sup>1</sup>, M. Laabir<sup>1</sup>, C. Vincent<sup>1</sup>, A. Fiandrino<sup>2</sup>, Y. Collos<sup>1</sup> and A. Pastoureaud<sup>2</sup>

<sup>1</sup>Laboratoire Ecosystèmes Lagunaires UMR CNRS et Université Montpellier II n°5119 case courrier 093 Place Eugène Bataillon 34095 Montpellier Cedex 05 France; genovesi@univ-montp2.fr; vaquer@univ-montp2.fr; laabir@univ-montp2.fr; cvincent@univ-montp2.fr; <sup>2</sup>Ifremer LER/LR BP 171 Boulevard Jean Monnet 34203 Sète France; Annie.Fiandrino@ifremer.fr; apastour@ifremer.fr

### Abstract

*Alexandrium catenella* causes recurrent toxic blooms ( $> 10^6$  cells L<sup>-1</sup>) in the Thau Lagoon (France). Bloom developments occur only in the creek of l'Angle, which is a small, shallow and semi-enclosed embayment. We mapped the resting cyst distribution, which revealed a low average abundance of 40 cysts cm<sup>-3</sup> of sediment and few accumulation patches. Our experimental investigation on cyst biology revealed synchronic excystments with a cumulated rate reaching 85% in 4 days. However, the survival of germling cells and their capacity to form a new population were very low. These results suggest a very low realised seeding ratio in the creek ( $< 20\%$ ). At the same time, biological and hydrodynamical numerical models are being developed for bloom simulations in the area. In order to reconcile our observations with numerical simulations, it appears that the low inoculum resulting from excystments must remain concentrated close to the bottom, i.e the sediment surface, as a condition for successful bloom initiation. Less than 5 days of water column stability associated with a frontal structure reduces the dispersion, supports the cohesion of the bottom cell clusters and allows the seeding population to reach a critical density ( $5 \times 10^4$  cells L<sup>-1</sup>) for blooming.

### Introduction

The accumulation of resting cysts in the sediment, i.e cyst banks, determines “hot spots” for potential dinoflagellate bloom initiation (Kim *et al.* 2002) whose success depends on the seeding population. Its size depends on resting cyst density, excystment success, survival and viability of germling cells. The aim of this study was to integrate data related to resting cyst abundance and biology with numerical simulations to propose a scenario for *Alexandrium catenella* bloom initiation in a shallow lagoon.

### Material & Methods

#### Study area

Thau lagoon (France) with a mean depth of 4.5 m has a surface area of 75 km<sup>2</sup>. Sampling was carried out in the creek of l'Angle where *A. catenella* blooms had developed. This shallow (mean depth  $< 2.5$  m) and semi-enclosed area has a surface of 2 km<sup>2</sup>. For the survey of vegetative cell dynamics and resting cyst mapping, a sampling grid (100 m) was applied and 27 stations located by GPS were established.

#### Water sampling & vegetative cell dynamics

Water samples were collected weekly from spring and autumn 1998, using a pump system. They were fixed in formaldehyde (2 % final concentration) for

microscopic enumeration of vegetative cells using the Utermöhl method.

#### Sediment sampling & resting cyst mapping

At each station, three sediment cores were collected by scuba diving on April 2004, before the onset of spring blooms. The first 3 cm of each core were sliced out and stored at 6 °C in the dark. In addition, one core (40 cm long) was sampled every 3 cm to estimate cyst density along a vertical profile. Resting cysts were separated from the sediment according to a modified gradient density method using Ludox (Yamaguchi *et al.* 1995). The analysed sediment involved the 20-80 µm fraction size. Resting cyst density was expressed in cysts per cm<sup>3</sup> of sediment.

#### Excystment & germling cell viability

Samples (1 g) of sediment were removed from the core with the highest cyst density, and suspended in ESNW medium (Harrison *et al.* 1980) prior to sonication in a water bath (1 min, 100 H). The sediment fraction was resuspended, and resting cysts were isolated by mouth-pipetting under an inverted microscope. Resting cysts were then distributed in individual wells of a 96-multiwell plate filled with 200 µl of ESNW for 30 d at 20 °C, 100 µmol photons m<sup>-2</sup> s<sup>-1</sup> and at 12:12 L:D cycle. Experiments were conducted in triplicate, each containing 30-60 cysts. The germling cell was considered as viable only when it was able to

divide and participate to inoculum growth. The realised seeding ratio, i.e. the percentage of resting cysts whose excystment produced a germling cell with the capacity to divide, was estimated in order to quantify the inoculum size.

#### *Quantification of the seeding phase*

The cyst bank of a sediment unit of  $300 \text{ cm}^3$  ( $10 \times 10 \times 3 \text{ cm}$ ) was considered. In order to simplify quantification, the overlying water column was divided into cube of 1 litre ( $10 \times 10 \times 10 \text{ cm}$ ) and was roughly measured to a height of 1 m. The inoculum size was calculated as a function of the cyst bank density and its associated realised seeding ratio.

#### *Biological & hydrodynamical simulations*

A primary production model (Chapelle *et al.* 2000) was developed to simulate the simultaneous competition growth of 4 phytoplankton (ppk) groups: [PP] Picoppk, [NP] Nanoppk, [MP] Micropk and [Alex] *A. catenella*. The MARS 3D hydrodynamic model (Lazure 1992) reproduces the circulation of water masses within the Thau lagoon under given meteorological conditions, morphological characteristics of the lagoon, exchanges with the sea and inputs from the watershed. Simulation results were regular grids ( $169 \times 78$  cells of 100 m size) of vertically integrated currents with a time step of four hours. Both simulations took into consideration the environmental conditions during bloom periods.

## Results

#### *Vegetative cell dynamics*

*Alexandrium catenella* was mainly observed in the water during autumn at temperatures of 10-22 °C. Highest abundances were in October 2003 ( $4.5 \times 10^6$  cells  $\text{L}^{-1}$ ) and November 2004 ( $16 \times 10^6$  cells  $\text{L}^{-1}$ ). The distribution of vegetative cells was patchy. Blooms occurred mainly in the creek of l'Angle and could extend to others parts of the lagoon during favourable hydrodynamical conditions.

#### *Resting cyst vertical distribution & mapping*

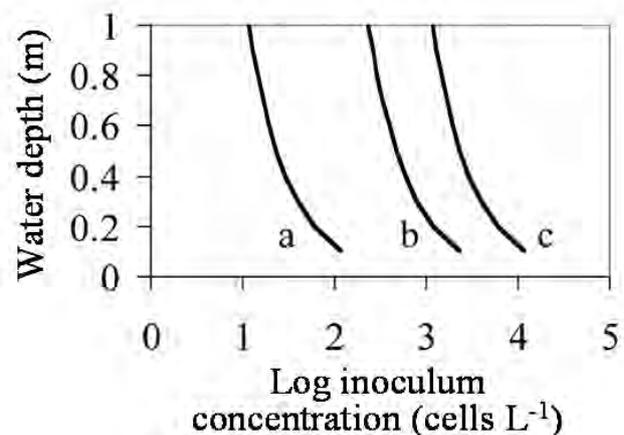
Resting cysts were more concentrated in the upper sediment layers (97 % down to 12 cm depth), particularly in the first 3 cm of sediment where 30 % of the cysts were observed. Density in the creek reached an average of 40 cysts  $\text{cm}^{-3}$  and varied from 2 to 200 cysts  $\text{cm}^{-3}$ . The resting cyst distribution was heterogeneous with several accumulation patches.

#### *Excystment & germling cell viability*

Excystments were synchronous and occurred during a short period of 4 days. The cumulated excystment rate reached 85 %. A proportion of 30 % of germling cells survived and 75 % of them divided. This means a realised seeding ratio of 20 %.

#### *Modelling of the seeding phase*

The inoculum size strongly depended on the water column mixing and the density of the cyst bank (Fig. 1). For example, in the situation of an unmixed water column we estimated that a cyst bank of 200 cysts  $\text{cm}^{-3}$  could potentially inoculate the first ten centimeters of water above the sediment surface with up to  $1.2 \times 10^3$  cells  $\text{L}^{-1}$ . However, the dilution of the inoculum size down to  $1.2 \times 10^2$  cells  $\text{L}^{-1}$  was linked to a dilution in the overall overlying water column when wind induced mixing (Chapelle *et al.* 2001).



**Figure 1.** Concentration of cells in the inoculum in the simulated water column with consideration of water mixing at three different cyst concentrations: (a) 2, (b) 40 and (c) 200 cysts  $\text{cm}^{-3}$ .

#### *Biological & hydrodynamical simulations*

Independent of inoculum size, simulations suggested that *A. catenella* competed with picoppk. Its inoculum size had to reach a density of  $5 \times 10^4$  cells  $\text{L}^{-1}$  to become competitive with both the nanoppk and the micropk (Fig. 2). During the bloom of 2003, a frontal structure linked to an abrupt change in bathymetry in front of the creek induced changes in current velocity and direction. It limited exchanges between the creek and the lagoon and suggested a water mass confined to the creek. The weak currents in the creek suggested that it was protected from wind influence, and the formation of gyre zones was linked with a decreasing of current velocity (Fig. 3).

## Discussion

The short time required by the hibernated resting cysts to reach an optimum excystment rate suggests a synchronisation in excystment and an opportunistic seeding strategy. It is counterbalanced by a low realised seeding ratio characterised by a “bottleneck” effect in which germling cell survival is the limiting factor. Less than 1/4 of the cysts in the bank participated in the seeding. The inoculum provided a density lower than the threshold critical density proposed by biological simulations. The situation is worsened by the low average density of resting cysts observed in our area. It is a striking situation which does not readily explain the proliferation of *A. catenella*. Bloom initiation was a result of both biotic and abiotic factors. It appears that the low inoculum resulting from excystments must remain concentrated close to the bottom as a condition for successful bloom initiation. Close to the bottom, the newly excysted vegetative cells may also take advantage of nutrient diffusion from the sediment (Souchu *et al.* 1998). Their swimming/motility (Doblin *et al.* 2006) and mucus secretion (Jenkinson 1986) leads to an accumulation of cells (cluster formation) and increases their competitive success compared to other phytoplankton groups through their allelopathic capacity (Laabir *et al.* 2006). In addition, a continuous seeding mechanism by the cyst bank of the surface sediment could reinforce the cell cluster size. This process is suggested by the vertical profile of resting cysts in the sediment (unpublished data), which reflects the influence of bioturbating macro-organisms (François *et al.* 1999) that could promote the upward vertical transport of buried cysts (Mizushima and Matsuoka 2004). Our hypothesis requires a few days (5) of unmixed-confined water column preventing the dispersion of germling cells and allowing the formation of the bottom cell clusters. Such periods of calm weather generally occur just prior to *A. catenella* blooms (unpublished data). In this situation, the seeding population of  $2.4 \times 10^2$  cells L<sup>-1</sup> (provided by a density of 40 cysts cm<sup>-3</sup>) with a growth rate of 1 division per day reaches  $5 \times 10^4$  cells L<sup>-1</sup> thus exceeding the critical density which determines the proliferation of *A. catenella*.

## Conclusions

This study showed the importance of germling cell survival for the quantification of the inoculum size and the resulting bloom initiation success. The scenario of the bottom cell clusters highlighted that a combination of both biotic and abiotic factors are

requisites for the inoculum to reach a critical density before blooming in a shallow lagoon. To validate our hypothesis, additional field and experimental studies will be needed, including: (1) taking into account the temperature to simulate *A. catenella* growth; (2) field hydrological data to demonstrate the occurrence of unmixed-confined periods during bloom initiation; (3) vertical sampling of the water column to confirm the existence of bottom cell clusters; and (4) estimation of natural excystment and germling cell viability to bring further insights regarding the seeding capacity of the cyst bank.

## Acknowledgements

This study was supported financially by the Region Languedoc-Roussillon and the Programme National d'Environnement Côtier (PNEC-France).

## References

- Chapelle, A., Lazure, P. & Souchu, S. (2001). *Oceanol. Acta* 24: 87-97.
- Chapelle, A., Ménesguen, A., Deslous-Paoli, J.M., Souchu, P., Mazouni, N., Vaquer, A. & Millet, B. (2000). *Ecol. Modelling* 127: 161-81.
- Doblin, M.A., Thompson, P.A., Revill, A.T., Butler, E.C.V., Blackburn, S.I. & Hallegraeff, G.M. (2006). *Harmful Algae* 5: 665-677.
- François, F., Dalègre, K., Gilbert, F. & Stora, G. (1999). In: *C. R. Acad. Sci. Paris Sciences de la Vie / Life Sciences* 322, Elsevier, pp. 339-345.
- Harrison, P.J., Waters, R.E. & Taylor, F.J.R. (1980). *J. Phycol.* 16: 28-35.
- Jenkinson, I. R. (1986). *Nature* 323 : 435-437.
- Kim, Y.O., Park M.H. & Han, M.S. (2002). *Aquat. Microb. Ecol.* 29: 279-86.
- Laabir, M., Masseret, E., Collos, Y., Vaquer, A. & Pastoureaud, A. (2006). 12th International Conference on Harmful Algae, Denmark, Sept. 2006 (Poster communication).
- Lazure, P. (1992). *Vie et Milieu* 42 : 137-45.
- Mizushima, K. & Matsuoka, K. (2004). *Phycol. Res.* 52: 408-13.
- Souchu, P., Gasc, A., Vaquer, A., Collos, Y., Tournier, H. & Deslous-Paoli, J. M. (1998). *Mar. Ecol. Progr. Ser.* 164: 135-146.
- Vincent, C., Ducharme, G., Fiandrino, A., Do Chi, T. & Aliaume, C. 2006. International Workshop, DITTY European Project, France, Feb. 2006 (Poster communication).
- Yamaguchi, M., Itakura, S., Imai, I. & Ishida, Y. (1995). *Phycologia* 34: 207-214.

## Top-down and bottom-up control of Harmful Algal Blooms (HABs)

Edna Granéli

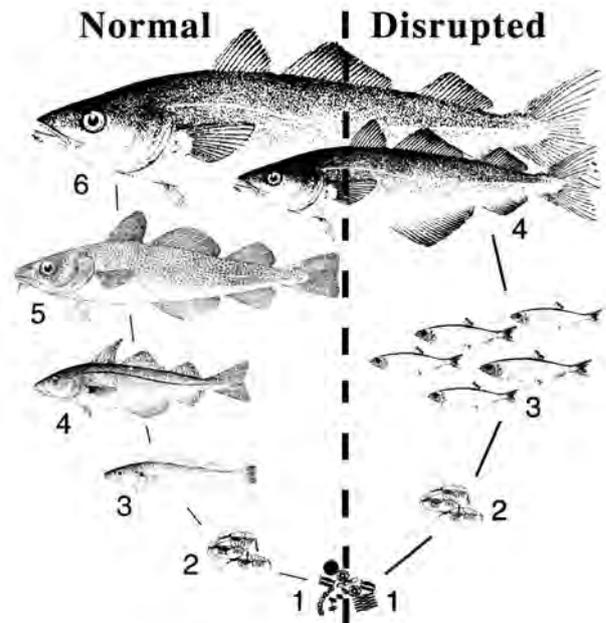
Marine Sciences Dept., Kalmar University, 39182 Kalmar, Sweden, edna.graneli@hik.se

### Abstract

The systematic over-exploitation of marine resources through fishing has led to changes at the top level of food chains. These changes have cascaded down. From lakes it has been known since approximately 1980 that a reduction in the number and size of larger piscivores (predators on smaller fishes) leads to an increase in the biomass of smaller planktivorous fishes. This, in turn, leads to higher predation pressure on larger zooplankton and as a consequence, grazing pressure on phytoplankton will decrease. This mechanism is especially strong in nutrient-rich waters. Thus, over-fishing and eutrophication may work synergistically to increase phytoplankton biomass. HAB species, in addition, have the advantage over other phytoplankton groups in being unpalatable (through the production of toxins and/or through body shape/size) and therefore - to a variable extent - grazing resistant. Diminished grazing in combination with higher availability of inorganic nutrients, the capability to use nutrients in dissolved and organic form, and an increase in toxicity, allelopathy and grazer deterrence when growing under N and P unbalanced conditions, is certainly a scenario that gives HA-species an 'upper-hand' allowing growth and bloom development at the cost of grazer sensitive, non-HAB phytoplankton species.

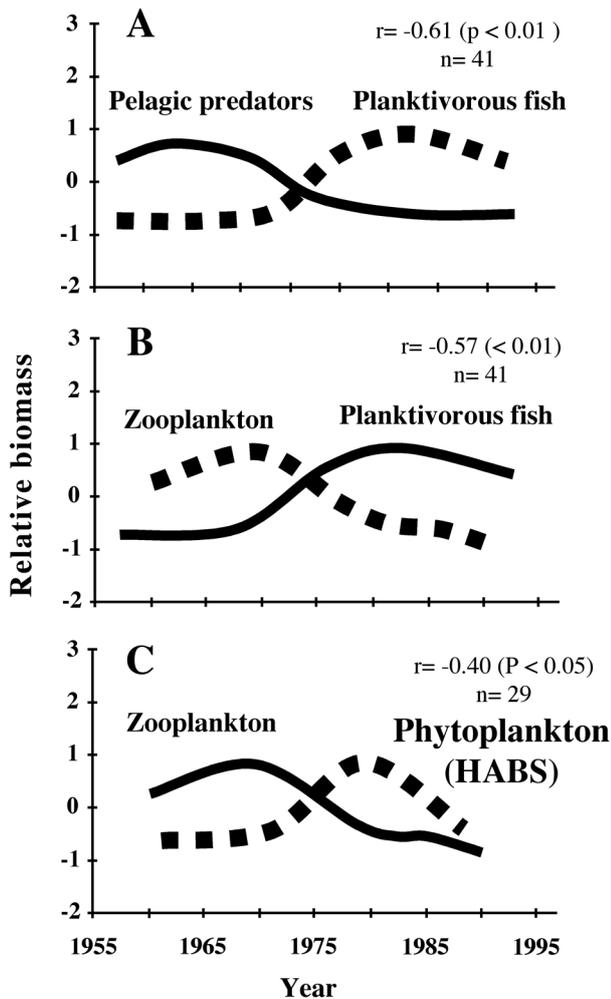
### “Overfishing” and its cascading effect on marine food webs

Reports of alarming decrease of important commercial fishes such as mackerel, tuna, cod, haddock, red-fish, hake, and whiting by over-fishing worldwide have gained the attention of the scientific community (Pauly and MacLean 2002) and the popular press (Guterl 2003). Myers and Worm (2003), using a meta-analysis approach, have estimated that the biomasses of large predatory fishes today are only 10% of the pre-industrial level in the global ocean. In a pristine system, the pelagic food chain contains several size classes between the top carnivorous fish and phytoplankton. Over-fishing disrupts the food web, causing shorter food chains with fewer trophic levels and leads to low diversity (Fig. 1). From lakes, coastal and shelf ecosystems, the effects from the “top-down” disruption induced by fisheries and other agents changing fish communities are evident (Jackson *et al.* 2001; Pauly and MacLean 2002; Worm and Myers 2003). In the Black Sea, for example, over-fishing has led to the disappearance of valuable commercial fish species with a total collapse of the fisheries as consequence; cascading effects of large predatory fishes can be seen down to zooplankton. While numbers of large fishes have decreased, those of planktivorous fishes have increased, and, in consequence, zooplankton has declined (Daskalov 2003; Fig. 2). Besides an increase in planktivorous fish, an invasion of the predatory ctenophore, *Mnemiopsis leidyi*, has also contributed to a disruption of the pelagic food web (Mee 1992; Daskalov 2002, 2003; Gucu 2002).



**Figure 1.** What fishing down the food web means for a typical predator. Left of the line is a food chain in pristine waters with many more levels (and size classes) between the top larger predators and the plankton. To the right is a disrupted food chain from over-fishing, with fewer steps, which also leads to smaller top predators (redrawn from Pauly and Maclean 2002).

Simultaneous to these events, there has also been an increase in HABs in these waters (Bodeanu 1993). Further, a jellyfish increase has also been reported for different parts of the world such as Africa's coast and the Bering Sea, the latter with a decrease in zooplankton biomass as a consequence (Lees *et al.* 2006; Fig. 3).



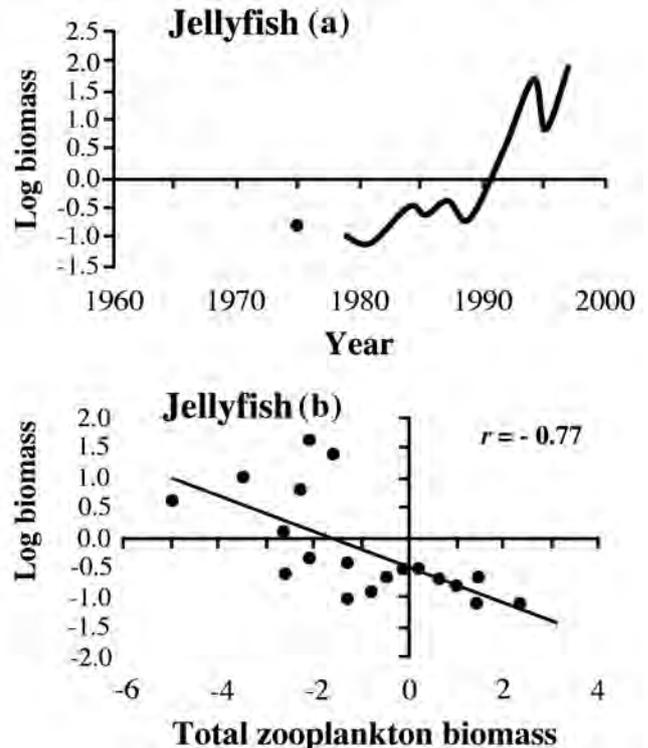
**Figure 2.** How over-fishing has driven a trophic cascade in the Black Sea. A diminishing of the large fish has increased the number of planktivorous fish, which as a consequence has decreased zooplankton and increased HABs (redrawn from Daskalov 2002).

### Eutrophication and HABs

The input of nutrients has increased several fold during the last five decades in almost all coastal waters close to human settlements (Cloern 2001; Anderson *et al.* 2002; Howarth and Marino 2006). However, although a high discharge of nutrients (mostly nitrogen and phosphorus) to coastal waters can be coupled to HABs, other anthropogenic impacts might be interacting with eutrophication to select for these problematic taxa. Aquaculture, represented by the shellfish industry and fish farming in many European countries, is of high local economic importance in some areas.

Aquaculture has suffered large fiscal losses during the last decades due to the occurrence of toxic/harmful phytoplankton blooms. However, it is not possible to disregard the possibility that aquaculture

itself (at least fish farming), by releasing nutrients (and using antibiotics), might be one of the factors contributing to HAB development in enclosed water bodies (see e.g., Davies *et al.* 2001). Besides, the increase of N and P inputs to coastal waters rarely approximates the optimal nutrient ratios for algal growth,



**Figure 3.** Increase in jellyfish biomass in the Bering Sea between 1965-1997 has decreased the zooplankton biomass. (a) Standardized log recruitment of jellyfish biomass and (b) Lagged relationship between fluctuations in jellyfish biomass and total zooplankton biomass (1-year lag) (redrawn from Lees *et al.* 2006).

thereby increasing or decreasing the limiting nutrient concentrations in receiving waters. Furthermore, many HAB species are able to not only use inorganic N and P for growth but can also use other sources of N as for example, urea and humic material (Carlsson *et al.* 1988; Granéli *et al.* 1999; Heil *et al.* 2005; Glibert and Legrand 2006). Land application of urea and the discharge of this form of N have increased in many parts of the world and can be, to some extent, coupled with HABs (Glibert and Burkholder 2006). Long-term trends show that the input of humic material to lakes, rivers, and the Baltic Sea has also increased in the last few decades, steadily increasing the concentrations of this form of organic N in the Baltic (W. Granéli pers. comm.).

Besides being able to use other forms of nutrients, HAB species have the advantage over other

phytoplankton groups in being, to a variable extent, grazing resistant. They might be unpalatable through the production of toxins and/or through their body shape/size (Turner and Tester 1997; Frangópulos *et al.* 2000). For toxin producing species, an increase as well as a decrease in the inhibition of zooplankton feeding by HAB species is considered to reflect varying toxin levels dependent on algal growth conditions (Nielsen *et al.* 1990; Turner and Tester 1997; Granéli and Johansson 2003) while allelochemical production also reflects growth conditions for some species (Granéli and Johansson 2003 a,b; Granéli and Flynn 2006). Toxicity can increase several fold when algae are grown under nutrient unbalanced conditions, with grazer deterrence and production of allelochemicals increasing as well, the latter chemicals deterring or even killing their grazers and other phytoplankton species (Granéli and Flynn 2006; Granéli and Hansen 2006; Stoecker *et al.* 2006).

For HA cells escaping grazing, some refuge is obtained where increased nutrient availability (through eutrophication) will divert nutrients from grazing-sensitive to the grazer-resistant HA phytoplankton species. These mechanisms, coupled with the over-fished top predator-induced HA expansion from reduced mesozooplankton biomass and size distribution described in sections above and below, may thus select for HAB species. Therefore, diminished grazing from top-down control or HA-produced deterrents, in combination with increasing amounts of nutrients from anthropogenic activities, might affect the occurrence of and contribute to an increase in HABs in marine systems.

### **Examples from “Top Down” “Bottom-Up” control in fresh-waters food webs**

For freshwater ecosystems, a great deal of experimentation and polemic discussions have been devoted to the question of “top-down” versus “bottom-up” control of primary producers. The concept of “cascading trophic interactions” now forms the centre of a thriving field of research. Several of the freshwater studies have involved not only mesocosm experiments (Andersson *et al.* 1978; McQueen *et al.* 1986; Mazumder *et al.* 1990), but also manipulation of whole lakes by addition or removal of planktivorous as well as piscivorous fish (Carpenter *et al.* 1987; Mazumder *et al.* 1990).

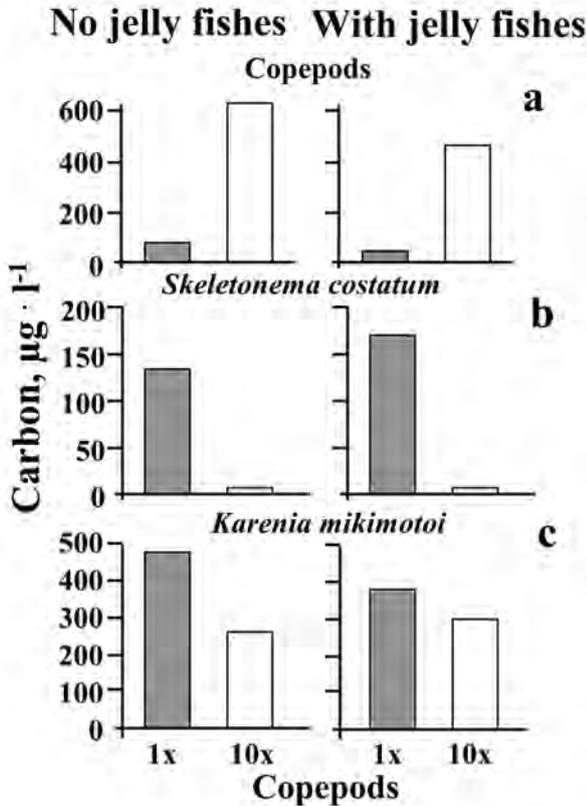
The studies have shown that changes at higher trophic levels will have an impact not only on zooplankton, but ultimately, even phytoplankton biomass

and species composition will be altered (Andersson *et al.* 1978; Lynch and Shapiro 1981; Mazumder *et al.* 1990). A reduction in the biomass of piscivorous fish leads to an increase of smaller, planktivorous fish, which then exert a strong predation pressure on zooplankton, thereby reducing their biomass and increasing phytoplankton biomass and species composition (Carpenter *et al.* 1987; Vanni 1987). Horsted *et al.* (1988) have shown that zooplankton production can be strongly controlled by fish predation in an estuarine system. However, the “top-down” effect seems to be stronger in oligotrophic compared to eutrophic lakes (McQueen 1998). On the basis of these results, attempts have been made to counteract eutrophication in lakes (high phytoplankton biomass) in North America and in Europe by addition of piscivores or reduction of zooplanktivorous taxa. Such measures can increase herbivore numbers and thus increase grazing pressure on the phytoplankton (Dorazio *et al.* 1987). Elser and Goldman (1991) have shown that HABs, represented by large size cyanobacteria dominating Clear Lake, a eutrophic lake in California, were not heavily grazed by zooplankton.

### **Top-Down versus Bottom-Up control of phytoplankton in marine waters**

From marine waters, only little information exists on top-down versus bottom-up control of phytoplankton. Experiments on the structuring of marine phytoplankton communities through zooplanktivores are rare, in contrast to experiments in lakes. Granéli *et al.* (1993) have shown in a mesocosm experiment that under low nutrient conditions, high copepod densities (up to six times natural densities), can keep phytoplankton biomass low and largely comprised of diatoms. Dinoflagellates, including the toxic *Karenia mikimotoi*, were little affected by grazing pressure in un-enriched treatments and were even less grazer impacted in treatments enriched with N and P. Deason and Smayda (1982) found an increase in the diatom biomass when the jellyfish *Mnemiopsis leidyi* was present in high numbers in Narragansett Bay. Heavy predation of *M. leidyi* on copepods reduced grazing on diatoms. In mesocosm experiments enriched with N and P, and where the food web was manipulated by introducing jellyfish (Granéli and Turner 2002), the diatom *Skeletonema costatum* increased as result of diminished grazing from lower copepod densities produced by high jellyfish demand; copepod densities in these experiments were near normal ambient densities. In the treatments where ten times the normal

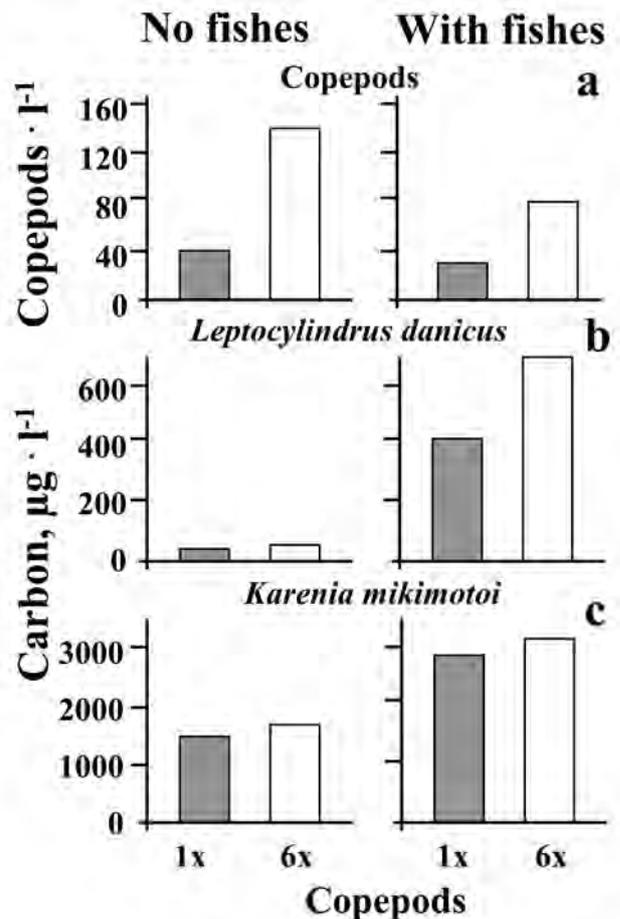
densities of copepods were created, predation by the jellyfish on the copepods was not sufficient to release the diatoms from being grazed (Fig. 4).



**Figure 4.** Top-down cascading effects from predation by jellyfishes (ctenophores) on two copepod densities (a), and the further effect on the biomass of a diatom (b) and a HA species (c) in nutrient-enriched mesocosms (redrawn from Granéli and Turner 2002).

The addition of planktivorous fish on the other hand, could release the diatoms from copepod grazing even when these were added at high densities (Fig. 5; Granéli *et al.* 1993). For a more classical HA species, predator-induced changes in copepod predation on HA species was also detectable. The non-toxin producer HA species *Ceratium* spp. were to some extent grazed when copepods were found in higher numbers, but grew even better when grazing pressure decreased after introduction of planktivorous fish (Granéli *et al.* 1993).

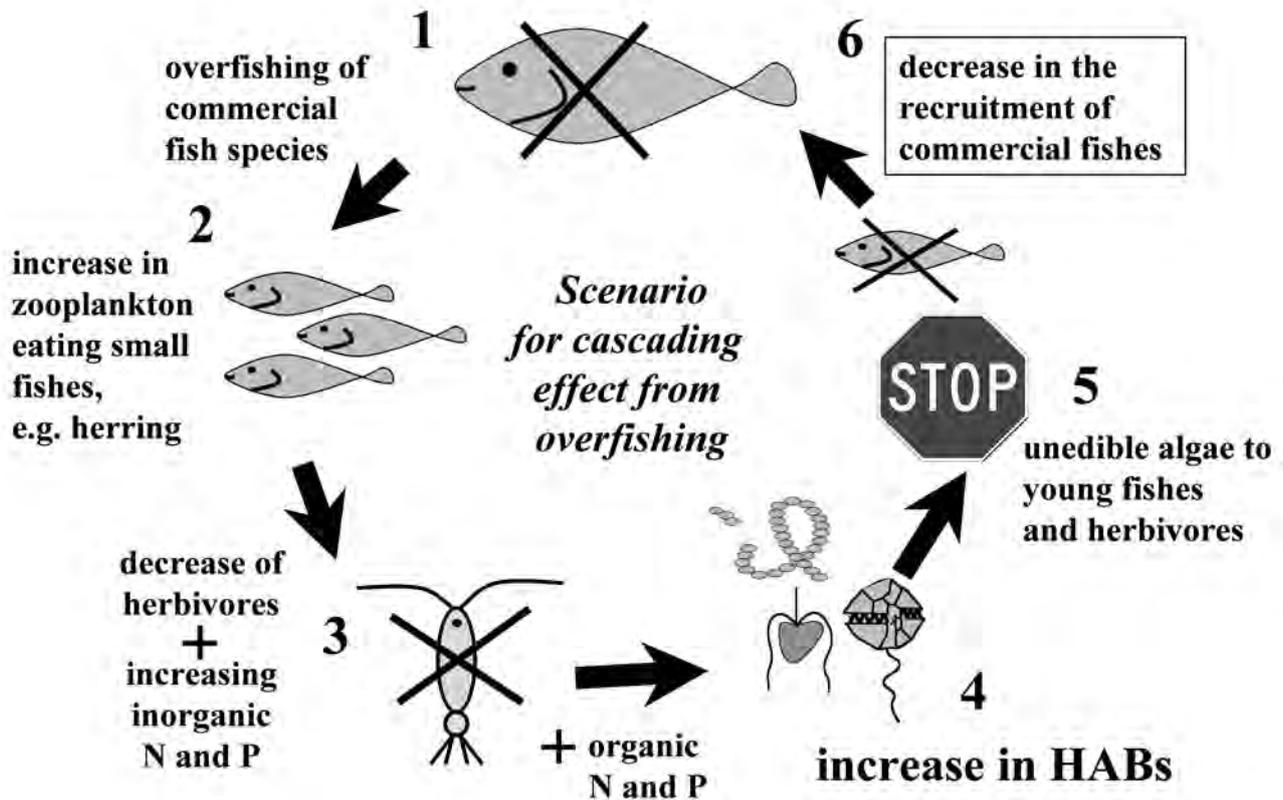
Other HA taxa became dominant, independent of potential grazers. The ichthyotoxic HAB former, *K. mikimotoi*, grew to very high cell densities independently of whether jellyfish or fish were present in mesocosm experiments, and even when copepods were added to the tanks at ten times the normal copepod biomass (Figs 4 and 5; Granéli *et al.* 1993; Granéli and Turner 2002). Similarly, the ichthyotoxic haptophyte *Chrysochromulina polylepis* also developed in the ex-



**Figure 5.** Top-down cascading effects from predation by planktivorous fish on two copepod densities (a) and the further effect on the biomass of a diatom (b) and a HA species (c) in nutrient-enriched mesocosms (redrawn from Granéli *et al.* 1993).

perimental tanks with high copepod densities, either with or without jellyfish (Granéli and Turner 2002). Both *K. mikimotoi* and *C. polylepis* form toxins with allelopathic effects and act as grazer deterrents (Fistarol *et al.* 2003; Granéli and Johansson 2003 a,b). These experiments confirmed what is generally found, i.e., these HA species are similar to many other toxic, grazer deterrent species: they are not effectively controlled from the top, and can probably grow even better because of reduced competition for nutrients from more palatable species. Together with the elevated nutrients and a situation when the grazers exert an even higher pressure on the accompanying palatable algal species, these types of HA can grow and accumulate to high densities.

In conclusion, we can expect that certain HA species may be favoured to the detriment of other non-HA species when changes higher up (i.e., a decrease in larger carnivorous fishes by over-fishing re-



**Figure 6.** Scenario on how the disruption of a marine food web by over-fishing might interact with eutrophication and cause an expansion of HABs.

sulting in an increase in smaller planktivorous fishes) in the food web occur (Fig. 6). A lower abundance of remaining grazers, or those of smaller size, will preferentially prey on non-HA species instead. This coupled to the higher availability of inorganic nutrients, the capability to use nutrients in dissolved and organic form, and an increase in toxicity, allelopathy, and grazer deterrence when growing under N and P unbalanced growth conditions, is a scenario that favours growth of HA species and bloom development. However, our knowledge concerning to what extent, and under which conditions, these anthropogenic impacts favour specific HAB species is still limited. Much more research is required, at least in marine and coastal waters. Climate change is ultimately another anthropogenic factor that cannot be excluded from perpetuating this phenomenon as it can interact with eutrophication and over-fishing, complicating the proposed impacts even more than suggested in this treatise.

#### Acknowledgements

I am most grateful to Christina Esplund for redrawing of the figures and to the unknown referees whose suggestions greatly improved the content and the Eng-

lish. This work was financed by The National Swedish Research Council, The Swedish Research Council Formas, and the European Commission (GLOBAL-2, contract number 003933 - Project Thresholds of Environmental Sustainability).

#### References

- Anderson, D.M., Glibert, P.M. & Burkholder J.M. (2002). *Estuaries* 25: 704-726.
- Andersson, G., Berggren, H., Cronberg, G. & Gelin, C. (1978). *Hydrobiologica* 59: 9-15.
- Bodeanu, N. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda T.J. (ed.), Elsevier Sci. Publ., Amsterdam, pp. 203-209.
- Carlsson, P., Edling, H. & Bechemin, C. (1998). *Aquat. Microb. Ecol.* 16: 65-80.
- Carpenter, S.R., Kitchell, J.F., Hodgson, J.R., Cochran, P.A., Elser, J.J., Elser, M.M., Lodge, D.M., Kretchmer, D., He, X. & von Ende, C.N. (1987). *Ecology* 68: 1863-1876.
- Cloern, J.E. (2001). *Mar. Ecol. Progr. Ser.* 210: 223-253.
- Daskalov, G.M. (2002). *Mar. Ecol. Progr. Ser.* 225: 53-63.
- Daskalov, G.M. (2003). *Mar. Ecol. Progr. Ser.* 255, 259-270.

- Davies, I.M., Rodger, G.K., Redshaw, J. & Stagg, R.M. (2001). *ICES J. Mar. Sci.* 58: 477-485.
- Deason, E.E. & Smayda, T. J. (1982). *J. Plankton Res.* 4: 219-236.
- Dorazio, R.M., Bowers, J.A. & Lehman, J.T. (1987). *J. Plankton Res.* 9: 891-899.
- Elser, J. & Goldman, C.R. (1991). *Limnol. Oceanogr.* 36: 64-90.
- Fistarol, G.O., Legrand, C. & Granéli, E. (2003) *Mar. Ecol. Prog. Ser.* 255: 115-125
- Frangópulos, M., Guisande, C., Maneiro, I., Riveiro, I. & Franco, J. (2000). *Mar. Ecol. Prog. Ser.* 203: 161-169.
- Glibert, P.M. & Burkholder, J.M. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer Verlag, Berlin Heidelberg, pp. 341-354.
- Glibert, P.M. & Legrand, C. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer Verlag, Berlin Heidelberg, pp. 163-176.
- Granéli, E. & Turner, J.T. (2002). *Mar. Ecol. Prog. Ser.* 239: 57-68.
- Granéli, E. & Johansson, N. (2003a). *Mar. Ecol. Prog. Ser.* 254: 49-56.
- Granéli, E. & Johansson, N. (2003b). *Harmful Algae* 2: 135 -145.
- Granéli, E. & Flynn, K. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer Verlag, Berlin Heidelberg, pp. 229-242.
- Granéli, E. & Hansen, P.J. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer Verlag, Berlin Heidelberg, pp. 189-202.
- Granéli, E., Olsson, P., Carlsson, P., Granéli, W. & Nylander, C. (1993). *J. Plankton Res.* 15: 213-237.
- Granéli, E., Carlsson, P. & Legrand, C. (1999). *Aquat. Ecol.* 33: 17-27.
- Gucu, A.C. (2002). *Est. Coast. Shelf Sci.* 54: 439-451.
- Guterl, F. (2003). *Newsweek* July 14 2003: 46-51.
- Heil, C.A., Glibert, P.M. & Fan, C. (2005) *Harmful Algae* 4: 449-470.
- Horsted, S.J., Nielsen, T.G., Riemann, B.J. & Bjørnsen, P.K. (1988). *Mar. Ecol. Prog. Ser.* 48: 217-224.
- Howarth, R.W. & Marino, R. (2006). *Limnol. Oceanogr.* 51: 364-376.
- Jackson, J.B.C., 18 other authors. (2001). *Science* 293: 629-638.
- Lees, K., Pitois, S., Scott, C., Frid, C. & Mackinson, S. (2006). *Fish and Fisheries* 7: 104-127.
- Lynch, M. & Shapiro, J. (1981). *Limnol. Oceanogr.* 26: 86-102.
- Mazumder, A., Taylor, W.D., McQueen, D.J., Lean, D.R.S. & LaFontaian, N.R. (1990). *J. Plankton Res.* 12: 109-124.
- McQueen, D.J., Post, J.R. & Mills, E.L. (1986). *Can. J. Fish. Aquat. Sci.* 43: 1571-1581.
- McQueen, D.J. (1998). *Lakes Reserv. Res. Management* 3: 83-97.
- Mee, L.D. (1992). *Ambio* 21: 278-286.
- Myers, R.A. & Worm, B. (2003). *Nature* 423: 280-283.
- Nielsen, T.G., Kiørboe, T. & Bjørnsen, P.K. (1990). *Mar. Ecol. Prog. Ser.* 62: 21-35.
- Pauly, D. & Maclean, J. (2002). In *a Perfect Ocean - the State of Fisheries and Ecocystems in the North Atlantic Ocean*. Island Press, Washington, 175 pp.
- Stoecker, D., Tillmann, U. & Granéli, E. (2006). In: *Ecology of Harmful Algae*, Granéli, E. & Turner, J.T. (eds), Springer Verlag, Berlin Heidelberg, pp. 177-188.
- Turner, J.T. & Tester, P.A. (1997). *Limnol. Oceanogr.* 42: 1203-1214.
- Vanni, M.J. (1987). *Ecology* 68: 624-635.
- Worm, B. & Myers, R.A. (2003). *Ecol. Soc. America* 84: 162-173.

## Rheological properties of exopolymeric secretions in HABs may be functions of length scale

Ian R. Jenkinson<sup>1</sup> and Tim Wyatt<sup>2</sup>

<sup>1</sup>Agence de Conseil et de Recherche Océanographiques, Lavergne, 19320 La Roche Canillac, France ian.jenkinson@wanadoo.fr, <sup>2</sup>Instituto de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain twyatt@iim.csic.es

### Abstract

Measurements were made of the rheology of seawater, algal cultures and HABs by two methods. The first used concentric cylinders separated by a gap of 500  $\mu\text{m}$ , while the second used fish-gills of pore size  $\sim 25\text{-}35 \mu\text{m}$ . More recently the yield stress of lumpy, organic floc suspension has been shown to depend strongly on the length scale of the measurements. Measuring the rheology of HABs and natural waters at different length scales now has the potential to elucidate the scales of polymer structures associated with bioengineering by plankton, particularly in HABs.

### Introduction

Many planktonic algae secrete exopolymers, which form networks, directly or by spontaneous assembly (Chin *et al.* 1998). These structures can locally thicken, and even gel, the water. We have argued earlier (Jenkinson & Wyatt 1995; Wyatt & Ribera 2006) that rheological changes wrought by these polymers alter physical and chemical processes in the local environment, and can thus be viewed as engineering tools in the sense pioneered by Jones *et al.* (1994). Local thickening is generally correlated with phytoplankton biomass (Jenkinson 1986, 1989; Jenkinson and Biddanda 1995; Seuront 2006), and may be uniform or quasi-fractal. Observed and measured by different techniques, this patchiness in both phytoplankton and exopolymer abundance is evident from ocean-basin ( $10^6 \text{ m}$ ) down to macromolecular ( $10^{-9} \text{ m}$ ) scales (refs in Jenkinson & Biddanda 1995; in Passow 2000; in Higgins *et al.* 2003; in Wyatt and Ribera 2006; in Žuti and Svetli i 2006).

Attempts have previously been made to model the effects of this rheological thickening on ocean turbulence (Jenkinson 1986, 1993b). Although care was taken to match the length scales used for measurement to those of the turbulence as closely as possible (mm-scale), however, it was not then known how strongly the polymeric component of the rheological properties might vary with length scale.

The aim of this communication is to illustrate how the hierarchically flocculated nature of algal exopolymers leads to any measured rheological property of being a function of the characteristic length-scale of the geometry of the flow system used to measure it.

We hope that such better understanding of rheological thickening and of the aggregation scales in

slimy harmful algal blooms (HABs) will help research into the mitigation of its harmful effects.

### Methods

Like seawater, but in more concentrated form, sewage sludge is a suspension of flocculated exopolymers (Li & Ganczarzyk 1986) so its physical structure is closely comparable. Yield stress  $Y$  in sewage sludge was measured by the EU standard method of pouring sludge into a Kasometer (Fig. 1), and allowing it to flow out through a capillary tube. When the flow stops,  $Y$  is measured as the height difference between the sludge-air surface and the capillary tube times the sludge density times the acceleration due to gravity. Spinosa and Lotito (2003) investigated the effects of both capillary diameter and sludge concentration on  $Y$ .

To investigate the effects of polymeric thickening in water on ventilation-flow in fish, an ichthyovis-

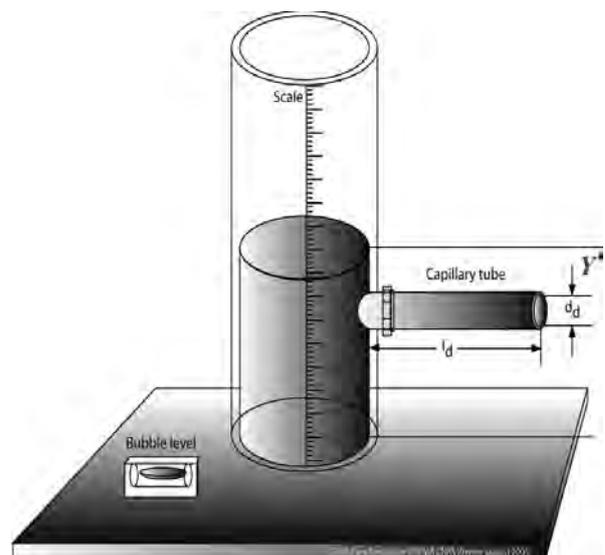


Figure 1. The Kasometer (yield-stress viscometer).

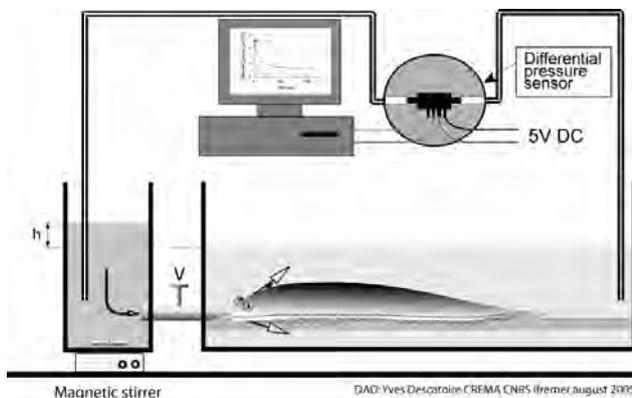
cometer was used to measure rheological properties, including  $Y$ , in seawater, algal cultures and intertidal organic fluff. This apparatus resembles the Kasometer, except that a dead fish replaces the capillary tube, and the water is directed through its gill passages. The range of hydrostatic pressure imposed spans produced by the same fish in life, thus minimising any mismatch of scales between those in the measurement apparatus and those in the process of interest, gill ventilation (Jenkinson and Arzul 1998; Jenkinson *et al.* 2007) (Fig. 2).

## Results

The data of Spinosa and Lotito (2003) show that in sewage sludge, not only is  $Y$  a function of sludge concentration but that

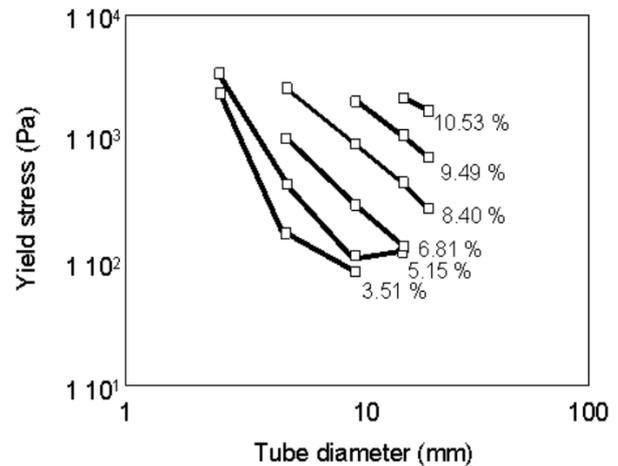
$$Y \sim 1/D^{-2}$$

where  $D$  is the capillary tube diameter (Fig. 3). The data of Jenkinson *et al.* (2007), who used an ichthyoviscometer incorporating a dead ~26-g sole to measure  $Y$  in organic fluff overlying the mud on intertidal flats, at scales relevant to ventilation by living sole, showed a strong positive relationship with the particulate organic matter (POM) concentration (Fig. 4). They showed that  $Y$  would exceed the maximum hydrostatic pressure developed by 26-g soles, ~30 Pa, thus preventing the sole from ventilating, at a POM concentration of 3 to 4 g.L<sup>-1</sup>.

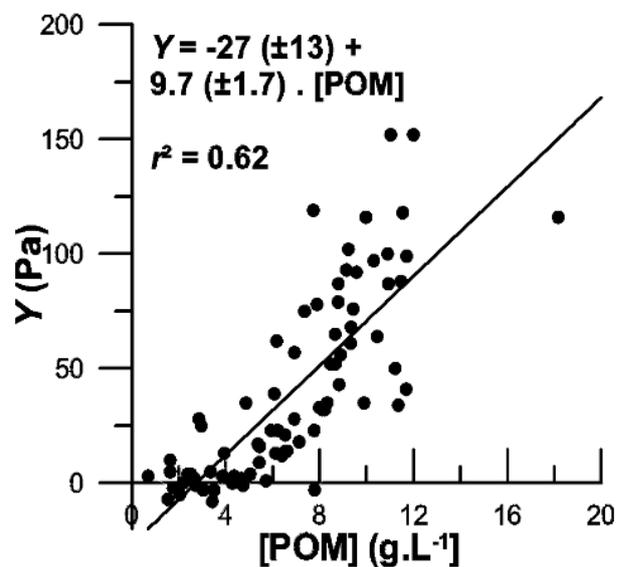


**Figure 2.** An ichthyoviscometer (from Jenkinson *et al.*, in press)

Jenkinson and Arzul (1998), using an ichthyoviscometer with 100-200-g largemouth bass or trout and a culture of 27,000 cells.mL<sup>-1</sup> of the ichthyotoxic dinoflagellate, *Karenia* (as *Gymnodinium*) *mikimotoi*, found  $Y$  equal to 50 to 90 Pa in fresh culture and 400 to 600 Pa in culture that had stood for 12 h. Extracellular organic matter concentrations in these cultures are likely to have been ~3 orders of magnitude less than in the organic fluff, yet they gave higher values of  $Y$ .



**Figure 3.** Yield stress of sewage sludge for different sludge concentrations (%) as a function of tube diameter. Note the difference in log scales on the x- and y-axes (drawn from data in Spinosa and Lotito 2003.)



**Figure 4.** Yield stress of intertidal organic fluff (Pa) vs. POM concentration (g.L<sup>-1</sup>) measured with an ichthyoviscometer incorporating a dead juvenile sole (Jenkinson *et al.* in press)

Finally, Jenkinson and Arzul (2002) used an ichthyoviscometer with dead ~80-g seabass to measure the effect of cysteine compounds on  $Y$  values produced by two ichthyotoxic dinoflagellates *K. mikimotoi* and *Gymnodinium cf maguelonnense*, isolated from southern Tunisia. At concentrations of 21,000 and 23,000 cells.mL<sup>-1</sup>, *K. mikimotoi* gave  $Y$  values of 33 and 38 Pa respectively, while at a concentrations of 10,000 and 21,000 cells.mL<sup>-1</sup>, *G. cf maguelonnense* gave respective values for  $Y$  of 7 and 22 Pa. Addition of 0.01 mM acetyl cysteine (= ethyl L-cysteine ester) reduced  $Y$  values in these cultures by 65-84 %.

## Discussion and Conclusions

Scales used in measurement should be matched to those of the environmental processes of interest as closely as possible. However, if these two length scales plus the function relating the particular rheological value to length scale are known, the value acting at the length scale of a process can be predicted from the value measured at a different (but not too distant) length scale. This case illustrates how a rheological property, in this case  $Y$ , of a lumpy material can show a very strong negative relationship with a critical length scale in the measurement apparatus.

The critical length scale for ventilation-flow is likely the gill-pore size. Even if water can be diverted into larger channels around the gills, such diverted water will not ventilate the respiratory surfaces. Pore widths for 26 to 200-g seabass range from 25 to 35  $\mu\text{m}$  (Langille *et al.* 1983), and were thus those pertinent to the ichthyoviscometer experiments mentioned above.

By comparison, Jenkinson (1986, 1993a,b) and Jenkinson and Biddanda (1995) used a gap width of 500  $\mu\text{m}$  (~15 times larger) in a rotary (Couette) system to measure the properties of algal cultures and seawater. Comparative measurements are thus required on the same blooms and cultures using measurement systems incorporating such different length scales.

That organic fluff of POM content several  $\text{g.L}^{-1}$  showed  $Y$  values comparable to those of *Karenia mikimotoi* and *Gymnodinium cf. maguelonnense* cultures ~3 orders of magnitude poorer in extracellular organic matter (POM/DOM) suggests that the structure of the polymers in the fluff may have been far smaller than those in *Karenia* and *Gymnodinium* cultures. Light microscopy of cultures and blooms stained with Alcian Blue might help resolve whether this is so.

Allelopathic action of *Karenia mikimotoi* may act through labile polyunsaturated fatty acids (PUFAs) diffusing out from the cell. As the PUFAs decay, it is proposed this gives a radius of action, here ~175  $\mu\text{m}$ . However, PUFAs secreted by *K. mikimotoi* (Gentien, this conference) may be mechanically linked to the secreted exopolymers.

Phytoplankton can secrete hierarchically aggregated polymers that modify the viscosity of the surrounding water as a function of their composition, their concentration and length scale. Polymer abundance and its correlation with phytoplankton abundance are scale-dependent, and can also vary with the stage of the life cycle. Exopolymers may be tools by which genes engineer niches by managing their physical environment. In addition, secondary effects

may result, such as air-sea surface reinforcement and increased vertical flux

## Acknowledgements

We thank Patrick Gentien for fruitful discussions, and Yves Descatoire for drawing Figs 1 and 2.

## References

- Chin, W.-C., Orellana, M.V. & Verdugo, P. (1998). *Nature* 391: 568-572.
- Higgins, M.J., Sader, J.E., Mulvaney, P. & Wetherbee, R. (2003). *J. Phycol.* 39: 722-734.
- Jenkinson, I.R. (1986). *Nature* 323: 435-437.
- Jenkinson, I.R. (1989). In: Okaichi, T., Anderson, D.M. & Nemoto, T. (eds), *Red Tides*, Elsevier, New York, pp. 435-438.
- Jenkinson, I.R. (1993a). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J., Shimizu, Y. (eds), Elsevier, Amsterdam, pp. 757-762.
- Jenkinson, I.R. (1993b). *Oceanol. Acta* 16: 317-334.
- Jenkinson, I.R. & Arzul, G. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (eds), UNESCO, Paris, pp. 425-428.
- Jenkinson, I.R. & Arzul, G. (2002). In: *Harmful Algal Blooms 2000*, Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. & Lewis, R.J. (eds), Unesco, Paris, pp. 461-464.
- Jenkinson, I.R. & Biddanda, B.A. (1995). *J. Plankt. Res.* 17: 2251-2274.
- Jenkinson, I.R. & Wyatt, T. (1995). In: *Harmful Marine Algal Blooms*, Lassus, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou-Le Baut, C. (eds), Lavoisier, Paris, pp. 603-608.
- Jenkinson, I.R., Claireaux, G. & Gentien, P. (2007). *Mar. Biol.*, 150: 471-485.
- Jones, C., Lawton, J. & Shachak, M. (1994). *Oikos* 69: 373-386.
- Langille, B.L., Stevens, E.D., Antantaraman, A. (1983). In: *Fish Biodynamics*, Webb, P.W. & Weihs, D. (eds), Praeger, New York, pp. 92-139.
- Li, D.-H. & Ganczarczyk, J.J. (1986). *Crit. Rev. Env. Control* 17: 53-87.
- Passow, U. (2000). *Mar. Ecol. Prog. Ser.* 192: 1-11.
- Seuront, L., Vincent, D. & Mitchell, J.G. (2006). *J. Mar. Sys.* 61: 118-133.
- Spinosa, L. & Lotito, V., (2003). *Adv. Env. Res.* 7: 655-659.
- Wyatt, T. & Ribera d'Alcalà, M. (2006). *CIESM Workshop Monographs* 28: 13-24.
- Žutić, V. & Svetličić, V. (2006). *CIESM Workshop Monographs* 28: 45-48.

## Life-strategies of viruses that infect *Heterosigma akashiwo*

J. Lawrence and C. Brown

Biology Department, University of New Brunswick, PO Bag Service 45111,  
Fredericton, NB, Canada, E3B 6E1, jlawrenc@unb.ca & cmbrown@mta.ca

### Abstract

A number of unrelated viruses infect the harmful bloom-forming raphidophyte *Heterosigma akashiwo*. These lytic viruses are distinct in their biochemical composition; they contain different nucleic acids and proteins and are therefore genetically diverse. Their life-strategy traits, such as latent period, burst-size and multiplication rate are also varied and therefore the characteristics of *H. akashiwo* infection and mortality are unique for each virus. These viral life-traits interact with the physiology and ecology of the host alga to influence the rate of propagation of infection throughout a bloom, and therefore the proliferation of each virus. The result is a complex, dynamic role of viruses in shaping the formation, maintenance and demise of harmful algal blooms *in situ*. These factors will be examined and used to demonstrate the role of viruses in phytoplankton mortality, and the importance of isolating and characterizing novel viruses to understand the role of viruses in phytoplankton population dynamics.

### Introduction

Viral infection has only recently been appreciated for its impact on population dynamics of phytoplankton. It is now recognized that infections become especially important during bloom conditions since the rate of infection depends on the density of host cells. *Heterosigma akashiwo* is a harmful bloom-forming alga against which numerous distinct viruses have been isolated (Nagasaki and Yamaguchi 1997; Lawrence *et al.* 2001; Tai *et al.* 2003). The viruses were all isolated from North Pacific waters, a number of them from the same geographic area. The isolates originating from the same region (Strait of Georgia, BC, Canada) all infect the same strain of host alga.

Lytic infections are marked by a number of events: replication of progeny, lysis of the host and concomitant release of progeny and organic material. This cycle inherently means viruses impact host abundance and nutrient cycling (reviewed in Fuhrman 1999). The 'arms race' is not limited to virus-host interactions; the co-existence of viruses that infect the same host suggests that each possesses traits that results in successful propagation and therefore persistence in the environment. It was our objective to examine and compare the characteristics and life-strategies of each virus to elucidate the variables that result in such diversity among viruses infecting the same host.

### Materials and Methods

We wished to compare the reproductive and life-strategies of known *H. akashiwo* viruses. We there-

fore compiled data from the available literature and from previously unpublished research by J. Lawrence to collate estimates of virus size, nucleic acid type and size, subcellular replication site, latent period and in some cases burst size, for four unrelated viruses that infect *H. akashiwo* (Table 1).

Burst-size estimates reported in the literature are calculated by a variety of means, such as bioassays to determine the number of infectious units released per lysed cell (ie. Nagasaki *et al.* 1999), fluorescence assays to determine the number of nucleic acid-containing particles produced per host cell (Lawrence *et al.* 2006), and electron microscopy to enumerate the number of viruses in a typical infected cell just prior to lysis (Lawrence *et al.* 2001). Since burst-size estimates were of primary concern, we sought to compare estimates obtained using the same method. This was most parsimoniously achieved using the latter approach; a collection of TEM images were used to enumerate the number of viruses in thin-sectioned *H. akashiwo* cells. The total number of particles in individual cells was then estimated by geometric calculations (Table 1). For example, in micrographs from Nagasaki *et al.* (1999), we counted 170 HaV particles in a section of virioplasm having a 4- $\mu\text{m}$  diameter and an estimated volume of 33.5  $\mu\text{m}^3$ . The section is not a plane, but rather a cylinder with a volume of approximately 2.5  $\mu\text{m}^3$ , or 7.5 % of the total virioplasm volume. We therefore divided 170 by 0.075, for an estimate of 2267. Similar principles were used to calculate burst sizes for other virus.

In some instances the estimates were much larger than those achieved with other methods, such as the fluorescence-based assay for HaRNAV (Lawrence *et al.* 2006;  $2 \times 10^5$  vs.  $2 \times 10^4$ ). However, in each case there was a reasonable explanation for the deviation. For this example, the fluorescence assay produces an underestimate because particles released as aggregates or attached to cellular debris, and incompletely assembled particles without nucleic acid will not be enumerated. The TEM method provides an estimate of the total number of viruses produced during one lytic cycle, independent of whether this progeny is successful or has the capacity to be successful. This allows us to compare reproductive effort.

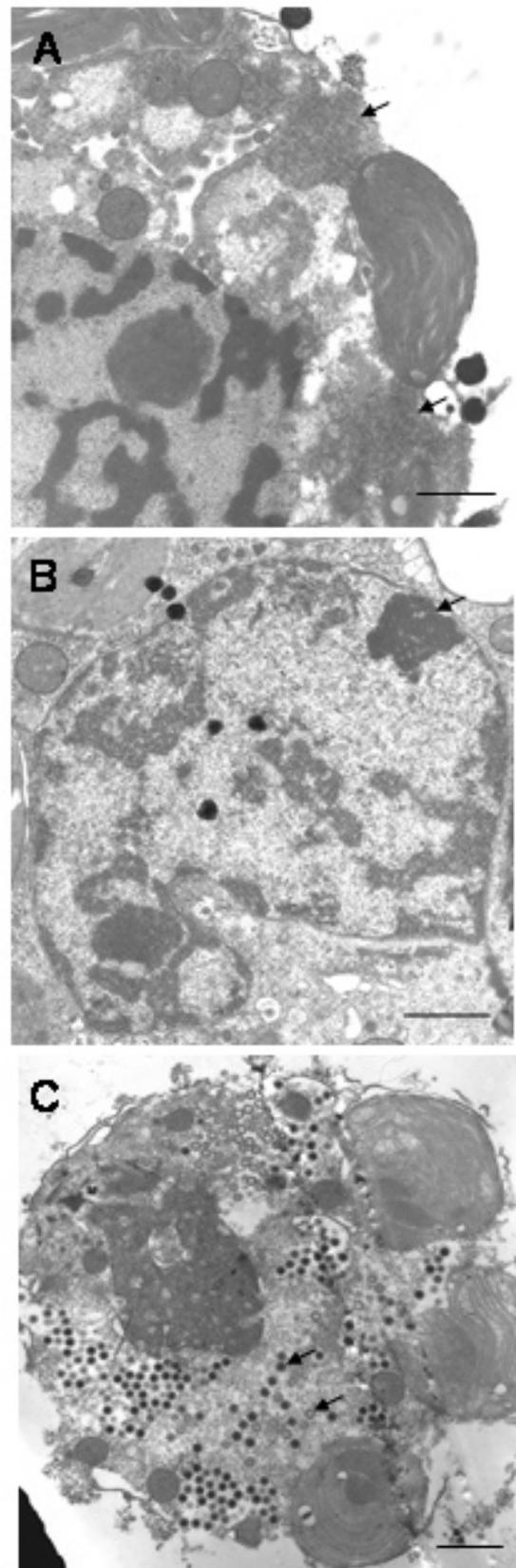
### Results and Discussion

A diverse group of viruses infect *H. akashiwo*. Indeed, with genomes comprised of ssDNA, ssRNA and dsDNA ranging in size from 9.1 kb to 294 kb, the viruses are phylogenetically unrelated (Table 1). The number of viruses produced inside an individual *H. akashiwo* cell varies over two orders of magnitude (Table 1). The most proliferate virus is HaNIV with an estimated burst-size of  $5 \times 10^5$ , and least is HaV with  $2 \times 10^3$  per cell. The length of time between infection and host lysis is much less variable, ranging from 30 to 53 hours (Table 1).

The multiplication rate of a virus can be determined by dividing the burst size by the latent period of a virus (De Paepe and Taddei 2006). This estimate allows us to compare the rate of resource utilization during viral replication, accommodates for the exponential reproduction of viral progeny, and is independent of host density (De Paepe and Taddei 2006). The multiplication rates of the four viruses examined in this study varied from  $67 \text{ h}^{-1}$  to  $1.2 \times 10^4 \text{ h}^{-1}$  (Table 1).

Production of a large number of small viruses rather than a small number of large virus particles might be advantageous if all virus particles have equal potential to reinfect a susceptible host. The stable coexistence of at least 4 different viruses capable of infecting the same host suggests that such an advantage is somehow balanced by disadvantages.

A form of viral niche-partitioning may best describe viral coexistence. In coliphage systems (De Paepe and Taddei 2006), the phage taxa with the highest multiplication rates also have the highest decay rates. However, rapid production and decay need not cancel each other out in all situations. For instance, at high host densities, decay rates become less important



**Figure 1.** TEM micrographs of *Heterosigma akashiwo* cells infected with A) HaRNAV, B) HaNIV and C) Ha2V. Arrows point to inclusions of viruses in A and B, and individual particles in C. Scale bar = 1  $\mu\text{m}$ .

since contacts between phage and hosts become more frequent, reducing the time spent drifting between susceptible hosts.

Decay rate estimates are not available for all *H. akashiwo* viruses at present. However, we may still study host density-dependent niche-partitioning to examine whether it may explain multiple co-existing virus types and virus strategies. To determine how this might be possible, we need to consider some of the variables that influence virus and host numbers: contact rate,  $k$  ( $\text{cm}^{-3} \text{d}^{-1}$ ); viral burst size,  $B$ ; host population,  $P$  ( $\text{cells cm}^{-3}$ ); virus concentration,  $V$  ( $\text{particles cm}^{-3}$ ); and virus decay rate,  $D$ . Viruses are added to a system by contact with and replication within a host, and are lost through decay, so that

$$kBPV = DV \quad (\text{Mann 2003})$$

We can cancel  $V$  when the rates of these opposing processes are equal. Therefore,

$$kBP = D$$

$k$  is estimated as  $4\pi RCf$ , where  $R$ =host radius (cm);  $C$ =diffusion constant of virus particle;  $f$ =proportion of contacts leading to an infection.

At very low cell densities, contact between viruses and hosts are less frequent than at high densities. For each virus type, there is a threshold host cell density below which contact is too infrequent for viral proliferation to occur. The advantage of a larger burst, therefore, is that given constant contact and decay rates, propagation can occur at a lower cell density. Conversely, at higher cell densities, the advantage conferred by a large burst size is diminished, so that a virus having a slower multiplication rate and a smaller burst size can proliferate. In essence, from the vantage point of the virus, the host cell density is the niche, and what serves as an advantage at a particular cell density may not work at another.

In order for this theory to work, a larger virus with a smaller burst size and lower multiplicative rate must, under certain conditions, have an advantage over a smaller more prolific competitor. The additional genetic information harboured by these viruses may

hold the key. Perhaps larger viruses have slower decay rates, larger host ranges, larger diffusion constants or a higher proportion of successful contacts than their genetically more diminutive counterparts. Viruses can influence the clonal composition of a host population (Tarutani *et al.* 2000), which may in turn advantage or disadvantage a virus, depending on its host range. This highlights the need to further characterize, both physico-chemically and genetically, known algal viruses to gain insight into the interactions of viruses with bloom-forming phytoplankton.

### Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada through a Discovery Grant to J.L.

### References

- Brown, C.M., Lawrence, J.E. & Campbell, D.A. (2006). *J. Mar. Biol. Ass. U.K.* 86: 491-498.
- De Paepe, M. & Taddei, F. (2006). *PLoS Biol.* 4: 1248-1256.
- Fuhrman, J.A. (1999). *Nature* 399: 541-548.
- Lang, A.S., Culley, A.I. & Suttle, C.A. (2004). *Virology* 320: 206-217.
- Lawrence, J.E., Brussaard, C.P.D. & Suttle, C.A. (2006). *Appl. Env. Microbiol.* 72: 7829-7834.
- Lawrence, J.E., Chan, A.M. & Suttle, C.A. (2001). *J. Phycol.* 37: 1-7.
- Mann, N.H. (2003). *REMS Microbiol. Rev.* 27: 17-34.
- Nagasaki, K., Shirai, Y., Tomaru, Y., Nishida, K. & Pietrokoviski, S. (2005). *Appl. Env. Microbiol.* 71: 3599-3607.
- Nagasaki, K., Tarutani, K. & Yamaguchi, M. (1999). *Appl. Env. Biol.* 65: 898-902.
- Nagasaki, K. & Yamaguchi, M. (1997). *Aquat. Microb. Ecol.* 13: 135-140.
- Tai, V., Lawrence, J.E., Lang, A.S., Chan, A.M., Culley, A.I. & Suttle, C.A. (2003). *J. Phycol.* 39: 343-352.
- Tarutani, K., Nagasaki, K. & Yamaguchi, M. (2000). *Appl. Env. Microbiol.* 66: 4916-4920.

## Succession pattern of HAB species before large-scale blooms of dinoflagellates in the East China Sea in spring 2004/2005

D. Lu<sup>1</sup>, J. Göbel<sup>2</sup>, Y. Gao<sup>3</sup>, Y. Qi<sup>4</sup>, J. Zou<sup>5</sup>, P. Xia<sup>1</sup> and W. Du<sup>1</sup>

<sup>1</sup>Lab of Marine Ecosystem and Biogeochemistry, SOA, Second Institute of Oceanography, SOA, Hangzhou 310012 China, doudinglu@126.com; <sup>2</sup>Landesamt für Natur und Umwelt des Landes Schleswig-Holstein, Hamburger Chaussee 25, 24220 Flintbek, Germany, jgoebel@lanulandsh.de; <sup>3</sup>School of Life Sciences, Xiamen University, Xiamen 361005, China; <sup>4</sup>Institute of Hydrobiology, Jinan University, Guangzhou 510632 China; <sup>5</sup>Institute of Oceanology, Chinese Academy of Science, Qingdao, 266071, China

### Abstract

Large-scale blooms of *Prorocentrum donghaiense* in May have been a recurrent phenomenon for the last decade in the East China Sea. During the first ten days of April 2004, the density of *P. donghaiense* exceeded 10,000 cells/L and it was the dominant species of phytoplankton in the subsurface layer. The highest concentration was 100,000 cells/L near the 50-m isobath at some stations. These subsurface populations were the source of subsequent bloom developments. During 2005 the situation was different. Following a diatom bloom dominated by *Skeletonema costatum* and *Thalassiosira curviseriata* in late April, *Karenia mikimotoi* became dominant, followed by *P. donghaiense* and *Scrippsiella trochoidea* in the subsurface. This led to the development and outbreak of the first large-scale bloom of this ichthyotoxic species recorded in the ECS in late May. The change in oceanographic and nutrient conditions in winter and early spring of 2005 obviously influenced the succession pattern of phytoplankton, inducing the unusual proliferation of *K. mikimotoi*.

### Introduction

Harmful algal blooms have had serious economic impact on the resources of marine fisheries, public health and the aquatic environment throughout the coastal areas of the world (Hallegraeff *et al.* 2003). The coastal waters in the East China Sea (ECS) are influenced by several water masses such as Taiwan warm current (TWC) and Yangtze (Changjiang) River (Su 2001; Zhu *et al.* 2003) and have become one of the most frequent HAB areas in China (Zhou *et al.* 2001, 2003). During the last decade, *P. donghaiense*, a recurrent and high biomass bloom-forming dinoflagellate species, was recorded in the Yangtze river estuary and coastal waters of Zhejiang province, China. It formed massive blooms in the convergence zone of the river estuary and along the coastal waters of Zhejiang province during spring (Lu and Goebel 2001; Lu *et al.* 2002, 2003). However, the dynamic process leading to large dinoflagellate blooms in the ECS are not clear. A number of comprehensive cruises have been undertaken during the spring of 2004 and 2005 in order to examine successional patterns of targeted HAB species and to understand the ecological and oceanographic processes of large-scale blooms caused by *Prorocentrum* and other dinoflagellates.

### Materials and Methods

Phytoplankton was collected in the East China Sea during spring 2004 and 2005 (Fig. 1). Sampling sta-

tions were designed to cross the isobaths from 20 to 70 m. Samples were fixed with acidified Lugol's iodine solution (final concentration of sample 3 %). Olympus BH-2 and Leitz 20EBF light microscopes equipped with digital cameras were employed to analyze both living and preserved samples. Sedgewick-Rafter chambers were used for counting of cells. Temperature and salinity data were measured by the SBE37-CTD. Fluorescence measurements were made by a YSI-Sensor.

### Results and Discussions

After a small early spring bloom of diatoms dominated by *S. costatum* in March 2004, the population of *P. donghaiense* started to increase. During the first ten days of April 2004, the density of *P. donghaiense* exceeded 10,000 cells/L and it was the dominant phytoplankton species in the subsurface microthin layers. The highest concentration recorded was 100,000 cells/L near the 50-m isobath, and it is considered a source for subsequent development of massive blooms (Fig. 2a) In the spring of 2005 the situation was quite different. The succession from diatoms to dinoflagellates was delayed almost one month. The spring diatom bloom, dominated by *S. costatum* and *T. curviseriata*, started at the beginning of April and the scale of the bloom was much more extensive than in previous years (Fig. 3).

After the diatom bloom, *P. donghaiense* was not the dominant species as in previous years (Fig. 2b). In-

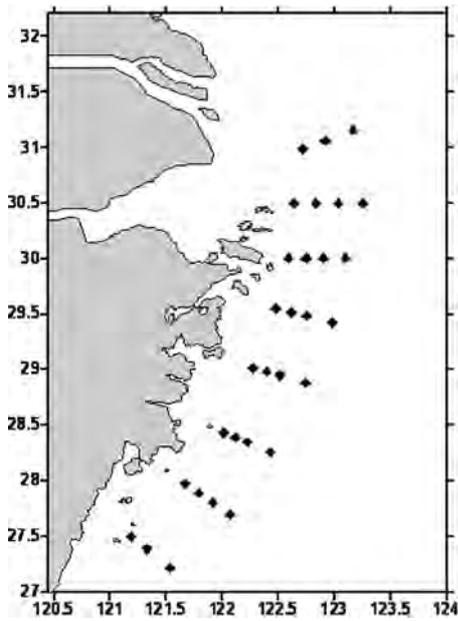


Figure 1. Sampling site.

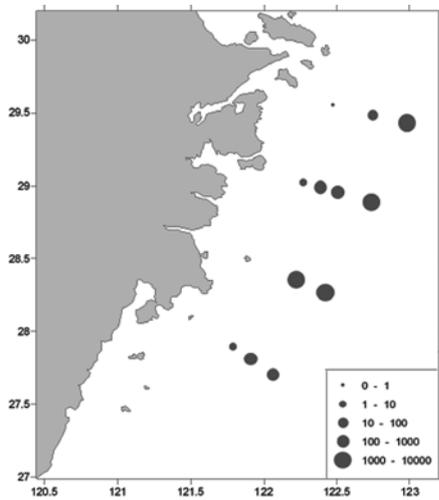


Figure 3. Massive diatom bloom dominated by *Skeletonema* in early April, 2005 ( $\times 10^3$  cells/L).

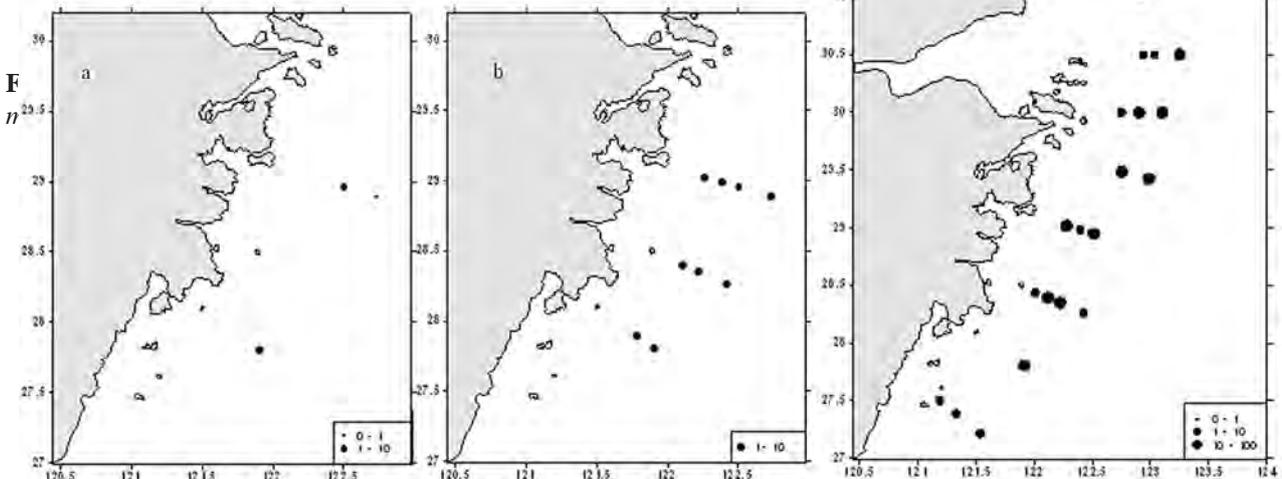


Figure 4. Cell concentration of *Karenia mikimotoi* at 10 m before its massive bloom ( $\times 10^3$  cells/L) a: 4-7 April; b: 11-12 April; c: 3-9 May.

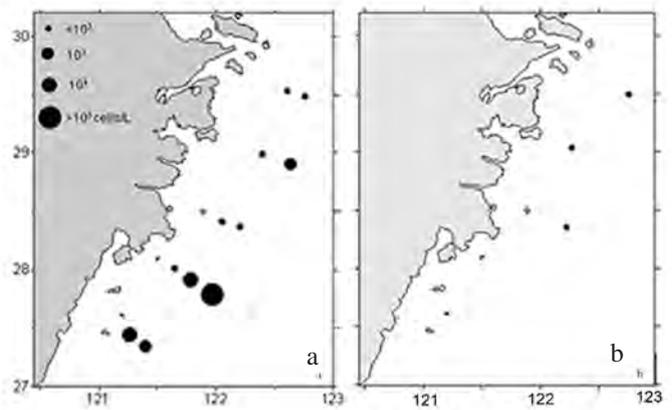
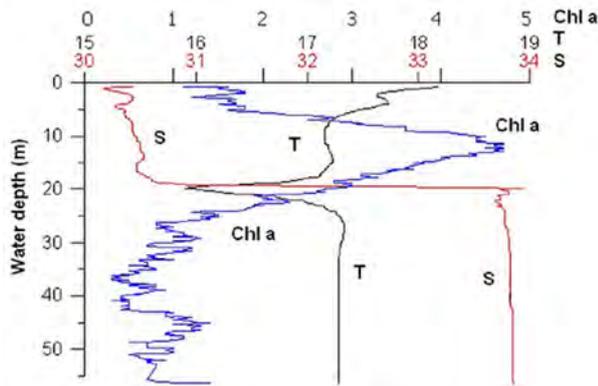
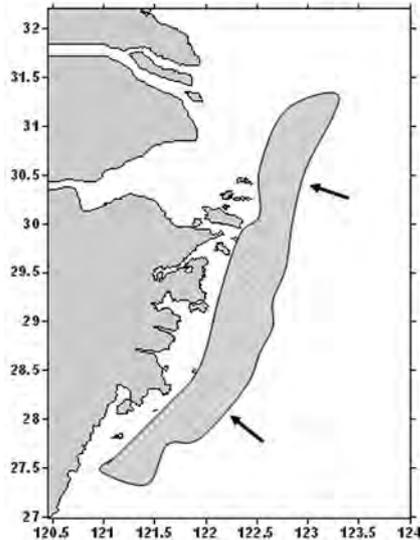


Figure 2. Occurrence of *Prorocentrum donghaiense* at 10 m in early April in the study area (a: 2004; b: 2005).

stead the *K. mikimotoi* concentration increased in the water column. At the beginning of April, only low cell concentrations were detected at a few stations (Fig. 4a). *Karenia mikimotoi* appeared at more stations one week later (Fig. 4b). During the first ten days of May, *K. mikimotoi* became the dominant species, followed by *P. donghaiense* and *S. trochoidea* and it occupied the main part of the study area (Fig. 4c). The high chl *a* concentrations were distributed between 10 and 20 m in thin subsurface layers (Fig. 5), where the cell density of *K. mikimotoi* was 20,000 cells /L and *P. donghaiense* was 8000 cells/L. These thin zones were considered the initial incubators for subsequent massive blooms of *K. mikimotoi* and *P. donghaiense*, leading, in late May, to the development and outbreak of the first large-scale bloom of the ichthyotoxic species *K. mikimotoi* in the ECS (Fig. 6).



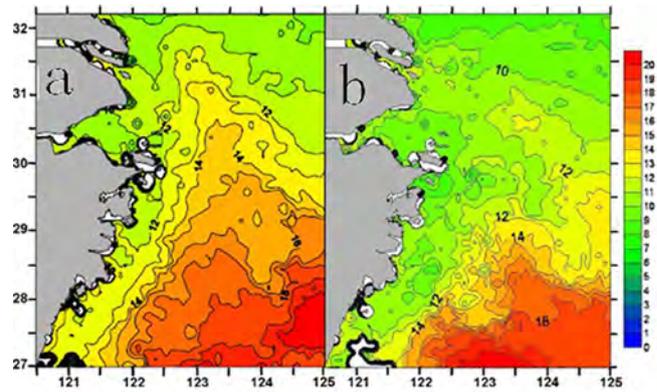
**Figure 5.** Vertical distribution of Chl *a* ( $\mu\text{g/L}$ ), temperature and salinity (thin layer is located above the pycnocline).



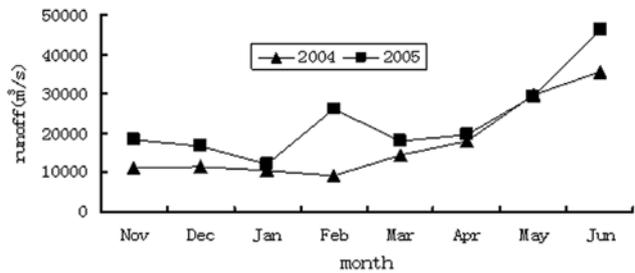
**Figure 6.** The massive bloom of *Karenia mikimotoi* occupied an area of 15,000  $\text{km}^2$  (arrows).

The unusual cold and rainy winter (temperature was much lower than in previous years, runoff of Yangtze River increased by 40 % during the first three months) changed the basic environmental conditions for the phytoplankton in early spring. The water temperature at the beginning of April in 2005 was 3 °C lower compared to 2004. This phenomenon was also observed from MODIS SST satellite data (Fig. 7).

The temperature increased fast in the middle of April and the salinity was higher in TWC compared to previous years (Zhu, 2005, personal communication). Nutrient levels were higher and nutrient ratios differed in early spring of 2005 compared to the corresponding periods of 2004. DIN and DIP increased 32 % and 33 %, respectively while Si increased 35 % due to greater input of runoff from Yangtze (Fig. 8). The higher concentration of DIN and Si extended to offshore waters. The change in oceanographic and nutrient conditions mentioned above in winter and early spring of 2005 obviously influenced the succes-



**Figure 7.** MODIS-SST at the beginning of April (a: 2004; b: 2005).



**Figure 8.** Runoff from Yangtze 2004 and 2005.

sion pattern of phytoplankton, inducing the unusual proliferation of *K. mikimotoi*.

### Acknowledgements

This work was supported by CEOHAB project (2001CB409701), exchange project between Germany and China (CHN 06/009) supported by the International Bureau of Ministry of Education and Research at DLR. Thanks are expressed to Dr. Dedi Zhu for processing of MODIS-SST images.

### References

- Hallegraeff, G.M., Anderson, D.M. and Cembella, A.D. (2003) (eds), Manual on Harmful Marine Microalgae, UNESCO, Paris.
- Lu, D. & Goebel, J. 2001. Chin. J. Oceanogr. Limnol. 19: 337-344.
- Lu, D., Göbel, J., Qi, Y., Zou, J. & Gao, Y. (2002) IOC Newsletter on Toxic Algae and Algal Blooms 23: 1-5.
- Lu, D., Göbel, J., Qi, Y., Zou, J. & Gao, Y. (2003). Chin. J. Appl. Ecol. 14: 1060-1064
- Su, J. (2001). Acta Oceanol. Sin. 23: 1-16 (in Chinese).
- Zhu, D. (2003). Chin. J. Appl. Ecol. 14: 1131-1034.
- Zhou, M., Zhu, M. & Zhang, J., (2001). Life Sci. 13: 54-59 (in Chinese).
- Zhou, M., Yan, T. & Zou, J. (2003). Chin. J. Appl. Ecol., 14: 1031-1038.

## Microscopic digital holography imaging of dinoflagellate behaviour

J. Sheng<sup>1</sup>, E. Malkiel<sup>1</sup>, D.W. Pfitch<sup>1</sup>, J. Katz<sup>1</sup>, J. Adolf<sup>2</sup>, R. Belas<sup>2</sup> and A.R. Place<sup>2</sup>

<sup>1</sup>The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218 jiansh@poseidon.me.jhu.edu, maikiel@jhu.edu, pfitch@jhu.edu and katz@poseidon.me.jhu.edu

<sup>2</sup>UMBI Center of Marine Biotechnology, 701 E. Pratt St., Baltimore, MD 21202, adolf@umbi.umd.edu, belas@umbi.umd.edu and place@umbi.umd.edu

### Abstract

Predator-prey interactions are fundamental to a greater understanding of harmful algal bloom (HAB) dynamics; however, interactions between dinoflagellates and their prey are difficult to monitor using standard microscopy. The high magnification needed to distinguish between dinoflagellates and prey species results in shallow depths of field and prevents tracking the 3-dimensional paths of multiple swimming organisms. Microscopic digital holography overcomes this limitation by using numerical reconstruction to provide in-focus views of all the organisms within a 3-mm depth. The accuracy in the tracking procedure is sufficient to provide fully 3-dimensional trajectories from one view. The technique was applied to cultures of toxic and nontoxic strains of *Karlodinium veneticum* with and without a predator, *Oxyrrhis marina*, as well as *Pfiesteria piscicida* with and without its algal prey, *Rhodomonas*. Typical swimming behaviour includes helical swimming and conspecifics revolving around each other. *Karlodinium* individuals were observed to have swimming speeds ranging of 0.05-0.5 mm/s and *Pfiesteria* cells 0.1-1 mm/s. We also observed a change in the average swimming speed of *P. piscicida* from 0.5 mm/s in isolation to 0.9 mm/s in the presence of its prey, *Rhodomonas*.

### Rationale and Background

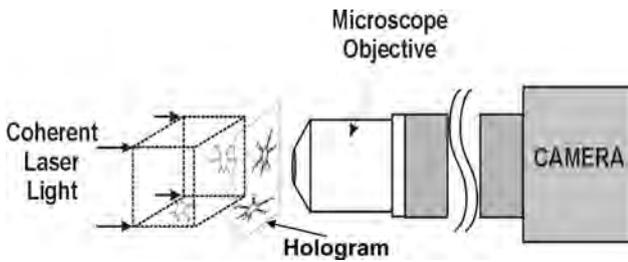
Harmful algal blooms (HABs) are proliferations of microscopic algae that harm the environment by producing toxins that accumulate in shellfish or fish, or through accumulation of biomass that affects co-occurring organisms and alters food webs. Most of the world's nearshore marine waters have experienced increases in frequency and type of HABs (Ramsdell *et al.* 2005). Substantial research on dinoflagellates (Taylor 1987) indicates that occurrence of HABs is a result of complex interactions between the causative species and (i) co-occurring organisms – preys, predators and symbionts, (ii) nutrients, and (iii) the physical environment - turbulence, temperature, light, gravity, diurnal cycle, etc. Resolving trends in this complex system is a major challenge, that limits our ability to understand and forecast development of HABs (Ramsdell *et al.* 2005).

Due to their size, 10-2000  $\mu\text{m}$  but mostly in the 10-30  $\mu\text{m}$  range, 3-D swimming behaviour of dinoflagellates must be investigated microscopically. Being limited by the narrow depth of field in conventional microscopy, most behaviour studies have been performed in “shallow” samples, where “wall effects” may affect behaviour. The triggering of imaging systems as subjects cross the in-focus plane, or use of 3-D traversing systems that follow organisms provide only limited solutions for making observations away from boundaries. Measurements are further compli-

cated by tendency of dinoflagellates to cluster in dense aggregations. Finally, inferring 3-D motion from 2-D projections involves unavoidable assumptions on geometric symmetry (Crenshaw *et al.* 2000). Consequently, many issues in dinoflagellate behaviour are still unclear. Recent advances in digital holographic microscopy (DHM) enable, for the first time, tracking of thousands of organisms in space and time within a volume containing a substantial depth.

### Digital Holographic Microscopy

Observations of dynamic processes at microscopic scales require tools for resolving short temporal and fine spatial scales. In an optical microscope, as lateral resolution increases, the depth of field decreases, e.g. to 12 mm at 10X and to 2 mm at 40X (Inoue and Spring 1997). Holography, on the other hand, maintains high lateral resolution over a substantial depth. With recent developments in digital holography, it is now possible to record holograms on digital media, and reconstruct them numerically (Schnars and Juptner 2002; Malkiel *et al.* 2003; Pan and Meng 2003). Inherently, these methods focus on isolated objects in shallow samples. To examine the spatial distribution and velocity of a dense aggregate of particles in a flow with an extended depth, we have recently introduced the in-line digital holographic microscope (DHM, Sheng *et al.* 2006). This method (Fig. 1) replaces the light source of a transmission microscope with a weak



**Figure 1.** Digital holographic microscope

(30 mW.cm<sup>-2</sup>), collimated laser beam. The objective focuses the hologram plane, which is located outside of the sample volume, onto a CCD array. The image is an interference pattern between light scattered from particles in the sample volume and the illuminating reference beam. It does not contain in-focus images of these particles. Analysis shows that the CCD plane is a magnified hologram plane (Sheng *et al.* 2006), drastically relaxing the spatial resolution requirement of the recording medium. Magnification then becomes a function of achieving the desired resolution. The 3-D particle field is reconstructed numerically using the Fresnel diffraction formula (Milgram and Li, 2002; Malkiel *et al.* 2006; Sheng *et al.* 2006). Each plane is reconstructed separately, typically in Fourier space. The coordinates of each particle are determined using an automated 3-D segmentation method (Sheng *et al.* 2006, and manuscript in press) and the 3-D velocity is determined from displacement of particle centroids. Implementation of DHM has included calibration tests and development of a series of data analysis software tools. We have successfully reconstructed samples containing 0.75–3 mm spherical particles at concentrations of ~2000 particles/mm<sup>3</sup> and a total number in the 10<sup>4</sup> range.

## Material and Methods

A cuvette (Spectrum Cell, Inc.) with an inner dimension of 3 mm × 3 mm and 40 mm in vertical direction was used as the measurement container. The large horizontal dimensions ensure that cells suspended in the cuvette were less likely influenced by the boundaries. Vertical length of the cuvette was intentionally chosen to be long for considerations on the possible vertical migration of cells due to phototaxis or gravitaxis. All outside surfaces of the cuvette were coated with anti-reflection coating to prevent interference from multiple reflections. A Q-Switched diode-pumped ND:YLF (=660 nm, multiple longitudinal modes, Crystal Lasers, Inc.) was used to illuminate the cuvette. The illumination was reshaped, filtered and collimated into a Gaussian beam of 3 mm in diameter

through a 10-mm pinhole assembly. A high-speed digital holo-graphic microscope (Sheng *et al.* 2006) was used to image the swimming cells in the cuvette. The high-speed digital holographic microscope consisted of a commercial 20X objective lens (Edmund Scientific) and a high-speed 1K × 1K CMOS camera (maximum speed at 2000 frame per second up to 3 s recording, Photron). The objective lens was located 160 mm away from the sensor and was configured in the same manner as a conventional light microscope. The pixel dimension of the sensor was 19 × 19 μm<sup>2</sup>, and thus a physical dimension of 19.5 mm × 19.5 mm. The spatial resolution was 0.975 μm on planes parallel to the hologram plane. The object plane was located at 100 μm away from the inner wall of the cuvette. The entire measurement volume was 0.8 mm × 0.8 mm × 3 mm. A right-handed coordinate system was used as a fixed reference frame with the positive y direction pointing downwards and the positive z direction into the paper. Holograms were recorded at the rate of 60 fps instead of 2000 fps and total duration of 13 s for each recording.

Cultures of *Pfiesteria piscicida* (CCMP 1830), *Rhodomonas* sp. (CCMP 768), *Karlodinium veneticum* (CCMP 2064) and *Storeatula major* in mid-log growth were placed in the cuvette individually and in combination (Predator:Prey; 2.5:1) and holograms collected. Each hologram was reconstructed at an interval of 10 mm in z direction (perpendicular to the hologram) over a range of 3 mm using Fresnel diffraction formula:

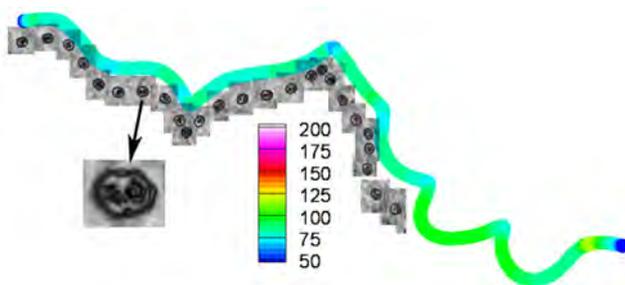
$$I_r(x_0, y_0, z_0) = \iint I_h(x_1, y_1) \frac{\exp\left\{j\frac{2\pi}{\lambda z} [(x_0 - x_1)^2 + (y_0 - y_1)^2]\right\}}{j\lambda z_0} dx_1 dy_1$$

where  $I_r$  is the reconstructed irradiance distribution at depth location,  $z_0$  and  $I_h$  is the recorded digital hologram. Once a stack of images were reconstructed, information on cell location were extracted using a hybrid two-step auto-focusing routine. 3-D Cell images were first processed by a 3-D segmentation algorithm (Sheng *et al.* 2006) to determine the 3-dimensional cell centroids. It was found that due to the unique scattering of cells, scattering intensity formed two peaks which often saddled the in-focus point. The aforementioned 3-D segmentation, primarily relied on the intensity, hence produced an in-focus centroid that was biased toward either of intensity peaks, about ±20 mm away. To improve the measurement accuracy in the z direction, a second procedure was performed. The procedure relied on the sharpness of cell image and was applied to the images within ±100 μm around the cell centroid determined by the first procedure. The

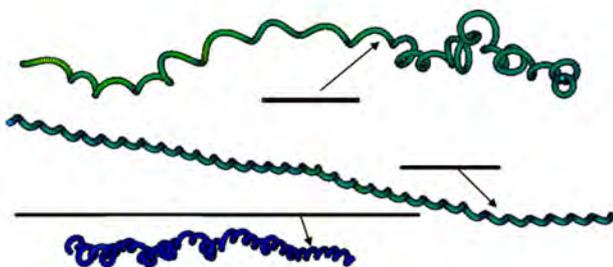
measurement of sharpness, i.e. surface integral of the Laplacian derivatives of the entire image, along the z direction was fitted with a Gaussian curve to achieve sub-pixel resolution. Centroid measurements in x and y directions were further computed by the intensity weighted averaging a 2-D segment of the in-focus cell image. Further information on each cell, such as cross section dimension, total reconstructed volume, and in-focus snapshots, were extracted as well for later use in Lagrangian cell tracking.

## Results and Discussion

Sample reconstructions are presented in Fig. 2 for *Karlodinium veneficum*. Each in-focus image is located in a different depth, i.e. the entire 3-D trajectory cannot be viewed by a conventional microscope but is easily reconstructed using the DHM. Fig. 2 reveals the complexity of a typical trajectory, and an increase in velocity prior to contact with another organism. Characteristic *Pfiesteria* trajectories in the presence of *Rhodomonas* (Fig. 3) demonstrate variability in velocity magnitude, direction of rotation, pitch and radius of helices. Typical swimming behaviour includes helical swimming and conspecifics revolving around each other. *K. veneficum* individuals are observed to have swimming speeds ranging of 0.05 - 0.5 mm/s and *P. piscicida* cells 0.1-1 mm/s. We also observed a



**Figure 2.** Trajectory of *Karlodinium* with velocity in  $\mu\text{m s}^{-1}$  colour-coded. Total duration of record: 20 s.



**Figure 3.** Sample *Pfiesteria* trajectories with velocity colour coded. Scale: 100  $\mu\text{m}$ . Combined image shows tracks over 1 mm depth (1/3 of total sample volume depth).

change in the average swimming speed of *P. piscicida* from 0.5 mm/s in isolation to 0.9 mm/s in the presence of its prey, *Rhodomonas*.

Using this technology we have begun a series of experiments to quantify swimming behaviour of heterotrophic (*Pfiesteria*), mixotrophic (*Karlodinium*) and autotrophic (*Karenia*) dinoflagellates in response to prey abundance, nutrients, shear, bacteria, and diurnal and other cycles.

## Acknowledgements

The JHU part of this research work was supported by NSF, in part by ECHOHAB grant No. OCE-0402792, and in part by grant No. CTS0625571. The equipment was purchased under MRI grant No. CTS0079674. This work was also funded in part by grants from NOAA Coastal Ocean Program under ECOHAB award NA04NOS4780276 to UMBI, and Grant U50/CCU 323376, Centers for Disease Control and Prevention and the Maryland Department of Health and Mental Hygiene. This is contribution # 07-164 the UMBI Center of Marine Biotechnology, and #212 from the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) programme.

## References

- Crenshaw, H.C., Ciampaglio, C.N. & McHenry, M. (2000). *J. Exp. Biol.* 203: 961-982.
- Inoue, S. & Spring, K.R. (1997). *Video Microscopy: the Fundamentals*. 48 pp.
- Malkiel, E., Sheng, J., Katz, J. & Strickler, J.R. (2003). *J. Exp. Biol.* 206: 3657-3666.
- Malkiel, E., Abras, J.N., Widder, E.A. & Katz, J. (2006). *J. Plankt. Res.* 28: 149-170.
- Milgram, J.H. & Li, W.C. (2002). *Appl. Optics* 41: 853-864.
- Pan, G. & Meng, H. (2003). *Appl. Optics* 42: 827-833.
- Ramsdell, J.S., Anderson, D.M. & Glibert, P.M. (2005). *Harness 2005, harmful algal research and response: a national environmental science strategy 2005-2015*.
- Schnars, U. & Juptner, W.P.O. (2002). *Measurement. Sci. Technol.* 13: R85-R101.
- Sheng, J., Malkiel, E. & Katz, J. (2006). *Appl. Optics* 45: 3893-3901.
- Sheng, J., Malkiel, E. & Katz, J. *J. Fluid Mechanics* (in revision)
- Taylor, F.J.R. (1987). *The Biology of Dinoflagellates*, Blackwells, Oxford, 768 pp.

## Copepods feeding on a thin-layered bloom of *Dinophysis acuta*

L. Sobrinho-Gonçalves and M.T. Moita  
IPIMAR, Av. Brasília, Lisbon Portugal, andresg72@yahoo.com

### Abstract

The abundance of *Dinophysis acuta* in the digestive contents of five copepod taxa was studied in situ during a thin-layer bloom on the NW upwelling coast of Portugal, when *D. acuta* reached  $24 \times 10^3$  cells  $L^{-1}$ . The copepod community, sampled in two depth strata, was abundant and reached a maximum of 17800 ind.  $m^{-3}$ . The concentration of *D. acuta* in the copepods was low (average 0.3 cells ind. $^{-1}$ ) and related to the dinoflagellate concentration in the water column. Only the larger copepods, *Calanus helgolandicus* and *Centropages chierchiae*, showed relevant ingestion rates, although restricted to locations with more than  $9 \times 10^3$  *D. acuta* cells  $L^{-1}$ . The *C. chierchiae* population showed the highest clearance rate (157 *D. acuta* cells  $L^{-1} day^{-1}$ ), indicating some degree of “active” feeding on the bloom associated with its omnivore-raptorial behaviour and/or with a possible immunity to the toxins. The three smaller taxa population, although very abundant, together presented a low clearance rate (120 *D. acuta* cells  $L^{-1} day^{-1}$ ) probably due to their weaker filtering capacities and/or to selective feeding on more edible phytoplankton. As the studied copepods accounted for 65 % of all meso-zooplankton, we speculate that, despite of a probable high grazing pressure, the thin-layer bloom of *D. acuta* did not seem to have been top-down controlled.

### Introduction

Large dinoflagellates (k-strategists) like *D. acuta*, necessarily have “special” protective mechanisms to avoid grazing and to reach high concentrations of cells since they compete with other microalgae of similar sizes and higher growth rates (Smayda 1997; Jansen 2006). The interactions between toxic microalgal blooms and the surrounding zooplankton remain insufficiently studied, e.g. the effects of these blooms on the feeding behaviour of grazers (Turner *et al.* 1998). Copepods, as one of the main grazers of microalgae, could play a key role in the control, structure and development of dinoflagellate blooms (Jansen *et al.* 2006). However, there are few studies on the interactions of copepods with *Dinophysis* (Carlsson *et al.* 1995; Maneiro *et al.* 2000; Jansen *et al.* 2006) and none concerning *D. acuta*.

Moita *et al.* (2006) observed, for the first time in northwestern Iberia (subject to seasonal up-welling), a bloom of *D. acuta* (DSP) forming a thin layer. The main objective of this work was to make a preliminary evaluation of the spatial and inter-specific variability of copepod feeding on that *D. acuta* bloom.

### Material and Methods

Meso-zooplankton samples were collected on the NW coast of Portugal during a cruise in September 2003, which covered a cross-shelf section off Figueira da Foz, between  $40^{\circ}12'N$   $8^{\circ}57'W$  and  $40^{\circ}12'N$   $9^{\circ}06'W$ ,

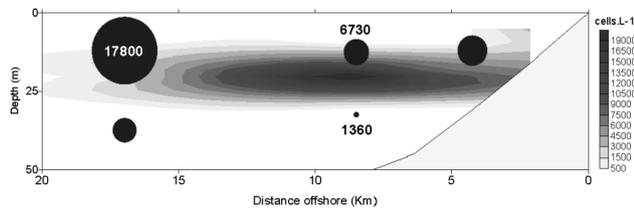
approximately. At each station, vertical hauls were taken at two depth strata (0-25 m and 25-50 m, respectively) using a WP2 net with 200- $\mu$ m mesh size. Meso-zooplankton samples were preserved in 4 % buffered formalin. This type of preservation could have induced some food regurgitation by copepods. Five taxa of abundant herbivore/omnivore copepods (of different size classes) were studied: *Calanus helgolandicus*, *Centropages chierchiae*, *Clausocalanus* spp., *Temora longicornis* and *Paracalanus parvus*. From these taxa, the digestive contents of 160 specimens (mostly adults), randomly selected, were analyzed by dissection and screened for remains of *D. acuta*.

### Results

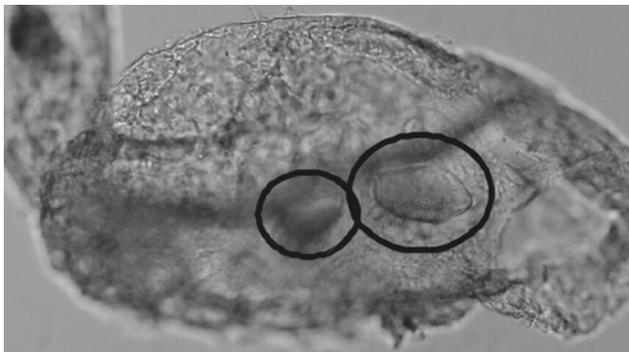
A thin-layered bloom of *D. acuta* occurred under upwelling relaxation conditions and strong water column thermal stratification (Moita *et al.* 2006). *D. acuta* maxima were distributed between 18 m and 20 m depth, reaching  $24 \times 10^3$  cells  $L^{-1}$  at 8.5 km offshore (Moita *et al.* 2006) (Fig. 2). *D. acuta* reached 44 % of the microphytoplankton community in the bloom core (coincident with fluorescence maxima) and accounted for 10 % of the community if we consider the whole area of study (Sobrinho-Gonçalves 2007). At 12 km offshore, the very high concentrations of Chl *a* detected by Moita *et al.* (2006) indicate an important presence of *D. acuta* cells in that location.

The copepod community reached a maximum of 17800 ind. m<sup>-3</sup>, with an average abundance of 8000 ind. m<sup>-3</sup> in the area (Fig. 1). The relatively small *T. longicornis* was the dominant species, accounting for 35 % of the meso-zooplankton, while the other four copepod taxa together represented 30 % (Sobrinho-Gonçalves 2007). The maxima of *D. acuta* and of copepods did not coincide (Fig. 1). Remains of *D. acuta* cells were detected in the digestive contents of all copepod taxa (Fig. 2) except *P. parvus*, resulting in an average ingestion of 0.3 cells ind.<sup>-1</sup>. The highest numbers of cells ingested by copepods occurred in the surface stratum (0-25 m) and coincided with the maximum concentrations of *D. acuta* in the water at 8 km and 12 km offshore (Fig. 3).

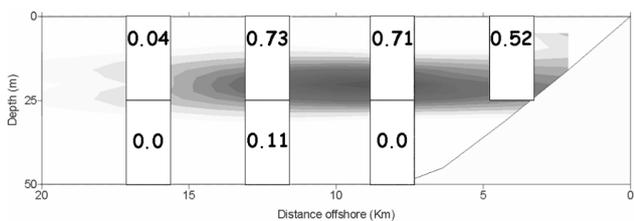
The two larger copepod species apparently ingested more *D. acuta* (average 0.71 cells ind.<sup>-1</sup>, S.E.=0.07) than the three smaller ones (average 0.05 cells ind.<sup>-1</sup>, S.E.=0.01) (Fig. 4). The most relevant results came



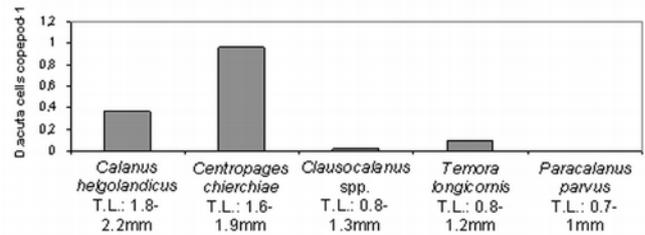
**Figure 1.** Spatial distribution of total copepods (ind. m<sup>-3</sup>) (circles in a linear scale) and of *Dinophysis acuta* (cells L<sup>-1</sup>) (shaded contours).



**Figure 2.** Micrograph of two *Dinophysis acuta* cells inside a *Centropages chierchiae* stomach.



**Figure 3.** Distribution of the number of *Dinophysis acuta* found in the digestive contents of copepods (average of cells ind.<sup>-1</sup>) for each vertical haul (rectangles). *D. acuta* distribution is presented in the background.



**Figure 4.** Average number of *Dinophysis acuta* found in the digestive contents of each studied copepod taxon (cells ind.<sup>-1</sup>). T.L. – total length of copepods.

from *C. chierchiae* since: i) it was the only species to ingest *D. acuta* at the deeper stratum (25-50 m), ii) it presented an average ingestion of more than 2 cells ind.<sup>-1</sup> at 8 km and 12 km offshore (0-25 m) (Fig. 3), with a maximum of 10 cells ind.<sup>-1</sup>.

According to Dam and Peterson (1988) the gut clearance time for calanoid copepods is 15-20 min when seawater temperature is 16 °C (this value was registered at 20 m depth, next to the *D. acuta* maxima) and when phytoplankton is abundant in the area. In this way, *C. helgolandicus* in the 0-25 m stratum showed an average ingestion rate of about 3.5 cells ind.<sup>-1</sup> h<sup>-1</sup> at 4-12 km offshore, close to the *D. acuta* bloom core, while *C. chierchiae* showed an average of 7.3 cells ind.<sup>-1</sup> h<sup>-1</sup>, with a maximum rate of 35 cells ind. 1 h<sup>-1</sup>. Regarding the three smaller copepods, their combined ingestion rate was approximately 1.1 cells ind.<sup>-1</sup> h<sup>-1</sup>.

## Discussion

According to Moita *et al.* (2006), the concentration of *D. acuta* cells (Fig. 1) was one of the highest for the region during the last decade. The abundance of the accompanying copepods (Fig. 1) was also high when compared with the observations of Villate (1991) and Bode and Alvarez-Ossorio (2004) on the NW coast of Iberia. Since the herbivores/omnivores were dominant (Sobrinho-Gonçalves 2007), the phytoplankton was apparently under a high grazing pressure in the study area.

The results presented in Fig. 3 show that the ingestion of *D. acuta* by copepods was directly associated with the concentration of this dinoflagellate and also probably related to the highest proportions in the micro-phytoplankton (>40 %). Although copepods were very abundant at 17 km offshore (Fig. 1), their ingestion values were low (Fig. 3). Thus, they probably did not control the offshore limit of the *D. acuta* bloom.

Peters and Downing (1984) stated that in calanoid copepods there is a general increase of ingestion rates

with animal size. This difference was also observed between the studied copepods, with the larger taxa ingesting more *D. acuta* cells than the smaller ones (Fig. 4). Jansen *et al.* (2006) found an average ingestion rate of 34 *Dinophysis norvegica* cells. female<sup>-1</sup> h<sup>-1</sup> by *C. helgolandicus* and considered that this animal was effectively feeding on that toxic dinoflagellate. In the present study, on the contrary, the only copepod species to approach those ingestion rates was the smaller *C. chierchiae* and not *C. helgolandicus*. The results suggest that this later species probably was passively filtering *D. acuta* together with other abundant phytoplankton. On the other hand, *C. chierchiae* seems to be able to actively feed on *D. acuta* because it is probably an omnivore species with a raptorial behaviour like the similar *Centropages typicus*. This last species has the ability to grasp large cells (>60 µm) captured by selective ambush (Caparroy *et al.* 1998).

Several studies have proven the feeding preference of calanoid copepods on large phytoplankton cells, resulting in accelerated filtering rates and metabolism (e.g. Dam *et al.* 1993; Kleppel 1993). In this way, *D. acuta* would be an attractive food particle. Our results are contrary to this assumption, as four of the five studied copepods, in particular the three dominant smaller ones, did not feed efficiently on that dinoflagellate (Fig. 4). Previous works mention that small/medium herbivore copepods like *Acartia* have a negative feeding selectivity on toxin-producing dinoflagellates (Donaghay and Small 1979; Maneiro *et al.* 2000). Thus, the ingestion rates observed for the three smaller copepods (Fig. 4) were possibly caused by a similar rejection behaviour or by a morphological inability to capture large cells with high swimming speeds like *D. acuta*. The efficient feeding of *C. chierchiae*, although restricted to the highest concentrations of *D. acuta* (Fig. 3), can be related to some kind of immunity to the protective mechanisms of *D. acuta*, namely toxins.

According to the copepod abundance reported for the study area by Sobrinho-Gonçalves (2007), the total clearance rate on *D. acuta* by the five copepods population was approximately 280 cells L<sup>-1</sup> day<sup>-1</sup> (157 by *C. chierchiae* alone). This total clearance by copepods most likely did not significantly affect the *D. acuta* bloom, since the thin layer (18-20 m depth) presented always more than 9 x 10<sup>3</sup> cells L<sup>-1</sup>. The clearance rates represented less than 3 % of daily *D. acuta* population, not considering *D. acuta in situ* division rates that can be around 0.20 day<sup>-1</sup> in NW

Iberia (Reguera *et al.* 2003). This indicates that the protective mechanisms of the dinoflagellate (possibly toxins) diverted the high grazing pressure in the area towards other more adequate and equally abundant phytoplankton. The studied copepod taxa accounted for 65 % of the meso-zooplankton, thus the thin layer bloom of *D. acuta* was probably not being directly top-down controlled. The results of the present work need future confirmation using ultra-freezing preservation of zooplankton samples preventing food regurgitation by copepods.

### Acknowledgements

This work was supported by QCAIII-POPesca MARE “Caracterização ecológica da zona costeira”.

### References

- Bode, A. & Alvarez-Ossorio, M.T. (2004). ICES J. Mar. Sci. 61: 563-571.
- Carlsson, P., Granéli, E., Finenko, G. & Maestrini, S.Y. (1995). J. Plankton Res. 17: 1925-1938.
- Dam, H.G. & Peterson, W.T. (1988). J. Exp. Mar. Biol. Ecol. 123: 1-14.
- Dam, H.G., Miller, C.A. & Jonasdottir, S.H. (1993). Deep-Sea Research II 40: 197-212.
- Jansen, S., Riser, C.W., Wassmann, P. & Bathmann, U. (2006). Harmful Algae 5: 102-112.
- Kleppel, G. (1993). Mar. Ecol. Progr. Ser. 99: 183-195.
- Maneiro, I., Frangópulos, M., Guisande, C., Fernández, M., Reguera, B. & Riveiro, I. (2000). Mar. Ecol. Progr. Ser. 201: 155-163.
- Moita, M.T., Sobrinho-Gonçalves, L., Oliveira, P.B., Palma, S. & Falcão, M. (2006). Afr. J. Mar. Sci. 28: 265-269.
- Peters, R.H. & Downing, J.A. (1984). Limnol. Oceanogr. 29: 763-784.
- Reguera, B., Garcés, E., Bravo, I., Pazos, Y., Ramilo, I. & González-Gil, S. (2003). Mar. Ecol. Progr. Ser. 249: 117-129.
- Smayda, T.J. (1997). Limnol. Oceanogr. 42: 1137-1153.
- Sobrinho-Gonçalves, L. (2007). MSc. Thesis. Univ. Lisboa, Portugal.
- Turner, J.T., Tester, P.A. & Hansen, P.J. (1998). In: Physiological Ecology of Harmful Algal Blooms, Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (eds), Springer Verlag, Berlin, pp. 453-474.
- Villate, F. (1991). J. Plankton Res. 13: 691-706.

## Discrimination and dynamics of naturally occurring mixed *Alexandrium* populations using rRNA-targeted fluorescent oligonucleotide probes

N. Touzet and R. Raine

Martin Ryan Institute, National University of Ireland Galway, Ireland, nicolas.touzet@nuigalway.ie, robin.raine@nuigalway.ie

### Abstract

Field investigations in Cork Harbour, Ireland have revealed a mixed population of *Alexandrium* comprising a non-toxic form of *A. tamarense* (West European ribotype) and a PSP toxin producing *A. minutum* (Global Clade). The latter species is now confirmed as the most probable causative organism responsible for historical occurrences of PSP contamination of shellfish in the area. The morphological similarity between these species has made comparative dynamics studies difficult when using conventional methodologies. This problem is compounded by the requirement to understand the processes involved in lifecycle transitions in order to determine the mechanisms that govern the initiation and termination phases of blooms. Here we report the use of LSU rRNA targeted taxa-specific fluorescent oligonucleotide probes for studies on comparative bloom dynamics of the two species within the same estuary. Morphological observations carried out with both culture and field samples are reported.

### Introduction

Harmful Algal Blooms (HABs) can cause a variety of severe environmental and public health concerns such as biofouling of recreational areas and seafood poisoning in humans (Shumway 1990; Hallegraeff & Hara 1995). Some species within the dinoflagellate genus *Alexandrium* produce neurotoxins that have been responsible for human intoxications after the consumption of shellfish contaminated by Paralytic Shellfish Poisoning (PSP) toxins (Kao 1993). Because of the high degree of morphological similarities between species, discrimination within the *Alexandrium* genus is not reliable when performed only by light microscopy, the method used routinely in most monitoring programmes. Moreover, the differentiation between *Alexandrium* spp. lifecycle stages in the water column currently resides in the examination of minute details such as the number of longitudinal flagella and cell dimensions (Probert 1999).

Molecular biology techniques relying on nucleotide variability within the rRNA molecule have proved reliable for the fast discrimination and quantification of *Alexandrium* spp., whose contribution to the phytoplankton community is often small (Scholin & Anderson 1994; Walsh *et al.* 1998; Galluzzi *et al.* 2004; Anderson *et al.* 2005; Gribble *et al.* 2005). Whole-cell fluorescent *in situ* hybridisation (WC-FISH) is a commonly used technique which has successfully been applied to HAB studies and which offers a compromise between morphological and genetic analyses (Miller & Scholin 1998; Anderson *et al.* 1999; John *et al.* 2005).

The use of taxa-specific rRNA-targeted probes employed in a WC-FISH assay for the simultaneous discrimination of the morphologically similar species *A. minutum* (Global clade, Lilly *et al.* 2005) and *A. tamarense* (West European ribotype, Scholin *et al.* 1995) in Cork Harbour, Ireland, is reported.

### Material and Methods

#### Culturing

Clonal cultures of *A. minutum* and *A. tamarense* were derived after isolating single cells obtained after the division of planomeiocytes originating from resting cysts collected in surface sediments from the North Channel of Cork Harbour. They were maintained in controlled conditions (f/2 medium minus silicate, 15 °C temperature, 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density and 14:10 L:D photoperiod). Compatible strains of *A. minutum* from the area were also inoculated together in 1:10 diluted culture medium (Guillard 1975) to induce sexual reproduction.

#### Probe design

D1-D2 domain LSU rDNA sequences obtained after PCR amplification and sequencing were compiled with those of other *Alexandrium* species imported from GenBank and aligned with GeneDoc. Oligonucleotide probes were designed by selecting sites displaying suitable degrees of specificity in the sequence alignment (at least two base pair mismatches with closely related species). Probes were obtained commercially (MWG Biotech) with the 5'-end labelled with CY3 (orange dye) and FITC (green dye) respectively for *A. minutum* (MinA: 5'-TTATATGGTTGATGTGG-GTGC-3') and *A. tamarense* (TamA: 5'-TAG-

GTTTTGGCTGTGGGTGA-3'). Cross-reactivity tests were carried out with a selection of *Alexandrium* species (*A. tamutum*, *A. ostenfeldii*, *A. peruvianum*, *A. andersonii*, *A. minutum* Pacific Clade and *A. tamarensis* North American ribotype) as well as other dinoflagellates commonly found in Irish coastal waters (*Scrippsiella* sp., *Glenodinium foliaceum*, *Gymnodinium splendens*, *Gonyaulax* sp. and *Prorocentrum* spp.).

#### Sample processing

Cultured *A. minutum* vegetative cells, planozygotes and resting cysts were fixed in formalin (1 h, 1 % final concentration v/v) and re-suspended after centrifugation (3000 rpm, 5 min) in 100 % ice-cold methanol to remove pigments and stabilise nucleic acids. Samples were stored at -32 °C until analysis. Field samples (2 l) were taken from Cork Harbour in summer 2005 for analysis with FISH probes, and were processed in a similar fashion as described above. Additional 50-ml taxonomic samples preserved in formalin were taken to compare the FISH-derived results with those obtained using an Utermöhl sedimentation chamber and calcofluor.

#### Hybridisation

Whole-cell FISH was carried out using a mixture of the two taxa-specific probes (1:1 volume) with a custom-made manifold according to the protocol described by Miller & Scholin (1998) with the following modifications. After hybridisation (1 h at 55 °C, buffer containing 20 % formamide), culture and field samples filtered on 13-mm diameter polycarbonate filters were removed from the manifold and placed onto slides. Before mounting coverslips, 5 µl of a mix of calcofluor (100 µg ml<sup>-1</sup>) and DAPI (3 µg ml<sup>-1</sup>) were added onto the filters as well as 10 µl of SlowFade® Light Antifade to prevent the fading of the dyes.

#### Microscopy

Membranes were examined with an epifluorescence microscope (100 W mercury lamp) with adapted short band pass fluorescent filter sets fitted on a three-way slider for the detection of the fluorescent signals of calcofluor, DAPI, FITC and CY.3. Observations were performed at ×200 magnification by scanning the entire filter and counting all positive signals. *Alexandrium* spp. quantification in taxonomic samples was also performed by using an Utermöhl sedimentation chamber. Samples were diluted with filtered seawater and aliquots (1-5 ml) containing 20 µg.ml<sup>-1</sup> of calcofluor settled in the chambers. Stained armoured dinoflagellates were examined under epifluorescence, and *Alexandrium* cell counts were carried out for each

sample, with species identification based on morphological characteristics of the thecal plates.

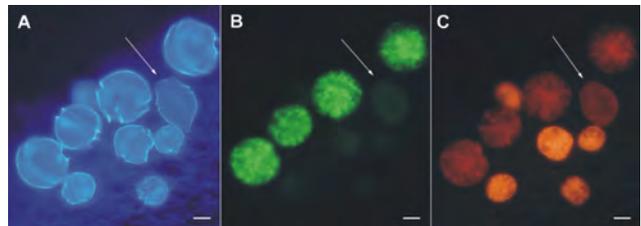
## Results

#### Probe specificity

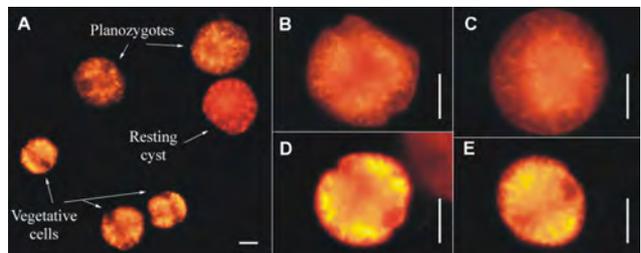
Strong fluorescent signals were observed after simultaneous hybridisation reactions of MinA and TamA with cultured vegetative cells of *A. minutum* and *A. tamarensis*. The probes did not react with any of the other *Alexandrium* and dinoflagellate species tested.

#### Application of WC-FISH to field samples

No matrix effects or apparent cross-reactivity with other phytoplankton species were observed after performing WC-FISH using MinA and TamA with natural field samples from Cork Harbour collected in July and September 2005 (Fig. 1). *A. tamarensis* was the most numerous *Alexandrium* species in the July samples (~81 %). However in September, *A. minutum* contributed to more than 98 % to the *Alexandrium* assemblage in the area. Cell concentrations derived with the WC-FISH method were compared to those obtained after using calcofluor and an Utermöhl chamber. A good correlation was found between the two methods ( $r^2=0.91$ ,  $n=20$ ) despite an underestimation of the *Alexandrium* spp. concentrations with the WC-FISH sample collection and analysis procedures by a factor of ~1.5. In the September samples, ~14 % (s.d.



**Figure 1.** Epifluorescence images of a field sample from Cork Harbour containing *A. tamarensis* and *A. minutum* cells labelled with WC-FISH probes MinA and TamA. Visualisation and discrimination of cells with calcofluor (A), FITC (B) and CY.3 (C) filter sets. Scale bars represent 10 µm. The arrow indicates a non-target *Scrippsiella* sp. cell.



**Figure 2.** WC-FISH epifluorescence images of *A. minutum* lifecycle stages labelled with MinA. Culture-derived auto-fluorescent cyst, bright vegetative cells and darker planozygotes (A). Enhanced views of planozygotes (B, C) and vegetative cells (D, E). Scale bars represent 10 µm.

3, n=5) of the *A. minutum* population in the North Channel displayed a granular cytoplasmic content with attenuated fluorescence after carrying out WC-FISH. Interestingly, those cells appeared extremely similar to planozygotes generated through sexual reproduction in the laboratory and observed after using the MinA FISH probe (Fig. 2).

## Discussion

Several molecular biology methods have been developed for the rapid detection and quantification of HAB species in the marine environment (Scholin *et al.* 2003). Despite a limited number of quantitative field studies, FISH probes have proved useful for the detection of *Alexandrium* spp. in various geographical locations (John *et al.* 2003; Anderson *et al.* 2005). MinA and TamA allowed for the fast discrimination of *A. minutum* and *A. tamarense* in culture and field samples. Calcofluor and DAPI also confirmed the probes' diagnostics and allowed the observation and enumeration of other HAB species such as *Dinophysis* spp. or *Prorocentrum* spp.

Mixed communities of *Alexandrium* spp. regularly develop in coastal waters worldwide, with sometimes the presence of both toxic and non-toxic species (McKenzie *et al.* 2004) as in Cork Harbour. The WC-FISH assay facilitated the determination of the abundances of *A. tamarense* and *A. minutum* in Cork Harbour, proving an ideal technique for future comparative population dynamics studies or as part of a phytoplankton monitoring programme. This method has also proved successful for the discrimination of cultured *A. minutum* and *A. andersonii* vegetative cells, species which occur in Irish waters (unpublished data).

The discrimination between *Alexandrium* spp. lifecycle stages in preserved samples is very complex when morphologically similar species co-occur. *A. minutum* cells were observed in field samples which showed a fluorescent pattern after WC-FISH similar to planozygotes derived from cultures. Those preliminary observations require additional data to verify that *A. minutum* planozygotes can be identified through WC-FISH. Their fast detection and enumeration may prove helpful for bloom termination studies. Future work will also consist in determining trends in the distributions and abundances of *A. tamarense* and *A. minutum* in Cork Harbour.

## Acknowledgements

Thanks to Hazel Farrell, Sandra Lyons and Aoife Ní Rathaille for assistance in field work. This work was

supported by the Higher Education Authority of Ireland (PRTL Cycle III) and by the EC 6th Framework Programme through the SEED project (GOCE-CT-2005-003375).

## References

- Anderson, D.M., Kulis, D.M., Keafer, B.A. & Berd-  
alet, E. (1999). *J. Phycol.* 35: 870-883.
- Anderson, D.M., Kulis, D., Keafer, B.A., Gribble,  
K.E., Marin, R. & Scholin, C.A. (2005). *Deep-Sea  
Research II* 52: 2467-2490.
- Galluzzi, L., Penna, A., Bertozzini, E., Vila, M.,  
Garcés, E. & Magnani, M. 2004. *Appl. Env. Micro-  
biol.* 70: 1199-1206.
- Gribble, K.E., Keafer, B.A., Quilliam, M.A., Cembella,  
A.D., Kulis, D.M., Manahan, A. & Anderson, D.M.  
(2005). *Deep-Sea Research II* 52: 2745-2763.
- Guillard, R. (1975). In: *Culture of Marine Invertebrate  
Animals*, Smith, W. & Chanley, M. (eds), Plenum  
Press, New York, pp. 29-60.
- Hallegraeff, G.M. & Hara, Y. (1995). In: *Manual  
on Harmful Marine Microalgae*, Hallegraeff,  
G.M., Anderson, D.M. & Cembella, A.D. (eds),  
UNESCO, Paris, pp. 365-371.
- John, U., Cembella, A., Hummert, C., Elbrächter, M.,  
Groben, R. & Medlin, L. (2003). *Eur. J. Phycol.*  
38: 25-40.
- John, U., Medlin, L.K. & Groben, R. (2005). *J. Plank-  
ton Res.* 27: 199-204.
- Kao, C.Y. (1993). In: *Algal Toxins in Seafood and  
Drinking Waters*, Falconer, I.R. (ed.), Academic  
Press, London, pp. 75-86.
- Lilly, E.L., Halanych, K.M. & Anderson, D.M. (2005).  
*Harmful Algae* 4: 1004-1020.
- McKenzie, L., de Salas, M., Adamson, J. & Beuzen-  
berg, V. (2004). *Harmful Algae* 3: 71-92.
- Miller, P.E. & Scholin, C.A. (1998). *J. Phycol.* 34:  
371-382.
- Probert, I.P. (1999). Ph.D. Thesis. University of West-  
minster, London.
- Scholin, C.A. & Anderson, D.M. (1994). *J. Phycol.*  
30: 744-754.
- Scholin, C.A., Hallegraeff, G.M. & Anderson, D.M.  
(1995). *Phycologia* 34: 472-485.
- Scholin, C., Vrieling, E., Peperzak, L., Rhodes, L. &  
Ruble, P. (2003). In: *Manual on Harmful Marine  
Microalgae*, Hallegraeff, G.M., Anderson, D.M. &  
Cembella, A.D. (eds), UNESCO, Paris, pp. 131-163.
- Shumway, S.E. (1990). *J. World Aquacult. Soc.* 21:  
65-104.
- Walsh, D., Reeves, R.A., Saul, D.J., Gray, R.D.,  
McKenzie, L., Bergquist, P.R. & Bergquist, P.L.  
(1998). *Biochem. System. Ecol.* 26: 495-509.

## Temporal changes in microcystin-producing and non-microcystin-producing *Microcystis* populations of a Japanese lake

Mitsuhiro Yoshida, Takashi Yoshida, Yukari Takashima, Naohiko Hosoda and Shingo Hiroishi  
Department of Marine Bioscience, Fukui Prefectural University, Obama, Fukui 917-0003, Japan,  
s0494004@s.fpu.ac.jp

### Abstract

Temporal changes in microcystin-producing and non-microcystin-producing *Microcystis aeruginosa* populations were examined in Lake Mikata, Japan. To identify potentially microcystin-producing and non-producing genotypes, we performed a genotypic analysis based on the sequence diversity of the 16S-23S rDNA internal transcribed spacer (ITS). Microcystin-producing genotypes were detected in different samples, when the relative abundance of the subpopulation with a microcystin synthetase gene to the total *M. aeruginosa* was relatively high. Moreover, the dynamics of microcystin-producing and non-producing populations showed a significant correlation with nitrate concentration in the lake. Our data suggest that multiple ecotypes, adapted to different ecological parameters, may coexist within the *M. aeruginosa* community.

### Introduction

Cyanobacteria frequently form blooms in eutrophic lakes, ponds and reservoirs. A major bloom-forming component, the genus *Microcystis*, can produce a potent hepatotoxin called microcystin. There have been several reports of deaths in wild and domestic animals as well as humans due to this acute poisoning that causes massive hepatic hemorrhage (Beasley *et al.* 1989; Pouria *et al.* 1998).

Previously, individual species of *Microcystis* were determined on the basis of morphological features, such as cell size and cell arrangement in colonies (Komárek 1991); however, these features change during cultivation. Recently, *Microcystis* species have been combined as *M. aeruginosa* following bacteriological taxonomic criteria (Otsuka *et al.* 2001). Both microcystin-producing and non-microcystin-producing strains may be isolated from the same water source. Several attempts have been made to link microcystin production to morphological characteristics or molecular phylogeny (Otsuka *et al.* 1999; Tillett *et al.* 2001); however, none of these revealed any correlation with toxicity. Therefore, the knowledge of the composition and variability of microcystin-producing and non-producing strains in the environment is very limited.

In this study, we investigated the temporal changes in microcystin-producing and non-microcystin-producing *M. aeruginosa* populations in Lake Mikata. ITS genotyping was employed for identification of the genotypes.

### Materials and Methods

Water samples were collected from Lake Mikata in September and October 2004, and August and September 2005. A 100-ml sample was sonicated gently, and the cells were harvested by centrifugation at 14,400 g for 10 min. DNA was extracted and purified using the methods described by Yoshida *et al.* (2003).

Each DNA extract was used as PCR template for the ITS clone library analysis. A partial ITS sequence of about 360 bp was obtained by the PCR amplification using oligonucleotide primers MITS-F (5'-AAG GGA GAC CTA ATT CVG GT-3') and MITS-R (5'-TTG CGG TCY TCT TTT TTG GC-3'). These primers were designed to be *M. aeruginosa*-specific based on a comparison of cyanobacterial ITS sequences available in the DDBJ/EMBL/GenBank databases. The primer sequence specificities were confirmed by using a BLAST search of the DDBJ/EMBL/GenBank databases. The ITS clone library analysis was conducted as described by Yoshida *et al.* (2005).

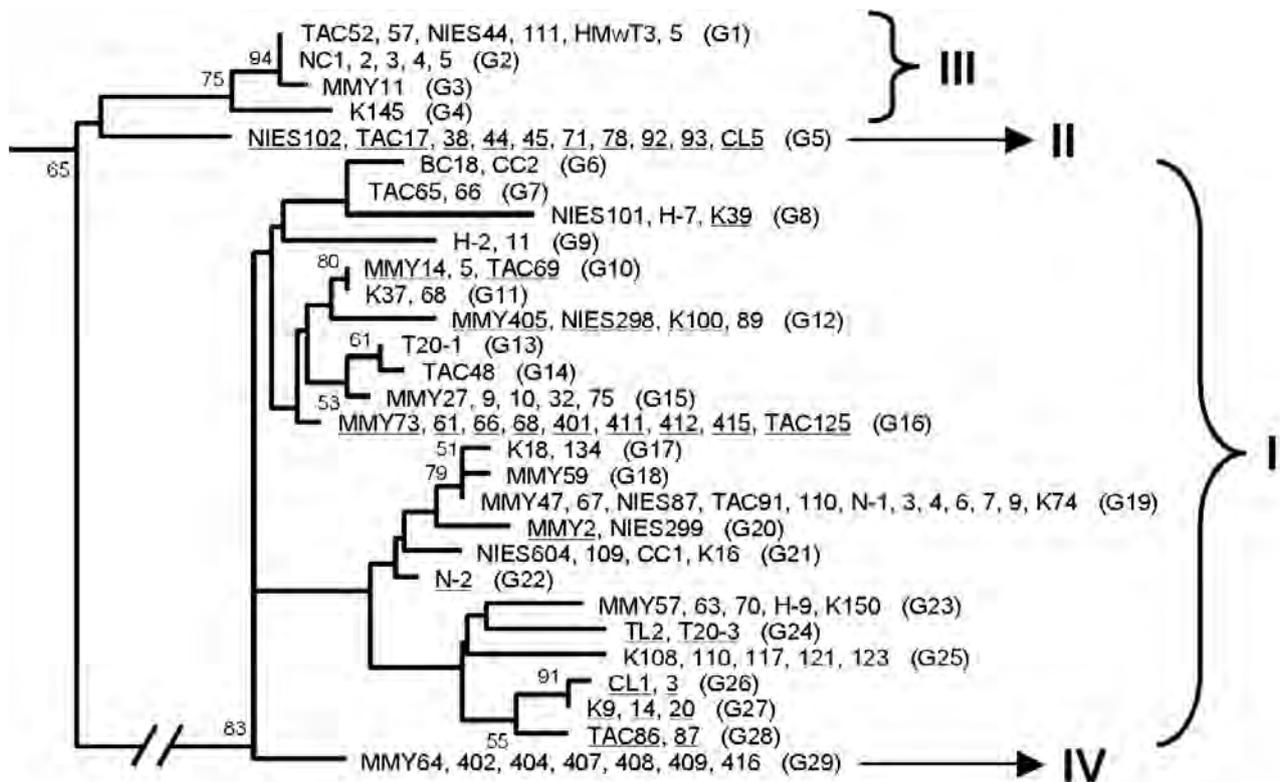
The nucleotide sequences were automatically aligned using GENETYX-WIN version 5.0 (Software Development) and subsequently corrected manually. The phylogenetic tree was inferred from 317 unambiguously aligned bases by the neighbor-joining method with GENETYX-WIN ver. 5. One thousand bootstrap trials were performed.

### Results and Discussion

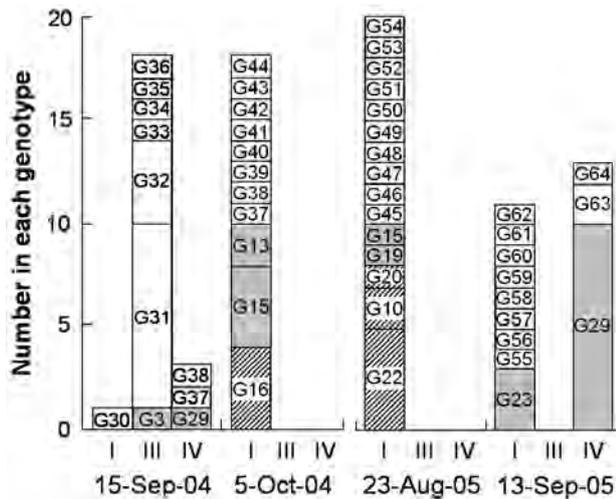
To recognize a genetic difference between potential microcystin and non-microcystin producers, a phylogenetic analysis and genotyping of the ITS sequenc-

es of *M. aeruginosa* isolates were conducted. Fig. 1 shows a neighbor-joining phylogenetic tree of the 29 different ITS genotypes (G1-G29) observed in this study with near complete ITS sequences obtained for all *M. aeruginosa* isolates, and for other *M. aeruginosa* sequences already in the database. Four major sequence clusters are indicated. Cluster designation is based on data from Otsuka *et al.* (1995). Clusters I to III have been previously described (Otsuka *et al.* 1995), while cluster IV is novel. All sequences of cluster IV were from Lake Mikata isolates. All the strains of cluster II were microcystin producers, and all strains of clusters III and IV were non-producers. Cluster I included both microcystin-producing and non-producing strains. With the exception of the three genotypes (G8, G12, and G20) comprising three, four, and two strains, respectively, all genotypes in this phylogenetic cluster corresponded with microcystin production. For example, G16 consisted of 11 microcystin-producers, and G19 of 14 non-producers. This indicates that the genotyping based on ITS sequences is effective for understanding *M. aeruginosa* diversity at the intra-specific level, as described by Otsuka *et al.* (1995).

A newly developed *M. aeruginosa*-specific PCR primer set, MITS, was used to amplify ITS fragments from samples collected from Lake Mikata, and ITS clone libraries were produced on four sampling dates (15 September and 5 October 2004, and 23 August and 13 September 2005). The ITS clones from each clone library were subjected to sequence comparison analysis and, in addition to information about the ITS genotypic diversity in *M. aeruginosa* isolates (Fig. 1), we investigated temporal changes in the composition of the microcystin-producing and non-producing genotypes belonging to the different ITS-defined clusters (Fig. 2). The distribution of the clones belonging to the different ITS clusters within *M. aeruginosa* communities of Lake Mikata (Fig. 2) revealed first that September 2005 was characterized by high dominance of clones belonging to cluster III. All clones obtained in October 2004 and August 2005 belonged to cluster I. In September 2005, clones belonging to clusters I and IV were found in very similar proportions. Only cluster I was found throughout these sampling months and showed high variation in regard to the sampling months. The observations indicate that the composition of the ITS clusters reveal temporal variability.



**Figure 1.** Neighbor-joining phylogenetic tree of the partial ITS sequences from *M. aeruginosa* strains. The outgroup (*Synechocystis* sp. PCC6803) is omitted. Only bootstrap values of > 50 % are indicated at the nodes of the tree. The underlined strains indicate microcystin producers. The letters in parentheses, following strain numbers, refer to genotype assignment as indicated by G followed by a number.



**Figure 2.** Temporal changes in the number of microcystin-producing and non-microcystin-producing genotypes of the different clusters defined by the phylogenetic analysis and genotyping of the ITS sequences from *M. aeruginosa* in Lake Mikata. The fractions 'G followed by a number' represent each genotype assigned, as shown in Fig. 1. The number within each genotype is based on the ITS clone library analysis. Genotypes in the shaded and gray fractions indicate potential microcystin and non-microcystin producers, respectively (cf. Fig. 1). Genotypes in the white fractions did not correspond to any known genotype and it was therefore unclear whether microcystin was produced.

Of the 47 different *M. aeruginosa* genotypes retrieved, seven and five belonged to ITS clusters III and IV, respectively, comprising all non-microcystin-producing strains (Fig. 2). For cluster I four genotypes corresponded to G10, G16, G20, and G22, derived from the potential microcystin producers; and four corresponded to G13, G15, G19, and G23, derived from non-microcystin producers. The others did not correspond to any known genotype, and it was unclear whether microcystin was produced. The distinct microcystin-producing genotypes were found in different samples (October 2004 and August 2005).

We have developed a quantitative real-time PCR assay, targeting the phycocyanin intergenic spacer and the microcystin synthetase gene *mcyA*, and this approach was applied on the same samples for relative quantification of the abundance of *M. aeruginosa* and the microcystin subpopulation, respectively (Yoshida *et al.* 2007). The proportion of the *mcyA* subpopulation to the total *M. aeruginosa* varied from 0.5 to 6 % in 2004 and from 35 to 2% in 2005. When the relative abundance of the *mcyA* subpopulation was high, microcystin-producing genotypes were also detected (Fig. 2). In addition, it was noticed that the dynamics of microcystin-producing and non-producing genotypes was correlated with nitrate concentration

(Yoshida *et al.* 2007). Thus, our data suggest that multiple ecotypes, which are adapted to different ecological parameters, may coexist within the *M. aeruginosa* community. In the future, clarification is needed to determine whether there is a difference in the growth response and, if so, whether it is linked to toxicity or phylogeny. From Lake Mikata, we recently isolated a cyanophage that specifically infected only a microcystin-producing *M. aeruginosa* strain (Yoshida *et al.* 2006). Therefore, biological factors may also cause seasonal shifts between microcystin-producing and non-producing *M. aeruginosa* populations.

## Acknowledgements

This study was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (JSPS Research Fellowships for Young Scientists), Japan.

## References

- Beasley, V.R., Cook, W.O., Dahlem, A.M., Hooser, S.B., Lovell, R.A. & Valentine, W.M. (1989). *Food Anim. Pract.* 5: 345-361.
- Komárek, J. (1991). *Arch. Hydrobiol., Suppl.* 92 (Algol. Stud. 64): 115-127.
- Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. & Watanabe, M.M. (2001). *Int. J. Syst. Evol. Microbiol.* 51: 873-879.
- Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. & Watanabe, M.M. (1999). *FEMS Microbiol. Lett.* 172: 15-21.
- Pouria, S., de Andrade, A., Barbosa, J., Cavalcanti, R.L., Barreto, V.T.S., Ward, C.J., Preiser, W., Poon, G.K., Neild, G.H. & Codd, G.A. (1998). *Lancet* 352: 21-26.
- Tillett, D., Parker, D.L. & Neilan, B.A. (2001). *Appl. Env. Microbiol.* 67: 2810-2818.
- Yoshida, T., Yuki, Y., Lei, S., Chinen, H., Yoshida, M., Kondo, R. & Hiroishi, S. (2003). *Microb. Env.* 18: 16-23.
- Yoshida, M., Yoshida, T., Takashima, Y., Kondo, R. & Hiroishi, S. (2005). *Env. Toxicol.* 20: 229-234.
- Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N. & Hiroishi, S. (2007). *FEMS Microbiol. Lett.* 266: 49-53.
- Yoshida, T., Takashima, Y., Tomaru, Y., Shirai, Y., Takao, Y., Hiroishi, S. & Nagasaki, K. (2006). *Appl. Env. Microbiol.* 72: 1239-1247.

## 6. MITIGATION



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Potential role of clay in mitigating Chesapeake Bay algal blooms

E.F. Brownlee<sup>1</sup>, S.G. Sellner<sup>2</sup> and K.G. Sellner<sup>3</sup>

<sup>1</sup>Hood College, 401 Rosemont Avenue, Frederick, MD, USA 21701, efb2@hood.edu, <sup>2</sup>Morgan State University Estuarine Research Laboratory, St. Leonard, MD, USA 20678, sgsellner@moac.morgan.edu, <sup>3</sup>Chesapeake Research Consortium, 645 Contees Wharf Road, Edgewater, MD, USA 21037, sellnerk@si.edu

### Abstract

Because of increasing concern for algal blooms in Chesapeake Bay and proximal coastal bays, laboratory studies were undertaken to examine the removal of several bloom species through the addition of treated kaolin clay. *Prorocentrum minimum*, *Chattonella subsalsa* and a small coccoid cyanobacterium were grown in the laboratory and exposed to 0.9 g clay L<sup>-1</sup>. *In vivo* fluorescence (IVF) was measured on 4 replicates for each taxon (control and treated) before clay additions, 2.5 h after clay addition, and 4 d later. There was a significant decrease in IVF in all clay treatments with largest reductions noted for *Prorocentrum* and *Chattonella* (99 % and 92 %, respectively) within 2.5 h of the addition; there was no further decline in IVF over the next four days. For the cyanobacterium, clay was not as effective, removing only 61 % and 38 % of total cells over the 4 d when initial densities were 10<sup>9</sup> and 10<sup>8</sup> cells L<sup>-1</sup>, respectively. These results suggest that cation polymer-treated kaolin may be an effective mitigation strategy for flagellates common to Bay blooms whereas coccoid cyanobacteria may persist following clay additions.

### Abstract

Because of increasing concern for algal blooms in Chesapeake Bay and proximal coastal bays, laboratory studies were undertaken to examine the removal of several bloom species through the addition of treated kaolin clay. *Prorocentrum minimum*, *Chattonella subsalsa* and a small coccoid cyanobacterium were grown in the laboratory and exposed to 0.9 g clay L<sup>-1</sup>. *In vivo* fluorescence (IVF) was measured on 4 replicates for each taxon (control and treated) before clay additions, 2.5 h after clay addition, and 4 d later. There was a significant decrease in IVF in all clay treatments with largest reductions noted for *Prorocentrum* and *Chattonella* (99 % and 92 %, respectively) within 2.5 h of the addition; there was no further decline in IVF over the next four days. For the cyanobacterium, clay was not as effective, removing only 61 % and 38 % of total cells over the 4 d when initial densities were 10<sup>9</sup> and 10<sup>8</sup> cells L<sup>-1</sup>, respectively. These results suggest that cation polymer-treated kaolin may be an effective mitigation strategy for flagellates common to Bay blooms whereas coccoid cyanobacteria may persist following clay additions.

### Introduction

Phytoplankton at high densities can discolour water, produce noxious odours and taste, yield poor quality food for fish, produce toxins for organisms in the surrounding area or that drink it, and fuel hypoxia/anoxia. A major focus has been to explore several

approaches to limit phytoplankton blooms, either through inhibition of phytoplankton growth or removal of phytoplankton from the water column. Asian scientists have studied the ability of clay to remove phytoplankton from the water column, successful along the Korean coast and Chinese lakes (Na *et al.* 1996; Pan *et al.* 2006). More recently, a number of other groups have explored clay control in the laboratory, mesocosms, and some field sites (Choi *et al.* 1998; Kim *et al.* 2001; Sengco *et al.* 2001, 2004; Archambault *et al.* 2003, 2004; Xihua and Zhiming 2003; Pierce *et al.* 2004, Yu *et al.* 2004; Hagström and Granéli 2005), with promising results for cell and toxin removal except for the potential negative effects on suspension-feeding bivalves (Shumway *et al.* 2003; Archambault *et al.* 2004). Because of increasing concerns over the effects of phytoplankton blooms on organisms in Chesapeake Bay, laboratory studies were undertaken to examine the potential for removal of regional bloom-forming phytoplankters *Prorocentrum minimum*, *Chattonella subsalsa* and a coccoid cyanobacterium from the water column by the addition of a treated kaolin clay. It is important to find readily available natural materials to control excess algal blooms in the region, as this is a common problem in increasingly nutrient-enriched systems like the Bay and nearby coastal bay systems.

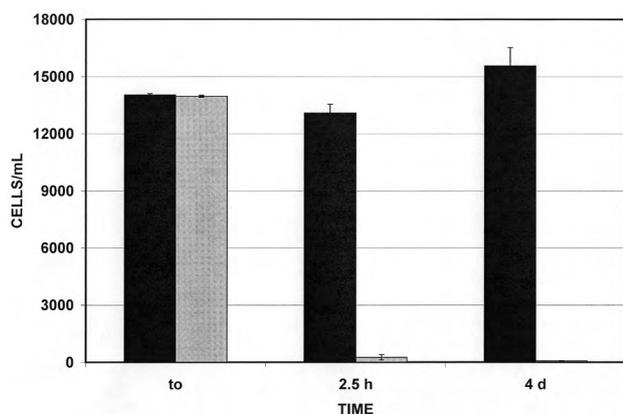
### Methods

Flocculation experiments were performed in 60-mL test tubes using four tubes for each taxon (*P. mini-*

*mum*, *C. subsalsa*, and a coccoid cyanobacterium), four tubes of each phytoplankton + clay (cation polymer-treated kaolin  $0.9 \text{ g}\cdot\text{L}^{-1}$ ), four tubes for media + clay, and four tubes for each type of medium. For each type of phytoplankton, 40 mL of phytoplankton diluted to bloom levels were added to the tubes designated phytoplankton and phytoplankton + clay; 40 mL of medium were added to tubes labeled media and media + clay. For the cyanobacterium, a high culture density and a lower density closer to field levels (bloom density found in summer waters) were used. For each tube, initial *in vivo* fluorescence (IVF) readings were performed. One ml of clay slurry (clay + deionized water) was added drop-by-drop to each of the phytoplankton + clay and media + clay tubes. The tubes were placed in an incubator at  $19 \text{ }^\circ\text{C}$  for 2.5 h. After this time, each tube was gently stirred with a thin spatula to resuspend settling but non-pelletized phytoplankton (the clay pellet at the bottom was barely swirled), capped, and the fluorescence read. The tubes, with caps loosened, were returned to the incubator and the previous steps were repeated after 4 d. After the experiment, the clay and clay + algae pellets were examined at 100X under light and fluorescent microscopes to note any differences in clay-algae associations. Regressions between IVF and cell numbers were derived and used for estimating cell densities in the experiments.

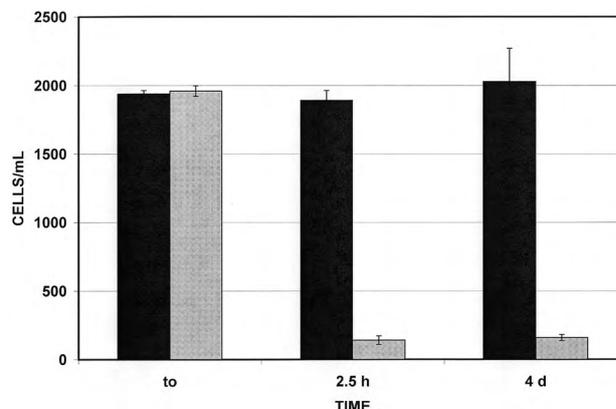
## Results

For *Prorocentrum*, the addition of clay resulted in almost complete removal of the cells after 2.5 h and after 4 d, IVF did not increase (Fig. 1). Untreated *P. minimum* increased from  $14.0$  to  $15.5 \times 10^3 \text{ cells}\cdot\text{mL}^{-1}$  while clay-treated algae declined from  $14.0 \times 10^3$  to  $60 \text{ cells}\cdot\text{mL}^{-1}$ .



**Figure 1.** *Prorocentrum minimum* in suspension with (gray bar) and without kaolin addition (black bar).

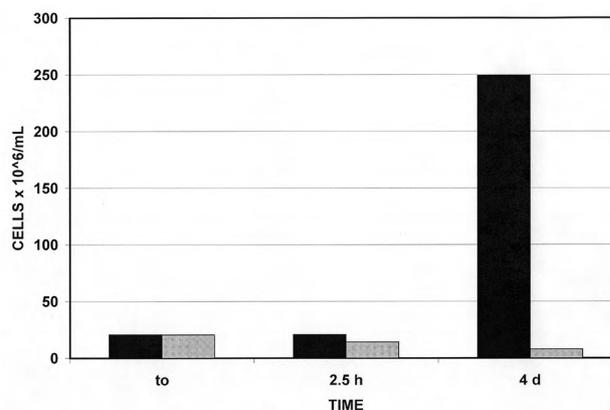
A similar pattern was noted for the raphido-phyte *C. subsalsa*. The alga was almost completely removed after 2.5 h when clay was added (Fig. 2), with a 93 % drop in IVF. Abundance of treated algae dropped from  $19$  to  $1.7 \times 10^2 \text{ cells}\cdot\text{mL}^{-1}$ , while untreated algae remained constant.



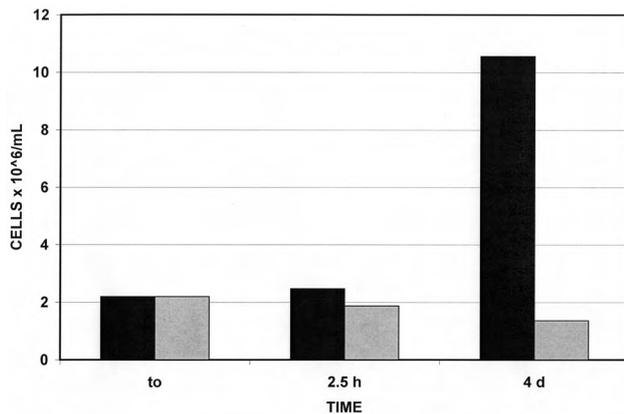
**Figure 2.** *Chattonella subsalsa* in suspension with (gray bar) and without kaolin addition (black bar).

Clay was not as effective at removing cyanobacteria when the prokaryote was present at high concentrations. Clay addition resulted in a slight but significant decrease (ANOVA,  $p < 0.001$ ) of IVF at 2.5 h (13 %) and a larger decrease (33 %) after 4 d (Fig. 3). The abundance of treated cells dropped from  $21$  to  $8 \times 10^6 \text{ cells}\cdot\text{mL}^{-1}$ . At lower cyanobacteria concentrations, clay was more effective in removing the cell from suspension. With clay treatment, there was a steady, significant decrease in IVF of 71 % (Fig. 4) and densities declined from  $2.2$  to  $1.3 \times 10^6 \text{ cells}\cdot\text{mL}^{-1}$ .

There were clear qualitative differences in sedimented clay ‘pellets’ in the treatments. Pellets in tubes with clay plus phytoplankton were hard and solid while particles in clay-only tubes were powdery and easily disturbed. Microscopy indicated that sedimented material in clay-phytoplankton treatments



**Figure 3.** The effects of kaolin addition on high densities of picocyanobacteria ( $21 \times 10^6 \text{ mL}^{-1}$  initially). Gray and black bars reflect with and without clay, respectively.



**Figure 4.** Kaolin effects on low picocyanobacteria abundances ( $2.2 \times 10^6 \text{ mL}^{-1}$  initially). Gray and black bars reflect with and without clay, respectively.

were large clumps of cells and clays (see [www.chesapeake.org/clays](http://www.chesapeake.org/clays) for plates).

### Discussion

Results of this study suggest that use of clay in controlling blooms is promising for routine use in short-residence time estuaries. It appears that for *P. minimum*, the ‘mahogany tide’ bloom former in late spring in the tidal Chesapeake and its tributaries, and for *C. subsalsa*, a summer bloom-forming taxon of Maryland and Virginia coastal bays, the algae are rapidly stripped from the water column and do not re-emerge, at least over 4 d, from the flocs to reconstitute blooms. Hence, clays are likely to be effective in removing these algae from the over-lying waters where they could reduce light penetration for early season growth of young submerged grasses (see Gallegos and Bergstrom 2005) or later season spreading beds in the littoral zones of the Bay and coastal bays.

For more abundant, small-celled cyanobacteria blooms, concentrations of clay relative to total cell number appear most critical to the removal of the prokaryote from suspension. This is not unrealistic and likely holds true for the eukaryote bloom formers as well: if the number of clay particles is saturated before aggregating with all available cells, some portion of the bloom-forming population will remain suspended (also seen by Sengco *et al.* 2005 for *Prymnesium parvum*), thereby insuring continuation of the bloom and potential regrowth to the high densities observed prior to clay addition.

Of concern are the effects of settled clay and phytoplankton on benthic oxygen concentrations and toxin transfer (Shumway *et al.* 2003; Archambault *et al.* 2004). The former condition, high oxygen demand, might be fostered by high bacterial activity

that could be associated with harder to erode settled, compacted aggregates, not unlike those documented by coagulated aggregates derived by Beaulieu *et al.* (2005). This is of particular concern for *C. subsalsa* as the raphidophyte is a common summer bloom former for dead-end canals of the rapidly developing coastal bays of Delaware and Maryland. The canals are low energy, poorly flushed systems and increasing supplies of clay-associated labile *C. subsalsa* to already organically-rich sediments in these areas could potentially foster even more problems, including anaerobic conditions and sulphidic poisonings of benthic and pelagic living resources. Additionally, a bloom of another similar species from these canals, *Chattonella* cf. *verruculosa* (possibly ‘*Chloromorium toxicum*’, Tomas 2005), was associated with brevetoxins in the field (Bourdelaïs *et al.* 2002) and killing *Gambusia* in laboratory bioassays (Tomas *et al.* 2002). If this population responds in a similar manner as *C. subsalsa* to clay addition, sedimentation of these cells in the area could exacerbate the low oxygen problems by exposing the benthos to these neuro- and ichthyotoxins.

Field assays in controlled settings, e.g., limnocorrals/mesocosms, should be considered as a next step in ascertaining potential success of local clays (or other particles) in regional bloom mitigation.

### Acknowledgements

The authors thank the Biomonitoring staff and M. Bundy of MSU for use of their equipment, W. Coats and S. Ribblett (Smithsonian Environmental Research Center) for the *Prorocentrum* culture, and D. Hutchins (University of Delaware) for the *Chattonella* culture. W. Coates is acknowledged for fluorescence photography of the cyanobacterium. Our thanks are also extended to M. Sengco (Woods Hole Oceanographic Institution) for the cationic polymer treated kaolin clay along with his helpful suggestions.

### References

- Archambault, M.-C., Bricelj, V.M., Grant, J. & Anderson, D.M. (2004). *Mar. Biol.* 144: 553-565.
- Archambault, M.-C., Grant, J. & Bricelj, V.M. (2003). *Mar. Ecol. Prog. Ser.* 253: 97-109.
- Beaulieu, S.E., Sengco, M.R. & Anderson, D.M. (2005). *Harmful Algae* 4: 123-138.
- Bourdelaïs, A.J., Tomas, C.R., Naar, J., Kubanek, J. & Baden, D.G. (2002). *Environ. Health Perspect.* 110: 465-470.
- Choi, H.G., Kim, P.J., Lee, W.C., Yun, S.J., Kim, H.G. & Lee, H.J. (1998). *J. Korean Fish. Soc.* 31: 109-113.

- Gallegos, C.L. & Bergstrom, P.W. (2005). *Harmful Algae* 4: 553-574.
- Hagström, J.A. & Granéli, E. (2005). *Harmful Algae* 4: 249-260.
- Kim, C.S., Bae, H.M. & Cho, Y.C. (2001). *Algae* 16: 67-73.
- Na, G., Choi, W. & Chun, W. (1996). *J. Aquacult.* 9: 239-245.
- Pan, G., Zou, H., Chen, H. & Yuan, X. (2006). *Environ. Pollut.* 141: 206-212.
- Pierce, R.H., Henry, M.S., Higham, C.J., Blum, P., Sengco, M.R. & Anderson, D.M. (2004). *Harmful Algae* 3: 141-148.
- Sengco, M.R., Hagström, J.A., Granéli, E. & Anderson, D.M. (2005). *Harmful Algae* 4: 261-274.
- Sengco, M.R., Li, A., Tugend, K., Kulis, D. & Anderson, D.M. (2001). *Mar. Ecol. Prog. Ser.* 210: 41-53.
- Shumway, S.E., Frank, D.M., Ewart, L.M. & Ward, E.J. (2003). *Aquacult. Res.* 34: 1391-1402.
- Tomas, C.R. (2005). Abstract, 3rd Symp. on Harmful Algae in the U.S. (MBARI, Moss Landing, CA), p. 63.
- Tomas, C.R., Ono, C., Yoshimatsu, S. & Göbel, J. (2002). In: *Harmful Algae 2002 Xth HAB*, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), FIO and UNESCO, St. Petersburg, FL, USA, pp. 425-427.
- Xihua, C. & Zhiming, Y. (2003). *Yingyong Shengtai Xuebao* 14: 1169-1172.
- Yu, Z., Sengco, M.R. & Anderson, D.M. (2004). *J. Appl. Phycol.* 16: 101-110.

## Minimizing economical losses with the help of “real-time” HAB surveillance

Edna Granéli\*, Christina Esplund, Catherine Legrand, Hanna Franzén and Christina Granéli  
Marine Sciences Dept., Kalmar University, SE-39182 Kalmar, Sweden, \*edna.graneli@hik.se

*Volunteers:* Allan Bachér, Ann-Margreth Bachér, Anders Barkewall, Solweig Bejerstrand, Stefan Carlsson, Sigurd Figoni, Birgitta Gerlofsson, Hans Gerlofsson, Bror Gustavsson, Henrik Hedén, Pelle Holmberg, Eva Hultenheim, Johan Hultenheim, Harald Janson, Viveka Johansson, Örn Johansson, Charlotte Karlsson, Stefan Lundqvist, Emmelie Nilsson, Carl Gustav Olderius, Arne Runsten, Anna Sandström, Nanna Serrander, Berit Sundström, Lasse Sörenson, Frida Villius, Sven Åhlin.

### Abstract

Cyanobacterial blooms covering almost the entire Baltic Sea is a normal feature in July-August. For the tourism industry at the island Öland, SE Sweden, the economical losses during the summer 2005 amounted to 16-21 million euros. Because remote sensing satellite images have a low resolution, it appears that all Öland beaches are covered with decomposing algae. In reality, these blooms rarely affect the western side of the island. To more accurately assess accumulation, we instituted intensive daily real-time surveillance of the algal accumulation on the beaches, by volunteers, who were trained at the University of Kalmar. By transmitting the resulting algal accumulation information to the public within hours, we showed that: 1) although, in the SMHI remote sensing images Öland is surrounded by the blooms, these blooms did not reach the beaches, 2) that the real-time warning system could boost public confidence in the local water quality and be used in advertising campaigns to increase tourism in Öland.

### Scientific background

In Sweden, satellite pictures showing cyanobacterial blooms covering almost the entire Baltic Sea during July-August, are daily placed by the Swedish Meteorological and Hydrological Institute in their homepage ([www.smhi.se](http://www.smhi.se)). The media report these events in a very negative way scaring tourists from the beaches. The economical tourism losses, for the island of Öland, SE Sweden, during the 2005 summer amounted to 16-21 million euros. Because of the low resolution provided by these satellite images it appears that all the beaches on Öland are covered by the blooms.

In reality, the blooms rarely affect the western side of the island, and even the east side, when affected, can within hours be suitable for swimming as wind and currents rapidly move the blooms offshore.

Since the problem with increased algal blooms will probably remain for years to come, we started a pilot project in order to decrease the economical losses due to unnecessary avoidance of the beaches by tourists. This was accomplished by an intensive daily real-time algal surveillance of the beaches in our homepage ([www.hik.se/alg](http://www.hik.se/alg)). Several types of graphics, the latest posted at 9.00 a.m. each day, indicated when the beaches were “algal free”. It was hoped that this information would generate sufficient confidence for the tourists to return to the beaches.

### How was the daily HAB surveillance done?

Volunteers were recruited in May (pensioners, youth, camping-place workers, etc.) and received the necessary training in simple research techniques at the University of Kalmar.

Daily, between 6 and 7:30 a.m. in July and August 2006, at least one volunteer per beach took photos of the beaches and measured physical data (salinity, water temperature, cloudiness and winds). These data in addition to the following 2 photographs:

- 1) of a water sample in a clear bottle ( $\approx 1$  liter)
- 2) of a filter containing the algae corresponding to 1 liter of seawater placed beside a colour scale, ranging from low to high densities of cyanobacteria

were sent to the coordinating scientist at Kalmar University.

The scientists, after validating the data placed, before 9.00 am, the photographs on the homepage, ([www.hik.se/alg](http://www.hik.se/alg)), and wrote a recommendation if the beach was fit for swimming (Fig. 1).

Every third day, the volunteers also sampled water for analyses of nutrients ( $\text{NO}_3$ ,  $\text{NH}_4$ ,  $\text{PO}_4$  and  $\text{SiO}_3$ ), chlorophyll and cyanobacteria pigments (phycocyanin and phycoerythrin). The volunteers preserved samples for phytoplankton counts; took samples and added the reagents for ammonia analyses and filtered water for later chlorophyll analyses.

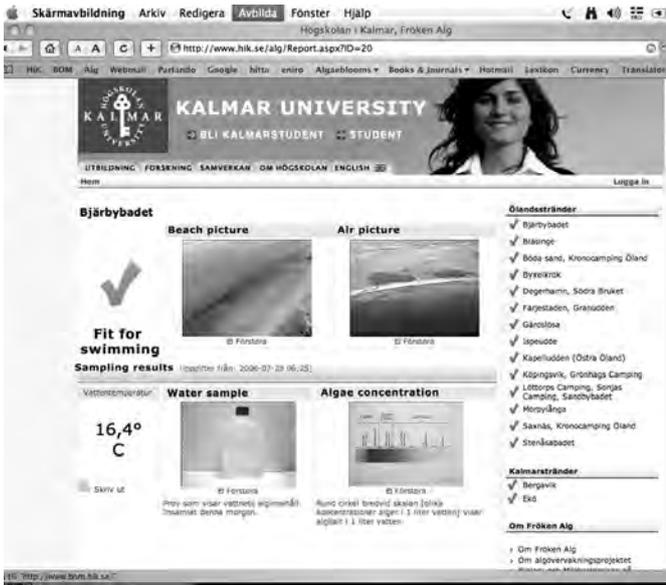


Figure 1. A homepage for each beach was updated daily at [www.hik.se/alg](http://www.hik.se/alg).

Also ten inverted microscopes equipped with a digital camera were placed around the island, so the volunteers could, in case of a bloom, send photos of the algae to University of Kalmar to count and identify.

**How did the real-time algal surveillance work?**

As anticipated, we could show that the Öland beaches were not affected by cyanobacteria although heavy cyanobacteria blooms covered the entire Baltic proper from the first week and throughout July 2006 (Fig. 2).

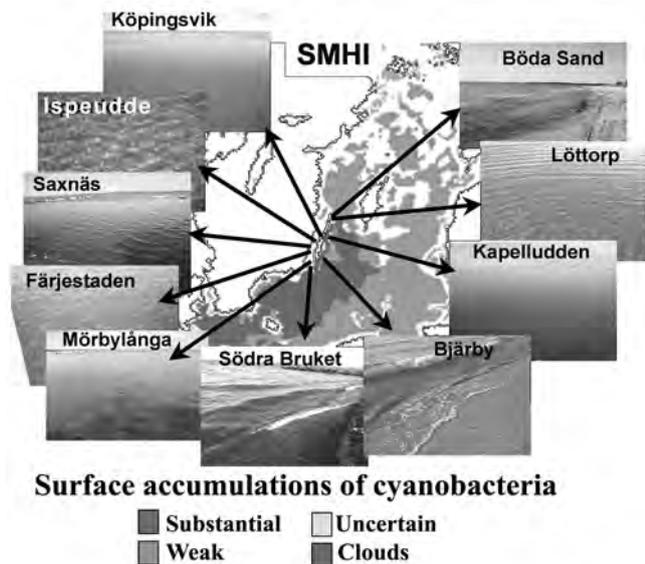


Figure 2. Pictures showing algae-free beaches at the same date that a remote sensing image shows substantial blooms around Öland. SMHI reports: [http://www.smhi.se/weather/baws\\_ext/balt/2006/BAWSDag\\_0607.htm](http://www.smhi.se/weather/baws_ext/balt/2006/BAWSDag_0607.htm)

Even when, on few occasions, some of the beaches were reached by cyanobacteria, we could very rapidly, with the help of the microscope photographs and quick transport of algal samples for us to count, inform the public that the toxin-producing species *Nodularia spumigena* was in low/undetectable cell densities, and that closing of the beach was not necessary. The major part of these blooms were made up by *Aphanizomenon* sp. and *Anabaena* sp. The highest cell densities for *Nodularia spumigena* (at Byxelkrok July 10) were still far below harmful levels (Fig. 3).

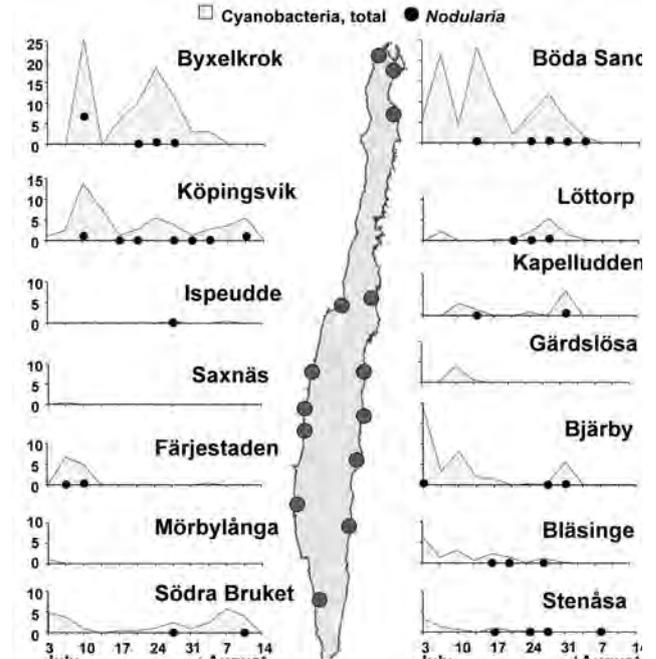


Figure 3. Cell counts of cyanobacteria for the beaches on Öland July-August 2006.

The project has also generated a large amount of data on the input of N and P to this coastal area which can be used for planning of wetlands to reduce N and P input to these coastal waters, etc.

**Impact of the project on stake holders**

During July 2006, 1000 - 1500 persons/day visited the algal monitoring project home page, [www.hik.se/alg](http://www.hik.se/alg). The highest numbers were on Mondays, declining during the week to the lowest number on Saturdays (Fig. 4). On the mobile service [mobil.hik.se](http://mobil.hik.se), there were 2618 page visits during the same period. For the telephone answering service "Miss Algae" (+49 480 446010) 1569 calls took place from 1 July to 15 August.

The project received a huge amount of positive publicity in the media, both regionally and nationally. The "catastrophic" headlines that during the summer of 2005 scared the tourists away turned to very positive during the summer of 2006 (Figs 5, 6).

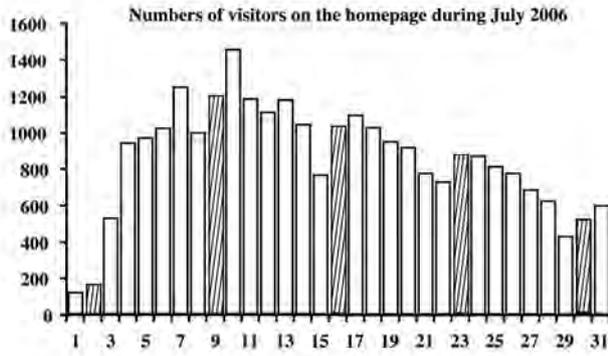


Figure 4. 1000-1500 visited www.hik.se/alg daily during July 2006.

**The aim and the most important results of the project**

One of the aims of the project was to recruit volunteers and train them in simple research techniques. The 25 successfully recruited and trained volunteers were able to conduct a daily surveillance and inform the public concerning algal abundance on at least 10 beaches on Öland and 2 in Kalmar. Some of the results were:

1. Information from the sites was made available daily before 9:00 a.m. on the web at www.hik.se/alg, by mobile service mobil.hik.se and by the automatic answering service "Miss Algae" +46 480 446010.
2. The surveillance showed that although cyanobacterial blooms according to SMHI's remote sensing pictures covered the entire Baltic proper, these blooms did not reach Öland's beaches during July-August 2006.
3. Even when cyanobacterial blooms reached some beaches we could rapidly establish that no or low numbers of the toxic *Nodularia spumigena* were present.
4. The positive media coverage of the beach monitoring program in Öland in 2006 reversed the adverse economic impacts from the negative 2005 headlines.
5. Importantly, feedback concerning the web page and mobile service indicated a high level of public trust in the information we provided.
6. The volunteers proved to be able to learn new techniques quickly, to perform their daily duties with enthusiasm, and to provide the public, through us, highly reliable data that restored the confidence of the tourists which then returned to the beaches.
7. Most of the volunteers will continue sampling once a month throughout the year (to build up a long-term data set for restoration purposes) and are interested in continuing the project next summer.
8. It is known that early retirement shortens one's life (British Medical Journal, BMJ, doi: 10. 1136/bmj. 38586.448704.E0, 21 October 2005) so this kind of project is of great social value as well.



Figure 5. Newspaper headlines from 2005.



Figure 6. Newspaper headlines from 2006.

**Acknowledgements**

We are most grateful to all the volunteers, or ALG-SPANARNA "Algal-Watchers" (see above for the names) for their tremendous effort in the daily pursuit to "detect" the cyanobacterial blooms on the Öland coast in all kinds of weather, and sampling at such "ungodly" hours. You are forever in our hearts! We thank the following agencies for their financial support: The Swedish Environmental Department, The Swedish EPA, Sparbanksstiftelsen Öland, The County Board in Kalmar County, Kalmar University, Swedish Agency for Economic and Regional Growth (Nutek), the Regional Council in Kalmar County, ÖlandsTurist AB, ÖlandsCamping, Borgholm and Mörbylånga municipalities.

## Preliminary assessment of *Bacillus mycooides* as a biological control agent for *Microcystis* blooms

J.R. Gumbo and T.E. Cloete

Department of Microbiology & Plant Pathology, University of Pretoria, Pretoria 0001, South Africa,  
jabulani\_gumbo@yahoo.co.uk

### Abstract

A flow cytometric technique was used to assess viability of *Microcystis* cells after exposure to *Bacillus mycooides* B16. The assessment involved two cellular functions, esterase activity and membrane integrity, after dual staining with fluorescein diacetate and propidium iodide. The results revealed three *Microcystis* subpopulations: living, membrane compromised and dead cells. In *B. mycooides* B16 treated samples, *Microcystis* biomass was reduced by 85 % on day 6, whereas an exponential increase was noted in the control samples. *B. mycooides* B16 lysed *Microcystis* cells, assessed by SEM micrographs. Copper sulphate, a known algicide, also lysed *Microcystis* cells. *B. mycooides* B16 may be considered as a potential biological control agent for the management of *Microcystis* algal blooms.

### Introduction

The technique of flow cytometry coupled with the use of fluorogenic probes is now well developed and is applied to the counting and viability assessment of aquatic microorganisms and cyanobacteria in particular (Phinney and Cucci 1989). Flow cytometry is a rapid, sensitive and precise technique that is used to count thousands of cells per second as they are carried within a fast moving fluid that passes a focused light beam (Franklin *et al.* 2004). Fluorescence emission and excitation characteristics are used to distinguish cyanobacteria with different subpopulations and from other microorganisms such as bacteria, based on accessory pigments (Franklin *et al.* 2004). This has led to the development of a tool to quantify viability in phytoplankton, in particular *Microcystis*, following exposure to different environmental stresses such as nutrient limitation (Brookes *et al.* 2000), nutrient enrichment (Latour *et al.* 2004), copper toxicity (Franklin *et al.* 2004), turbulence (Regel *et al.* 2004), acid mine drainage exposure (Regel *et al.* 2002), ultrasonic irradiation (Lee *et al.* 2000) and viral infection (Brusard *et al.* 2001).

The viability of *Microcystis* cells was assessed by flow cytometric analysis of two cellular functions, esterase activity and membrane integrity, after staining with fluorescein diacetate (FDA) and propidium iodide (PI), respectively (Joux and Lebaron 2000). During the screening of predatory bacteria from a *Microcystis* water bloom, *Bacillus mycooides* B16 was isolated. A number of *Bacillus* species have been found to be antagonistic towards *M. aeruginosa* (Rein *et al.* 1974; Wright and Thompson 1985; Nakamura

*et al.* 2003). The main objective of this study was to determine the potential of *B. mycooides* B16 as a biological control agent for *M. aeruginosa*. The specific objectives were to: use flow cytometry to assess *Microcystis* viability after exposure to *B. mycooides* B16, and to investigate the effect of *B. mycooides* B16 on the morphology of *Microcystis* by Scanning Electron Microscopy (SEM).

### Materials and Methods

#### *Culture of organisms*

Batch cultures of *Microcystis aeruginosa* PCC7806 were cultured in 2 L modified BG 11 medium (Krüger and Eloff 1977) under shaker incubation (80 rpm, 25 °C) and continuous light (24  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 17 d. *B. mycooides* B16 was cultured in 10 % Tryptic Soy Broth under shaker incubation, and lyophilized powder was prepared thereafter. Mass cell production for *Bacillus* spp. was followed (Korsten and Cook 1966).

#### *Experimental design*

To each of six 250-mL cotton plugged Erlenmeyer flasks containing 100 mL cyanobacterial suspension, 0.4 g of lyophilized powder were added. Another six flasks containing 100 mL cyanobacterial suspension only, served as controls. The flasks were subjected to similar culture conditions of host cyanobacteria. The lytic effect was determined by flow cytometric counts of *Microcystis* cells on a daily basis for a period of 6 days. Samples were then harvested for SEM.

#### *Flow cytometry*

For flow cytometric analysis, prior to fluorescent staining, *Microcystis* colonies were disrupted with

ultrasonication for 20 s. The FDA staining technique for *Microcystis* developed by Brookes *et al.* (2000) was followed. A procedure similar to that of Ross *et al.* (1989) and Franklin *et al.* (2004) was followed in the development of a PI staining technique for *Microcystis*. To 100  $\mu\text{L}$  of a *Microcystis* subsample, 100  $\mu\text{L}$  of FDA (120  $\mu\text{g mL}^{-1}$ ), 100  $\mu\text{L}$  of PI (60  $\mu\text{g mL}^{-1}$ ) and 100  $\mu\text{L}$  of FLOW-COUNT beads (Beckman Coulter, USA) were added. A Beckman Coulter Epics ALTRA flow cytometer (excitation: argon laser 15 mW, 488 nm) was used to count the *Microcystis* cells.

### SEM

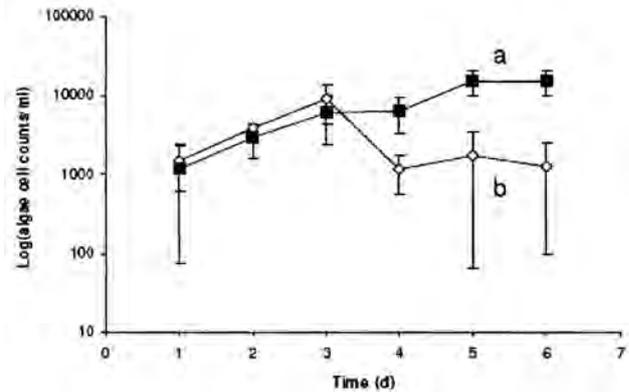
SEM morphological assessments of *Microcystis* cell membrane were derived from four samples. Two centrifuge tubes, an aliquot (1 mL) of copper sulphate (10 mg  $\text{mL}^{-1}$ ) and 1 mL of 10 % Triton X were separately added to 2 mL of *Microcystis* suspension. Two more samples, bacteria-treated and control, were subjected to SEM. Standard processing techniques were followed in the preparation of samples for SEM examination on a Jeol JSM 840 scanning electron microscope operating at 5.0 kV.

## Results and Discussion

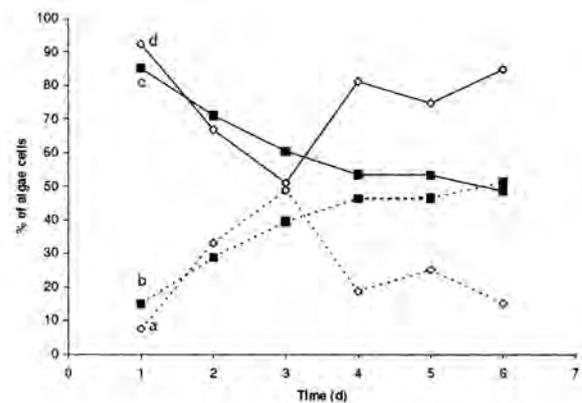
### Flow cytometric counts of *Microcystis* cells

The dual staining of *Microcystis* cells revealed three subpopulations: live, membrane compromised and dead cells. Both fluorescent stains (FDA and PI) were able to stain *Microcystis* cells with compromised membranes, and these cells have been classified as dead (Joux and Lebaron 2000). The *Microcystis* population heterogeneity was then narrowed to two groups, namely live and dead cells (Figs 1, 2). The aged *Microcystis* batch culture had an addition of fresh modified BG11 medium before subdividing into bacteria-treated and control samples. The purpose of introducing fresh nutrients was to stimulate growth of remaining cells. *Microcystis* growth was noted in both bacteria-treated and control samples for the first three days, increasing more than 10 times (Fig. 1). This also coincided with the lag phase where the bacteria had to adjust to the wet conditions associated with resuspending them in the aqueous environment. Thereafter on days 3 to 6, the population of live *Microcystis* cells (bacteria-treated) decreased by 85 % relative to the population level of day 3 (Figs 1b, 2a). In control samples, the population of live *Microcystis* cells almost doubled from days 3 to 6, an exponential increase (Figs 1a, 2b). High PI counts of dead cells on day 1 (Figs 2c, d) was found in the aged culture of *Microcystis*. This was followed by decrease in popu-

lation of dead cells to day 3. Thereafter *Microcystis* (bacteria-treated) showed an increase in the population of dead cells (Fig. 2d) after the lag phase. In contrast to the controls, which showed a continued decrease in the population of dead cells (Fig. 2c) in the absence of predatory bacteria, more cells were dividing than dying. Therefore, *B. mycoides* B16 may have contributed to growth inhibition of *Microcystis*.



**Figure 1.** Changes in populations of live *Microcystis* cells: (a) Control and (b) bacteria-treated samples. Mean values of six replicates.



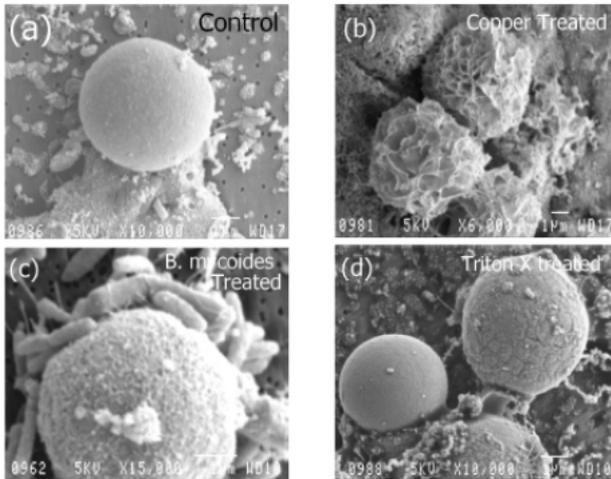
**Figure 2.** Percentage changes in population levels of *Microcystis* cells: (a) live (bacteria-treated); (b) live (Control); (c) dead (Control); and (d) dead (bacteria-treated) samples.

### Morphological assessment of *Microcystis* cell membrane damage

The SEM micrographs showed the extent of *Microcystis* cell membrane damage, most severe with copper, followed by *B. mycoides* B16 and lastly Triton X (Fig. 3). In the control sample, the *Microcystis* cell surface was smooth and spherical (Fig. 3a) (Lam *et al.* 1985). Copper sulphate is a well-known algicide chemical used to treat natural *Microcystis* algal blooms (Lam *et al.* 1985).

*B. mycoides* B16 may have released extracellular substances that caused damage to the *Microcystis*

cell as shown by perforations on the cell surface (Fig. 3c). Nakamura *et al.* (2003) isolated an unidentified extracellular substance from *B. cereus* N14, a close relative of *B. mycooides* B16 (Von Wintzingerode *et al.* 1997) that showed lytic activity towards *Microcystis*. It is possible that a similar extracellular substance of *B. mycooides* B16 may have resulted in cell lysis during this study.



**Figure 3.** SEM indicating the morphological changes to *Microcystis* cell membrane (a) control sample showing smooth cell structure, no visible damage; (b) copper-stripped membrane; (c) *B. mycooides* B16 caused perforations on membrane; and (d) Triton X caused ‘cracks’ on membrane.

### Conclusion

The results revealed three *Microcystis* sub-populations: living, membrane compromised and dead cells. In *B. mycooides* B16 treated-samples, *Microcystis* biomass was reduced by 85 % on day 6, whereas an exponential increase was noted in the control samples. *B. mycooides* B16 lysed *Microcystis* cells as assessed by SEM micrographs. Copper sulphate, a known algicide, also lysed *Microcystis* cells. Therefore, *B. mycooides* B16 may be considered as a potential biological control agent for the management of *Microcystis* algal blooms.

### Acknowledgements

Financial support for the study came from NRF and University of Pretoria (UP). I thank Prof T.E. Cloete, my promoter for his support; Mr A. Hall, Microanalysis and Microscopy Unit, UP for assistance with electron microscopy; Prof. R. Anderson, Drs R. Cockeran

and H. Steel, Dept of Immunology, UP for technical assistance with flow cytometry; and Dr. T. Downing, Nelson Mandela Metropolitan University, for provision of *Microcystis aeruginosa* PCC7806. JRG was awarded an ISSHA travel bursary to attend and present at the 12th International Conference on Harmful Algae, Copenhagen, Denmark.

### References

- Brookes, J.D., Geary, S.M., Ganf, G.G. & Burch, M.D. (2000). *Mar. Freshwater Res.* 51: 817-823.
- Brussaard, C.P.D., Marie, D., Thyraug, R. & Bratbak, G. (2001). *Aquat. Microb. Ecol.* 26: 157-166.
- Franklin, N.M., Adams, M.S., Stauber, J.L. & Lim, R.P. (2001). *Arch. Environ. Contam. Toxicol.* 40: 469-480.
- Franklin, N.M., Stauber, J.L. & Lim, R.P. (2004). *Environ. Toxicol. Chem.* 23: 1452-1462.
- Joux, F. & Lebaron, P. (2000). *Microbes and Infect.* 2: 1523-1535.
- Korsten, L. & Cook, N. (1996). *S. Afr. Avocado Growers' Assoc. Yearbook* 19: 54-58.
- Krüger, G.H.J. & Eloff, J.N. (1977). *J. Limnol. Soc. South Afr.* 3: 21-25.
- Lam, A.K.-Y., Prepas, E.E., David, S. & Hrudey, S.E. (1995). *Water Res.* 29: 184-554.
- Latour, D., Sabido, O., Salencon, M.J. & Giraudet, H. (2004). *J. Plankton Res.* 26: 719-726.
- Lee, T.J., Nakano, K. & Matsumura, M. (2000). *Biotechnol. Letters* 22: 1833-1838.
- Phinney, D.A. & Cucci, T.L. (1989). *Cytometry* 10: 511-521.
- Regel, R.H., Ferris, J.M., Ganf, G.G. & Griffiths, R.W. (2002). *Aquat. Toxicol.* 59: 209-223.
- Regel, R.H., Brookes, J.D., Ganf, G.G. & Griffiths, R.W. (2004). *Hydrobiol.* 517: 107-120.
- Ross, D.D., Joneckis, C.C., Ordóñez, J.V., Sisk, Am., Wu, R.K., Hamburger, A.W. & Nora, R.E. (1989). *Cancer Res.* 49: 3776-3782.
- Nakamura, N., Nakano, K., Sugiura, N. & Matsumura, M. (2003). *J. Biosci. Bioengin.* 95: 179-184.
- Von Wintzingerode, F., Rainey, F.A., Kroppenstedt, R.M. & Stackebrandt, E. (1997). *FEMS Microbiol. Ecol.* 24: 201-209.
- Reim, R.L., Shane, M.S. & Cannon, R.E. (1974). *Can. J. Microbiol.* 20: 981-986.
- Wright, S.J.L. & Thompson, R.J. (1985). *FEMS Microbiol. Lett.* 30: 263-267.

## HABs and clay flocculation: review and discussion of studies, impacts and future directions

M.R. Sengco<sup>1</sup> and K.G. Sellner<sup>2</sup>

<sup>1</sup>Smithsonian Environmental Research Center, P.O. Box 28, Edgewater, MD, USA, sengcom@si.edu, <sup>2</sup>Chesapeake Research Consortium, 645 Contees Wharf Road, Edgewater, MD, USA, sellnerk@si.edu

### Abstract

A symposium on the use of natural clays and other strategies to control harmful algal blooms (HABs) was convened as part of the international meeting. Six speakers from several countries gave short presentations on their research. Substantial progress was evident in laboratory and field tests examining clay flocculation as a control option to mitigate bloom effects. More examples of freshwater applications in lakes were presented, particularly in China. Participants suggested that further research is needed on post-clay application impacts. Following extensive discussion, it was concluded that international communication and potential data exchange should be formalized through a website. In addition, it was recognized that increased outreach to the scientific and management communities was crucial prior to the implementation of any mitigation strategy.

### Introduction

Over the past decade, there has been substantial international investigation of bloom mitigation strategies. Significant attention focused on clay application as early laboratory experiments and limited field demonstrations showed that clay-algal aggregation can remove microalgae from near-surface waters in many cases. This process reduced potential threats to surface-cultured marine taxa (fish, shellfish), but raised concerns for vertical displacement of algal toxins to bottom waters and benthic fauna, as well as accompanying biological oxygen demand from the settling bloom.

A symposium on the use of natural clays and other mitigation strategies to control harmful algal blooms (HABs) was convened at this conference. The goals were (1) to review past and current data on the use and impacts of clays to control HABs, (2) to evaluate and discuss these results and to identify gaps for further research, and (3) to provide insights into the cost of clay applications and the technical challenges of implementation. Due to the broad interest in mitigation, dialog expanded beyond clays.

### Session Results and Conclusions

Mario Sengco (Smithsonian Environmental Research Center, USA) summarized the current state of knowledge on the use of clays in the U.S. (Sengco *et al.* 2001; Sengco and Anderson 2004; Yu *et al.* 2004). Generally, very fine (< 2 µm) expanding clays with high surface charge density, like bentonite and montmorillonite, were broadly effective (>85 % cell

removal) against a variety of marine HAB species, with effectiveness further enhanced with the addition of various chemical flocculants, reducing the amount of clay needed to remove cells. Studies with *Karenia brevis* and phosphatic clays revealed that toxins were absorbed by the clay (Pierce *et al.* 2004) and direct contact could lead to cell mortality at moderately high loading, with no resuspension, and prolonged contact between the clays and cells (Sengco *et al.* 2001). Studies using mesocosms and limnocorrals showed moderately high (>60 %) cell removal in the absence of flow. Temperature, salinity, pH, and dissolved oxygen concentration were unaffected by clay dispersal. Elevated turbidity decreased rapidly within 4 h of treatment while phosphate release (as well as nitrate, nitrite, ammonium, and silicate) was noted with the use of phosphatic clay. In flume studies, cell removal decreases with increasing flow due to lower settling rates. However, cell damage (i.e., loss of motility and shape) in the presence of clay appeared to increase with increasing flow (Archambault *et al.* 2003). Erodibility decreased as the settled floc layer compacted over time, but the addition of flocculant increased the erodibility of the layer relative to untreated flocs (Beaulieu *et al.* 2005).

Monica Bricelj (National Research Council, Canada) presented results from benthic impact studies. The effects of phosphatic clay on the growth and survival of two commercially important bivalves (*Mercenaria mercenaria* and *Mya arenaria*) were examined. For *M. mercenaria*, clay dispersal in the field may be more detrimental in a high-energy environment, leading to prolonged *in situ* resuspension

of clay, rather than in lower flow areas that favored complete sedimentation (Archambault *et al.* 2004). Although these suspension-feeding bivalves were relatively tolerant of clay deposition, with ten repeated clay treatments small but significant growth inhibition occurred (unpubl. data). Also, the effects of suspended and clay-flocculated cells on toxin accumulation in the tellinid clam *Macoma balthica* were examined in suspension- and deposit-feeding mode (unpubl. data). The effects of a natural *K. brevis* bloom on the toxin uptake and composition of *M. mercenaria* and natural benthic macrofauna were studied. Results showed that comparable brevetoxins (PbTx) levels can be accumulated by clams whether suspension- or deposit feeding. Within the macrobenthos, molluscs (bivalves and gastropods) accumulated the highest toxin levels during a natural bloom. Significant toxin levels in carnivorous gastropods demonstrated that toxin transfer to a higher trophic level can occur. Flocculation of toxic cells with clay can cause a shift in the pathway of PbTx transfer from suspension- to deposit feeders, thus mitigating impacts on commercially important bivalves. PbTx-2 from *K. brevis* cells was rapidly transformed in clam tissues to higher potency metabolites, i.e. PbTx-3 and its cysteine derivatives.

Zhiming Yu (Institute for Oceanology, Chinese Academy of Sciences) presented work from China, focusing on improving flocculation through chemical modification of clays, and on some initial field studies. Laboratory studies showed that kaolinites were more effective than montmorillonites at removing a number of algal species by virtue of the kaolinite's surface charge and particle size (Yu *et al.* 1994a, b). The approach to improving removal efficiency was to use chemical flocculants, like polyhydroxy aluminium chloride, to increase positive surface charge, or to promote bridging between particles, through the use of long-chained, polymeric, organic flocculants, such as quarternary ammonium compounds. Field experiments were conducted in Xuanwu Lake (4 km<sup>2</sup>) in Nanjing City, where large *Microcystis aeruginosa* blooms occur (maximum biomass 10<sup>7</sup> cells mL<sup>-1</sup>). High cell removal and improved water quality were observed following treatment. Monitoring of bacteria, phytoplankton and zooplankton showed no significant effect on aquatic ecology in the short-term. However, there was no additional monitoring after the study to address long-term effects.

Gang Pan (Research Center for Eco-Environmental Science, Chinese Academy of Sciences) summarized research in freshwater lakes to control nu-

sance algae, and to restore lake ecosystems with local soils/flocculant treatments (Pan *et al.* 2006a, b; Zou *et al.* 2006). Instead of clays, clean local shore soils were used to minimize cost (purchase and transport) and to reduce introduction of exogenous pollutants to the lake. To improve removal ability, the soils were treated with chitosan, a natural flocculant from acid-solubilized chitin. Field tests in floating mesocosms showed high removal efficiency and improvements on water quality (i.e., reduced turbidity, chlorophyll, nutrients). This method was used successfully to settle a half-meter-thick *Microcystis* bloom that had accumulated along the shore of Lake Taihu. The most innovative use of the soil/flocculant method was in ecosystem restoration. In laboratory mesocosms, accumulated algal biomass was settled using the chitosan-treated soil. Oxygen-releasing chemicals and seeds of aquatic angiosperms were added to the matrix to reduce hypoxia as the algae decayed and additionally to assimilate recycled nutrients from algal decomposition, the latter preventing nutrient release and subsequent blooms in the upper water column.

Chang-Kyu Lee (National Fisheries Research and Development Institute, South Korea) presented studies on the impact of yellow clay dispersal to reduce the effects of *Cochlodinium polykrikoides* on penned fish (Na *et al.* 1996; Bae *et al.* 1998; Choi *et al.* 1998, 1999). In the first study, low doses of yellow clay (<1.25 % clay) did not affect respiratory rates (stress response) in rock fish (*Sebastes shlegelli*) nor respiratory rates of mussels and oysters; abalone were impacted. Analysis of the gut content of the three bivalves showed clay accumulation after treatment. However, after transfer to clean water and recovered feeding, clays were expelled within 4-6 h. Grazing rates on seaweed (*Undaria* sp.) by abalone were also unaffected by clay treatment. Biodiversity studies in field sites where yellow clay is frequently added showed little or no changes in benthic species abundance, biomass and composition in the three study areas. For South Korea, clay dispersal remains an inexpensive and effective method for protecting aquaculture from *C. polykrikoides* blooms.

Ian Jenkinson (ARCO, France) presented some data on the use of acetyl-L- and ethyl-cysteine to improve survival of fish when exposed to bloom species such as *Karenia mikimotoi* (Jenkinson *et al.* in press). The cysteine may help to fluidify algal mucus and reduce or destroy oxidative radicals. Pilot-scale studies are underway.

In summary, there was agreement that much more is now known about the application of clays, flocculants and other chemicals to treat blooms. There is a greater understanding about the mechanisms for removal and settling, the factors that influence flocculation rates (e.g., flow, salinity), as well as the impacts of clay dispersal on water quality and the benthic environment. There is concern for short-term impacts of clay dispersal on some benthic organisms, but longer-term studies in China and Korea showed little effect on the ecosystem after multiple clay dispersals. There were some estimates of mitigation costs for China and Korea. During the discussions, it was suggested that other control methods be emphasized, and that clay should not be the only focus as bloom control does not mean eradication. Instead, the goal is to minimize HAB impacts by reducing algal numbers to protect an important or valuable resource. The audience was also reminded to consider to what we compare the possible implications of bloom control. Instead of using non-bloom conditions as a baseline for comparisons, the effects and costs of clay dispersal should be compared with the impacts and cost of an untreated bloom. Finally, it was noted that the scientists' role is to provide critical data for evaluating efficacy and impacts of a mitigation strategy in order to inform the decision-making process. The decision to implement this (or any) method will involve all stakeholders (including managers, policy makers, and public).

To help in disseminating information on this topic for the community and the public as a whole, it was suggested that a database be constructed for all the available literature on this and other control methods, including papers, reports, and articles as well as summaries, pictures, data and a means to contact scientists working in this field to communicate with the public on clay dispersal.

## References

- Archambault, M.-C., Grant, J. & Bricelj, V.M. (2003). *Mar. Ecol. Prog. Ser.* 253: 97-109.
- Archambault, M.C., Bricelj, V.M., Grant, J. & Anderson, D.M. (2004). *Mar. Biol.* 144: 553-565.
- Bae, H.M., Choi, H.G., Lee, W.C. & Yoon, S.J. (1998). In: *Proceedings of the Korea-China Joint Symposium on Harmful Algal Blooms*, pp. 53-60.
- Choi, H.G., Kim, P.J., Lee, W.C., Yun, S.J., Kim, H.G. & Lee, H.J. (1998) *J. Korean Fish. Soc.* 31: 109-113.
- Choi, H.G., Lee, P.Y., Yun, S.J., Lee, W.C. & Bae, H.M. (1999). *Bull. Natl. Fish. Res. Dev. Inst. Korea* 57: 105-110.
- Beaulieu, S., Sengco, M.R. & Anderson, D.M. (2005). *Harmful Algae* 4: 123-138.
- Jenkinson, I, Claireaux & Gentien, P., 2006. *Mar. Biol.* in press
- Na, G.H., Choi, W.J. & Chun, Y.Y. (1996). *J. Aquaculture* 9: 239-245.
- Pan, G., Zhang, M.M., Chen, H., Zou, H. & Yan, H. (2006a). *Environ. Poll.* 141: 195-200.
- Pan, G., Zou, H., Chen, H. & Yuan, X. (2006b). *Env. Poll.* 141: 206-212
- Pierce, R.H., Henry, M.S., Hingham, C.J., Blum, P., Sengco, M.R. & Anderson, D.M. (2004). *Harmful Algae* 3: 141-148.
- Sengco, M.R., Li, A., Tugend, K, Kulis, D. & Anderson, D.M. (2001). *Mar. Ecol. Prog. Ser.* 210: 41-53.
- Sengco, M.R. & Anderson, D.M. (2004). *J. Euk. Microbiol.* 51: 169-172.
- Yu Z., Zou J., Ma X. (1994a). *Chin. J. Oceanol. Limnol.* 12: 193-200.
- Yu Z., Zou J. & Ma X. (1994b). *Chin. J. Oceanol. Limnol.* 12: 316-324.
- Yu, Z., Sengco, M.R. & Anderson D.M. (2004). *J. Appl. Phycol.* 16: 101-110.
- Zou, H., Pan, G., Chen H. & Yuan, X. (2006). *Environ. Poll.* 141: 201-205.

## 7. REGIONAL EVENTS



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## ***Microcystis aeruginosa* bloom and the occurrence of microcystins in a freshwater eutrophic lake in Comilla, Bangladesh**

M. S. Ahmed<sup>1</sup>, S. Hiller<sup>2</sup> and B. Luckas<sup>2</sup>

<sup>1</sup>Lab of Aquatic Resource Management, Department of Zoology, University of Dhaka, Dhaka 1000, Bangladesh, ms2ahmed@yahoo.com, <sup>2</sup>Institute of Nutrition, University of Jena, Dornburger Street 25, 07743 Jena, Germany

### **Abstract**

Bangladesh is a tropical country comprised of a large alluvial plain with 1.3 million fresh and brackish water ponds and lakes, and has an optimal environment for luxuriant growth of cyanobacteria. A bloom of *Microcystis aeruginosa* occurred recently in a lake in the Comilla district. Bloom samples were collected and filtered through a glass fibre filter. Methanol-water extracts of a fresh sample, filtered cells, and lyophilized cells were analyzed by high performance liquid chromatography (HPLC) with UV, MS, and MS-MS detection. Three types of microcystins were detected viz., Microcystin-RR, Microcystin-YR, and Microcystin-LR, and these were confirmed by HPLC/MS. In field collected samples of the bloom, the amount of MC-LR was highest (2120 µg l<sup>-1</sup>), followed by MC-RR (1400 µg l<sup>-1</sup>) and MC-YR (440 µg l<sup>-1</sup>). In lyophilized *M. aeruginosa* cells the amounts of MC-LR, MC-RR, and MC-YR were 1840, 590, and 580 µg l<sup>-1</sup>, respectively. Further investigations are needed to characterize other types of microcystins as well as their effects on human health and cultured fish.

### **Introduction**

Over 30 species of cyanobacteria are associated with toxic water blooms (Skulberg *et al.* 1993), with reports from at least 44 countries as well as the Baltic and Caribbean Seas, and the Atlantic, Pacific, and Indian Oceans (Carmichael 1989; Codd 1995). Eutrophication of freshwater and the appearance of cyanobacterial blooms have become a worldwide phenomenon that can become serious when such bloom-forming species release potent water soluble toxins (Watanabe and Oishi 1980; Vasconcelos *et al.* 1993; Carmichael 1994). Toxic cyanobacteria are now recognized as a hazard to human and animal welfare, and health assessments are being carried out to determine environmental health consequences (Skulberg *et al.* 1984; Carmichael 1994, 1995). The supply of clean and safe drinking water is one of the main challenges to public health in Bangladesh. Traditionally, surface water is the main source of drinking water and consumed either without any treatment or after boiling when fuel is available. There are about 1.3 million fresh and brackish water ponds (FRSS 1986) in the country and *Microcystis* blooms occur frequently in these ponds and lakes (Islam and Naher 1967; Aziz 1974; Islam and Uddin 1977; Islam 1991). Most of the pond waters are used for agricultural or domestic purposes (cooking, washing hands and mouths, bathing, drinking, etc.). The cyanobacterial blooms have been poorly studied. This paper deals with the isolation and characterization of microcystins from a natural bloom of *M. aeruginosa* in a freshwater lake.

tion and characterization of microcystins from a natural bloom of *M. aeruginosa* in a freshwater lake.

### **Materials and Methods**

The study lake, locally called 'Dharmo Sagor', is located in Comilla district (23°16' N latitude, 90° 40' E longitude) 98 km southeast of Dhaka. The lake is 12 ha in size and used mainly for domestic purposes. A bloom of *M. aeruginosa* began during the first week of March, 2005 and the highest cell dominance (95% *Microcystis*) was recorded on 4 March 2005. A bloom sample was collected using a 20-µm plankton net. An aliquot of the concentrated sample was filtered through a 0.45-µm glass fibre filter (Whatman GF/C, 47 mm diam) and dried in an oven at 60-80 °C. The remaining sample was freeze-dried. Dried filters covered with algal cells, lyophilized cells, and some fresh algae (frozen immediately after collection) were transported to the Alfred Wegener Institute, Sylt, Germany for analysis.

#### *Extraction*

GF/C filters and 1.0 ml of a mixture of water and methanol (50:50, v:v) were sonicated for 20 min and centrifuged (3000 x g). The supernatant was filtered through a 0.45-µm pore size nylon filter.

#### *Chemical analysis*

The HPLC/UV determination of microcystins follows Lawton *et al.* (1994) with some modifications

(Hummert *et al.* 2001a) - C18 column: Phenomenex Prodigy, ODS (3), 250 x 4.6 mm, 5  $\mu\text{m}$ ; mobile phase A: acetonitrile/0.05% TFA, mobile phase B: water/0.05% TFA. Microcystins were detected using a UV detector (Shimadzu SPD-10AV;  $\lambda = 238\text{ nm}$ ). HPLC/MS and HPLC/MS-MS analyses were conducted to confirm the identity of the toxin peaks in the chromatograms. The HPLC was coupled by an electrospray interface to a single quadrupole mass spectrometer (API 150, PE Sciex Instruments, Canada) and to a triple quadrupole mass spectrometer (API 365, PE Sciex Instruments, Canada). The detection was carried out in selected ion monitoring (SIM) mode using LC/MS and multiple reaction monitoring (MRM) mode using LC/MS-MS (Hummert *et al.* 2001b).

#### Microcystins and nodularin standards

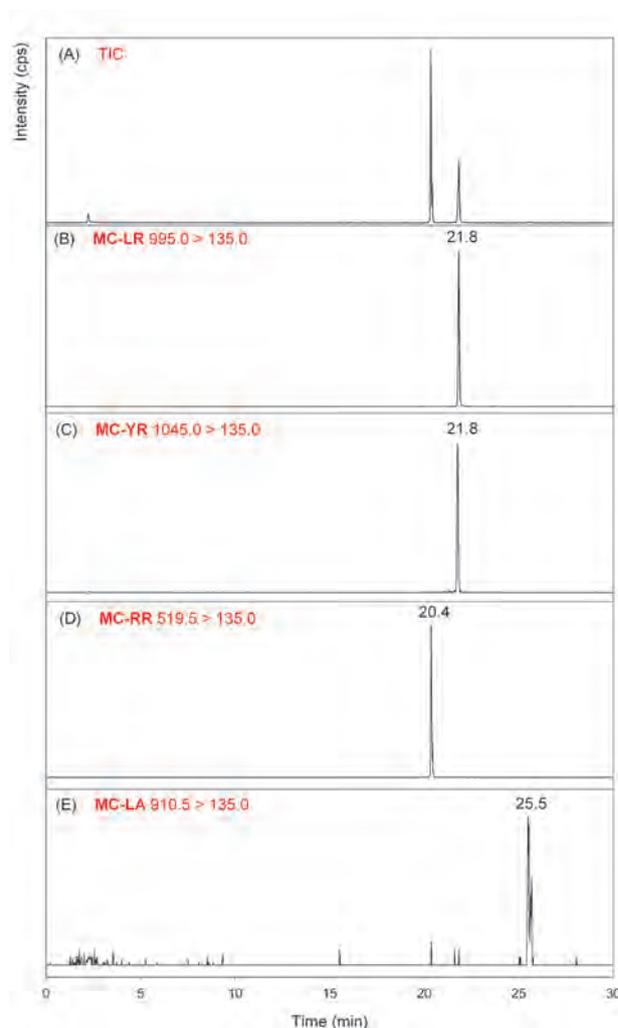
Standards of Microcystin-RR, Microcystin-LR, Microcystin-YR, Microcystin-LA, and Nodularin were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA).

#### Chemicals

HPLC grade acetonitrile and HPLC grade methanol were from Baker (Deventer, Netherlands). Water was purified to HPLC grade quality with a Millipore-Q RG Ultra Pure Water System (Millipore, Milford, USA).

## Results and Discussion

In the original bloom sample, the cell density of *M. aeruginosa* was  $6.153 \times 10^8$  cells  $\text{l}^{-1}$ . During the bloom, dissolved oxygen, free carbon dioxide, and nitrite nitrogen in the pond water were recorded as 4.43, 15.0, and 0.69  $\text{mg l}^{-1}$ , respectively. The pH was 8.4 and the water temperature 24–26  $^{\circ}\text{C}$ . HPLC analysis of the *M. aeruginosa* extract showed three peaks, the retention times of which agreed well with standard MC-RR, MC-YR, and MC-LR. The results of HPLC-MS identified three variants of microcystin (Fig. 1) according to their corresponding molecular weights: MC-LR (at  $m/z$  995.0  $[\text{M}+\text{H}]^+$ ), MC-RR (at  $m/z$  519.5  $[\text{M}+2\text{H}]^{2+}$ ), and MC-YR (at  $m/z$  1045.0  $[\text{M}+\text{H}]^+$ ). In the fresh *M. aeruginosa* sample the amount of MC-LR was highest (2120  $\mu\text{g l}^{-1}$ ), followed by MC-RR (1400  $\mu\text{g l}^{-1}$ ) and MC-YR (440  $\mu\text{g l}^{-1}$ ). In lyophilized cells the amounts of MC-LR, MC-RR, and MC-YR were 1840, 590, and 580  $\mu\text{g l}^{-1}$ , respectively (Table 1). A small amount of MC-LA was also detected. The total concentration of microcystins in fresh cells was 3960  $\mu\text{g l}^{-1}$ . Welker *et al.* (2004), in a study of three different regions in Bangladesh, detected microcystins in 39 ponds, mostly together with varying abundances of



**Figure 1.** HPLC/MS-MS chromatogram of microcystins detected from *Microcystis aeruginosa* (lyophilized sample). (A) TIC; (B) Microcystin-LR,  $[\text{MC-LR}+\text{H}]^+$  995.0 > 135.0; (C) Microcystin-YR,  $[\text{MC-YR}+\text{H}]^+$  1045.0 > 135.0; (D) Microcystin-RR  $[\text{MC-RR}+2\text{H}]^{2+}$  519.5 > 135.0; (E) Microcystin-LA  $[\text{MC-LA}+\text{H}]^+$  910.5 > 135.0.

potentially microcystin-producing genera such as *Microcystis*, *Planktothrix*, and *Anabaena*. Total microcystin concentrations in their study ranged between  $<0.1$  and  $>1000$   $\mu\text{g l}^{-1}$ , and more than half the positive samples contained concentrations exceeding 10  $\mu\text{g l}^{-1}$ .

Our results showed the concentration of microcystins (Table 1) to be well above the WHO provisional guideline value of 1  $\mu\text{g l}^{-1}$  MC-LR. In Australia, a safety factor for tumour promotion is 1.0  $\mu\text{g}$  microcystins or nodularins  $\text{l}^{-1}$  (Falconer *et al.* 1994). In Canadian drinking water, the maximum accepted concentration for MC-LR is 0.5  $\mu\text{g l}^{-1}$  and for other microcystins, 1  $\mu\text{g l}^{-1}$  of total microcystins (Carmichael 1995).

The occurrence of hepatotoxic *M. aeruginosa* blooms is particularly serious if the water is utilized as a drinking supply and/or for recreational purposes. The incidence rate of colorectal cancer was significantly higher in the population drinking river and pond water contaminated with cyanobacteria than in those using well and tap water (Zhou *et al.* 2002). In Bangladesh, local people use pond/lake water for domestic purposes even when a bloom or scum is formed, as they have no knowledge of toxicity and often no alternative.

Recently, use of surface water for human consumption has increased due to arsenic contamination of ground waters. In the Comilla district and perhaps other regions of Bangladesh, such a practice amounts to replacing one health hazard with another.

**Table 1.** Amounts of different microcystins detected from fresh algae, filtered algae, and lyophilized cells of a *M. aeruginosa* bloom.

Micro-cystins	Fresh $\mu\text{g (x10}^3\text{) l}^{-1}$	Filtered $\mu\text{g (x10}^3\text{) l}^{-1}$	Lyophilized $\mu\text{g (x10}^3\text{) l}^{-1}$
MC-RR	1.40	0.84	1.84
MC-YR	0.44	0.54	0.59
MC-LR	2.12	1.74	0.58
Total	3.96	3.12	3.01

Every year deaths are reported from villages and slum areas in Dhaka, Comilla, Chandpur, Sylhet, and Barishal following diarrhoea caused by contaminated drinking water. There is no official record of animal or human intoxication induced by cyanobacteria, but the role of microcystins in the deaths should be clarified.

### Acknowledgements

Financial support from the Intergovernmental Oceanographic Commission (IOC) of UNESCO to Dr Md. Sagir Ahmed for travel to Germany to analyze samples is greatly acknowledged. We thank two anonymous reviewers for helpful comments on the manuscript.

### References

Aziz, A.K.M. (1974). *Science* 183: 1206-1207.  
Carmichael, W.W. (1995). In: *Harmful Marine*

- Microalgae*, Hallegraff, G.M. Anderson, D.M. & Cembella, A.D. (eds), UNESCO, Paris, pp.163-175.  
Carmichael, W.W. (1994). *Sci. Am.* 270: 78-86.  
Carmichael, W.W. (1989). In: *Natural Toxins*, Ownby, C.A. & Odella, G.U. (eds), Peragon, Oxford, pp. 3-16.  
Codd, G. A. (1995). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernandez, M.L. & Wyatt, T. (eds), UNESCO, Paris, pp.13-17.  
Falconer, J.R., Burch, M.D., Steffensen, D.A., Choic, M. & Cloverdale, O.R. (1994). *Env. Toxicol. Water Qual.* 9: 131-139.  
Fisheries Resource Survey System (FRSS) (1986). (Directorate of Fisheries, Dhaka), Bulletin 3: 29.  
Hummert, C., Reichelt, M., Weiß, J., Liebert, H.-P. & Luckas, B. (2001a). *Chemosphere* 44: 1581-1588.  
Hummert, C., Dahlmann, J., Reinhardt, K., Dang, H. P.H., Dang, D.K. & Luckas, B. (2001b). *Chromatographia* 54: 569-575.  
Islam, A.K.M. & Nahar, L. (1967). *Sci. Res.* 4: 141-149.  
Islam, A.K.M. (1991). In: *Two Centuries of Plant Studies in Bangladesh and Adjacent Regions*, Islam, A.K.M. (ed.), Asiatic Society of Bangladesh, pp. 97-153.  
Islam, A.K.M. & Uddin, M.A. (1977). *J. Asiatic Soc. Bangladesh (Sci.)* 2: 75-81.  
Lawton, L.A., Edwards, C. & Codd, G.A. (1994). *Analyst* 119: 1525-1530.  
Skulberg, O. M., Carmichael, W.W., Codd, G.A. & Skulberg, R. (1993). In: *Algal Toxins in Seafood and Drinking Water*, Falconer, I. R. (ed.), Academic Press, pp. 145-164.  
Skulberg, O.M., Codd, G.A. & Carmichael, W.W. (1984). *Ambio B*: 244-247.  
Vasconcelos, W.R., Evans, W.W., Carmichael, W.W. & Namikoshi, M. (1993). *J. Env. Sci. Health A* 28: 2081-2094.  
Watanabe, M.F. & Oishi, S. (1980). *Jap. J. Limnol.* 41: 5-9.  
Welker, M., Chorus, I. & Fastner, J. (2004). Occurrence of cyanobacterial toxins (microcystins) in surface waters of rural Bangladesh-pilot study. WHO Report, 23 pp.  
Zhou, L., Yu, H. & Chen, K. (2002). *Biomed. Environ. Sci.* 15: 166-171.

## Seasonal occurrence of *Pseudo-nitzschia* species in the west coast and Shetland Isles, Scotland

L. Brown and E. Bresnan

Fisheries Research Services, FRS Marine Laboratory PO Box 101, 375 Victoria Road Aberdeen, UK  
AB11 9DB, brownl@marlab.ac.uk

### Abstract

Members of the genus *Pseudo-nitzschia* have been associated with extensive closures of offshore Scottish scallop fishing grounds due to the accumulation of high concentrations of domoic acid in the gonad tissue of *Pecten maximus*. FRS research cruises during spring and autumn 2005 surveyed the phytoplankton around the west coast of Scotland and Shetland Isles to investigate the diversity of *Pseudo-nitzschia* species in these areas. Light microscopy analysis showed two *Pseudo-nitzschia* morphotypes: (*P. seriata* 'type' cells (valve width >3 µm) and *P. delicatissima* 'type' cells (valve width <3 µm) to be present at high cell densities (>200,000 cells L<sup>-1</sup>). Detailed analyses of the samples by transmission electron microscopy identified *P. seriata*, *P. australis*, *P. pungens*, *P. fraudulenta* and *P. delicatissima*. A further unidentified species was also observed.

### Introduction

Members of the genus *Pseudo-nitzschia* have been associated with extensive closures of offshore Scottish scallop fishing grounds due to the accumulation of domoic acid (DA) in the gonad tissue of king scallops, *Pecten maximus* (Gallacher *et al.* 2001; Fehling *et al.* 2004). *Pseudo-nitzschia* species contribute significantly to the phytoplankton communities of Scottish waters with more than ten species identified (Bresnan 2003; Fraser *et al.* 2004, 2006; Fehling *et al.* 2006;). *Pseudo-nitzschia* communities have been observed

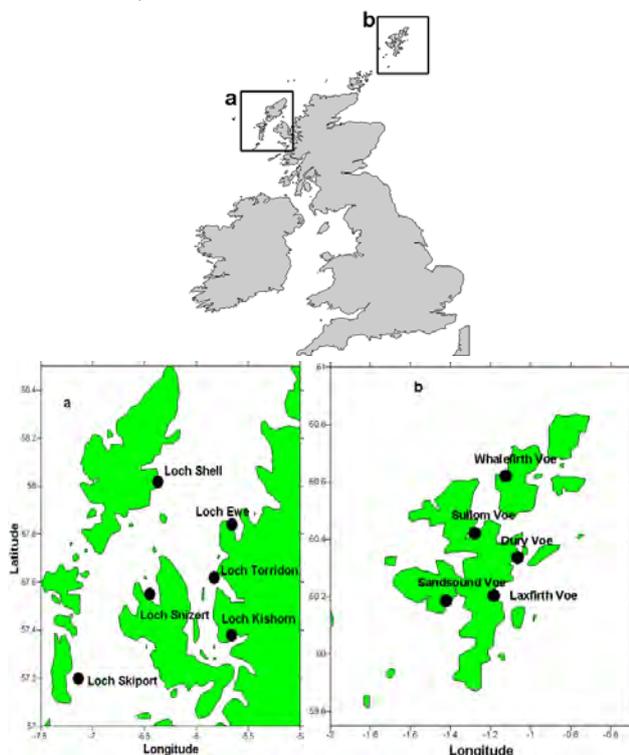
to be relatively diverse with up to five species co-occurring (Bresnan 2003). To investigate seasonality amongst *Pseudo-nitzschia* species around Scotland, a series of research cruises around sea lochs in the west coast and Shetland Isles took place during spring and autumn 2005 (Fig. 1). The aquaculture industry plays an important role in the economy of these areas.

### Materials and Methods

Water samples for cell counts by light microscopy (LM) were collected using a 10-m integrated tube system. Samples used for transmission electron microscopy (TEM) were collected using a horizontal surface phytoplankton net tow, mesh size 10 µm. LM analysis was performed on 10 ml sub samples using the Utermöhl technique (Utermöhl 1958). *Pseudo-nitzschia* cells were enumerated and classified into two size categories: *P. delicatissima* 'type' (width <3 µm) and *P. seriata* 'type' (width >3 µm). Samples for TEM analysis were acid cleaned (Christensen 1988), mounted onto 200 mesh formvar-covered grids and examined using a Philips CM10 transmission electron microscope. Cells were identified using guidelines in current literature (Skov *et al.* 1999; Lundholm *et al.* 2003, 2006).

### Results

LM counts showed *P. delicatissima* 'type' cells to dominate spring samples. West coast cell densities were much greater than those recorded in Shetland (Figs 2, 3). A maximum value of 226,200 cells L<sup>-1</sup> was observed at Loch Snizort. *P. seriata* 'type' cells occurred primarily in the autumn in both areas. Cell densities in Shetland exceeded those in the west coast



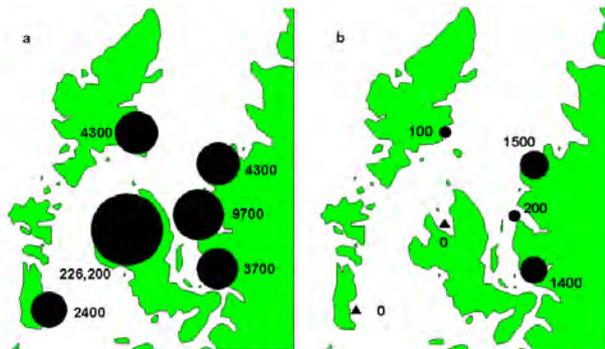
**Figure 1.** Locations of sea lochs surveyed: (a) West coast (b) Shetland Isles

(Figs 4, 5). A maximum of 148,900 cells  $L^{-1}$  was observed at Sullom Voe.

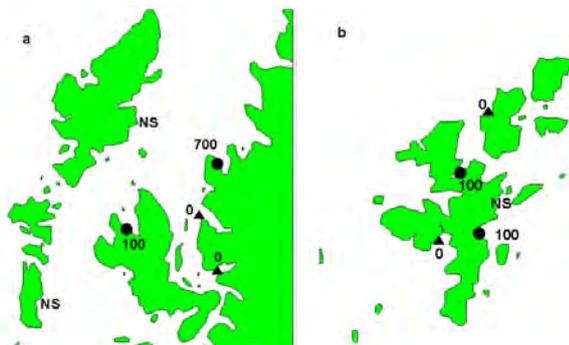
TEM identification was restricted to west coast spring samples and Shetland Isles autumn samples only. Low cell concentrations in other samples prevented TEM analysis. Five species were identified: *P. australis*, *P. fraudulenta*, *P. seriata*, *P. pungens* and *P. delicatissima*. A further unidentified species of the *P. delicatissima* 'type' was observed at Lochs Ewe and Kishorn. West coast waters were observed to exhibit a greater species richness with all five species identified in this location during the spring cruise. *P. delicatissima* was observed to dominate the *Pseudo-nitzschia* communities in this area at this time. Species of the *P. seriata* 'type' were observed to dominate samples taken from the Shetland area in autumn; *P. australis*, *P. fraudulenta*, *P. seriata* and *P. pungens*. *P. australis* was the most frequently observed species on the TEM grids. Morphological measurements used to identify each species and TEM micrographs of the central portion of each species observed are shown in Table 1 and Fig. 6. The unidentified *P. delicatissima* 'type' observed at Lochs Ewe and Kishorn in the spring cruise appeared similar to the unidentified Hobart species reported in Lundholm *et al.* (2003).

## Discussion

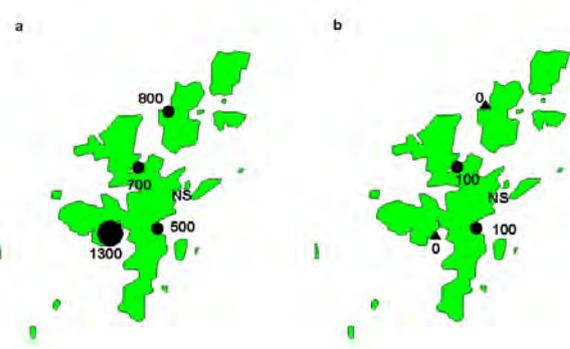
This study has shown *Pseudo-nitzschia* communities around the Shetland Isles and west coast of Scotland to be relatively rich and comprising up to five different species. These results concur with Fehling *et al.* (2006) and Bresnan (2003) where more than ten species were identified. LM results showed *Pseudo-nitzschia* to exhibit seasonal patterns of occurrence at the two sampling areas. *P. delicatissima* 'type' cells were observed to dominate in spring and *P. seriata* 'type' cells were observed to dominate during autumn. Ten years of data from the Toxic Phytoplankton Monitoring Programme at FRS support this seasonality and reflect cell densities recorded during the bloom periods. Also highlighted is the timing of the spring bloom with respect to latitude, first appearing around Shetland towards the end of April but not until the first week of May in west coast waters. The autumn bloom does not have such distinct timing and can develop through July and August in both areas (Bresnan 2003; Fraser *et al.* 2004, 2006). Comprehensive TEM studies of *Pseudo-nitzschia* communities throughout Scottish waters have identified species assemblages typical of those identified in this paper (Bresnan 2003). Species most frequently observed



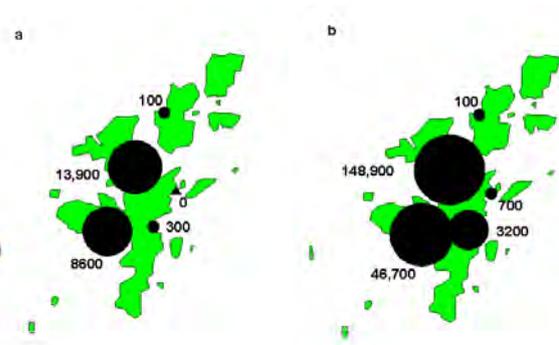
**Figure 2.** Western Isles spring communities: (a) *P. delicatissima* 'type' cells (b) *P. seriata* 'type' cells (all values in cells  $l^{-1}$ )



**Figure 4.** Western Isles autumn communities: (a) *P. delicatissima* 'type' cells (b) *P. seriata* 'type' cells (all values in cells  $l^{-1}$ , NS = no sample)



**Figure 3.** Shetland Isles spring communities: (a) *P. delicatissima* 'type' cells (b) *P. seriata* 'type' cells (all values in cells  $l^{-1}$ , NS = no sample)

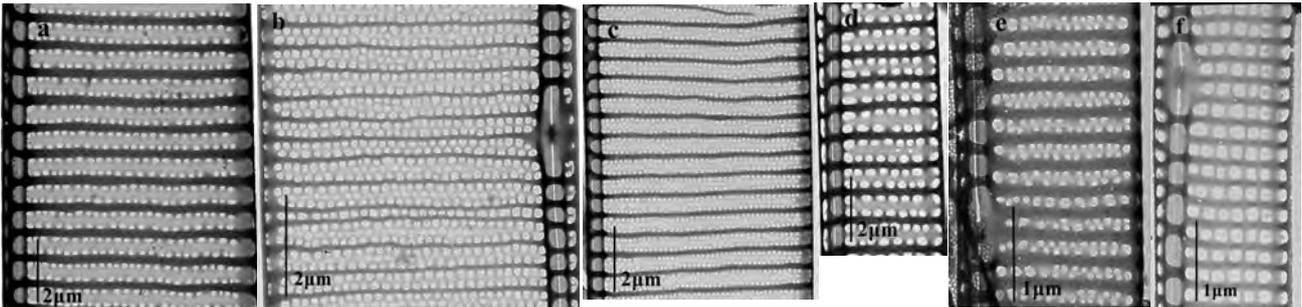


**Figure 5.** Shetland Isles autumn communities: (a) *P. delicatissima* 'type' cells (b) *P. seriata* 'type' cells (all values in cells  $l^{-1}$ )

**Table 1.** Morphological characters from TEM images used to identify *Pseudo-nitzschia* species in this study.

Species	Central nodule <sup>1</sup>	Interstriae per 10 µm	Fibulae per 10 µm	Rows of Poroids	Poroids per 1 µm	Width (µm)	Length (µm)
<i>P. australis</i> (n=26)	-	13-17	12-17	2	4-5	6.2-8.2	76.0-159.2
<i>P. fraudulenta</i> (n=11)	+	21-23	20-23	2-3	6	5.1-6.8	78.1-98.0
<i>P. seriata</i> (n=11)	-	15-19	15-20	3-4	7-8	6.6-8.0	128.2-155.1
<i>P. pungens</i> (n=9)	-	11-14	11-16	2	3-4	2.8-4.0	112.0-128.0
<i>P. delicatissima</i> (n=15)	+	36-40	22-26	2	8-10	1.5-1.9	50.0-85.3
<i>Pseudo-nitzschia</i> sp. (n=4)	+	39-42	22-24	1	5-6	1.4-1.5	62.5-66.6

<sup>1</sup> + = Central nodule present - = Central nodule absent



**Figure 6.** Micrograph images of *Pseudo-nitzschia* species identified from Scottish waters, (a) *Pseudo-nitzschia* cf. *australis*, (b) *Pseudo-nitzschia fraudulenta*, (c) *Pseudo-nitzschia* cf. *seriata*, (d) *Pseudo-nitzschia pungens*, (e) *Pseudo-nitzschia* cf. *decipiens*, (f) *Pseudo-nitzschia* sp.

during spring and autumn blooms have been *P. delicatissima* and *P. australis* respectively, as in this study. Confirmed DA producers *P. australis* and *P. seriata* (Fehling *et al.* 2004) were observed on the TEM grids during both seasons, showing that the potential for DA accumulation in shellfish remains a threat in these areas. *P. multiseries* was not observed and this species has been rarely recorded in Scottish waters (Bresnan 2003; Fehling *et al.* 2006) A study in Firth of Lorne, Scotland, has shown photoperiod to be the most significant environmental variable explaining seasonality among different *Pseudo-nitzschia* species in this area (Fehling *et al.* 2006). This is supported by laboratory studies (Fehling *et al.* 2004). Thus latitude should be considered when examining the dynamics of *Pseudo-nitzschia* in Scottish waters.

### Acknowledgements

The authors would like to thank Alastair McKinnon and Kevin Mackenzie, University of Aberdeen, and Jennifer Graham, Fisheries Research Services. This work was performed under the SEERAD funded project AE1191.

### References

Christensen, T. (1988). Alger i naturen og i laboratoriet. Københavns Universitet, Institut for Sporeplanter, 137 pp.

- Bresnan, E. (2003). FRS Contract Report 14/03, 53 pp.
- Fehling, J., Davidson, K., Bolch, C. & Tett, P. (2006). Mar. Ecol. Progr. Ser. 323: 91-105.
- Fehling, J., Green, D.H., Davidson, K., Bolch, C.J. & Bates, S.S. (2004). J. Phycol. 40: 622-630.
- Fraser, S., Bresnan, E. & Moffat, C. (2004). FRS Contract Report 12/04, 25 pp.
- Fraser, S., Brown, L. & Bresnan, E. (2006). FRS Contract Report 03/06, 28 pp.
- Fraser, S., Brown, L., McCollin, T. & Bresnan, E. (2006). FRS Contract Report 04/06, 16 pp.
- Gallacher, S., Howard, G., Hess, P., MacDonald, E., Kelly, M.C., Bates, L.A., Brown, N., MacKenzie, M., Gillibrand, P. & Turrell, W.R. (2001). In: Harmful Algal Blooms, Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. & Lewis, R.J. (eds), UNESCO, Paris, pp. 30-33.
- Lundholm, N., Moestrup, Ø., Hasle, G. & Hoef-Emden, K. (2003). J. Phycol. 39: 797-813.
- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. & Miller, P. (2006). J. Phycol. 42: 464-481.
- Skov, J., Lundholm, N., Moestrup, Ø. & Larsen, J. (1999). ICES Identification Leaflets for Plankton 158, 23 pp.
- Utermöhl, H. (1958). Zur Vervollkomnung der quantitativen Phytoplankton Methodik. Mitt. Int. Ver. Limnol. 9: 1-38.

## Dinoflagellate cysts from New Zealand ports and harbours with emphasis on the distribution of harmful species

F. Hoe Chang<sup>1</sup>, R. Stewart<sup>1</sup>, G. Inglis<sup>2</sup> and I. Fittridge<sup>2</sup>

<sup>1</sup>National Institute of Water & Atmospheric Research Ltd, Private Bag 14-901, Kilbirnie, Wellington, New Zealand; <sup>2</sup>National Institute of Water Atmospheric Research Ltd., Riccarton, Christchurch, New Zealand

### Abstract

Twenty two distinct cyst types were identified in sediment samples collected from fourteen ports and harbours in New Zealand. Six species are harmful or potentially harmful: four PSP-producing (*Gymnodinium catenatum*, *Alexandrium tamarense*, *A. cf. catenella* and *A. minutum*) and two yessotoxin and yessotoxin-like producing species (*Protoceratium reticulatum* and *Lingulodinium polyedrum*). Cysts of *G. catenatum* were confined to North Island ports and harbours, while those of *Alexandrium* spp. were more common on the North Island north-east coast than other parts of New Zealand. As yet, there is no evidence that harmful species have been introduced from overseas.

### Introduction

Some dinoflagellate species form resting cysts as part of their sexual life cycle (Dale 1983). Over time these cysts settle to the sea bed and provide a record of species existing in the area. Little is known about the distribution of harmful species, in particular non-bloom forming species, in New Zealand and whether any of these are non-indigenous. Between 2003 and 2005 surface sediment samples collected from fourteen ports and harbours were analyzed for dinoflagellate cysts with the aim of mapping the distribution and determining whether any of these are exotic species. These samples were part of large scale baseline surveys for the Ministry of Agriculture and Forestry (MAF) and Biosecurity New Zealand (BNZ).

### Materials and Methods

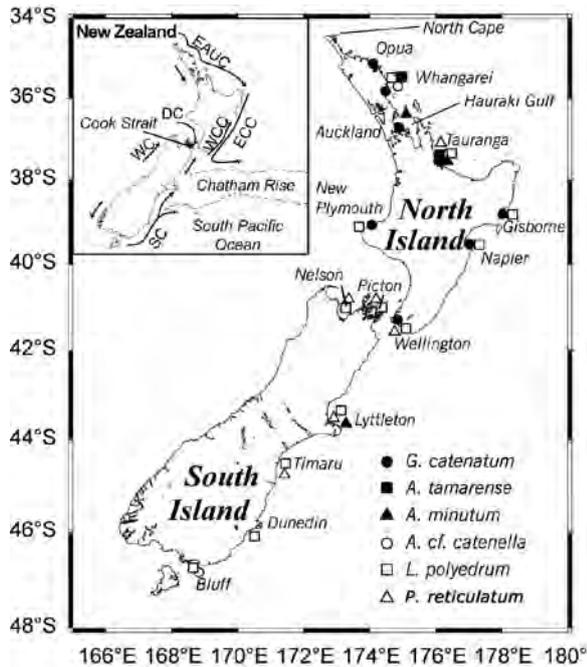
A total of 179 undisturbed surface sediment samples (4-5 cm) were collected by hand using SCUBA, or by using a harpoon corer in fourteen ports and harbours of New Zealand (Fig. 1). The samples were sealed in airtight plastic jars and kept cold at 4 °C in the dark until processing (Dale 1979). Subsamples were sonicated and processed as described by Bolch and Hallegraeff (2000); cysts were examined and counted using an inverted Nikon Diaphot light microscope. Selected cyst types were isolated for germination experiments; isolated cysts were maintained in f/2 medium (Guillard and Ryther 1962) at 18 °C with 80 μmol photons m<sup>-1</sup> s<sup>-1</sup> of cool-white fluorescent light under a 14:10 h L:D regime. Identification of cysts follows Matsuoka and Fukuyo (2000) and literature detailed by Bolch and Hallegraeff (1990). Determination of some species were confirmed by germination experiments.

### Results and Discussion

A total of 22 distinct cyst types were identified. Six are harmful or potentially harmful: four PSP- (*Gymnodinium catenatum*, *Alexandrium tamarense*, *A. cf. catenella* and *A. minutum*) (Figs 2-5) and two yessotoxin- and yessotoxin-like producing species (*P. reticulatum* and *L. polyedrum*) (Figs 7, 8). The PSP-producing gonyaulacoids *A. tamarense*, *A. minutum* and *A. cf. catenella*, appeared to be common on the north-east coast of North Island (Fig.1), while yessotoxin and yessotoxin-like producing species, were common in most sampling sites. Cysts of the only PSP-producing gymnodinioid, *G. catenatum*, was confined to ports and harbours of the North Island (Fig. 1). The rest were representatives of various protoperidinioid and gonyaulacoid groups (Figs 9-21) and also two unknown cyst types (Figs 22, 23). *A. minutum*, *A. tamarense*, *L. polyedrum*, *P. conicum* and *Scrippsiella trochoidea* were successfully germinated.

Dinoflagellate cyst types recorded in the present study are broadly similar to those identified in surveys of the Marlborough Sounds, New Zealand (Baldwin 1987), and Tasmania, Australia (Bolch and Hallegraeff 1990). However, the number of harmful or potentially harmful species found in this study is higher than those reported both in Marlborough Sounds (*P. reticulatum*, *L. polyedrum*) and Tasmania (*G. catenatum*, *A. tamarense* and *P. reticulatum*), reflecting the larger area and more diverse environments surveyed in this study.

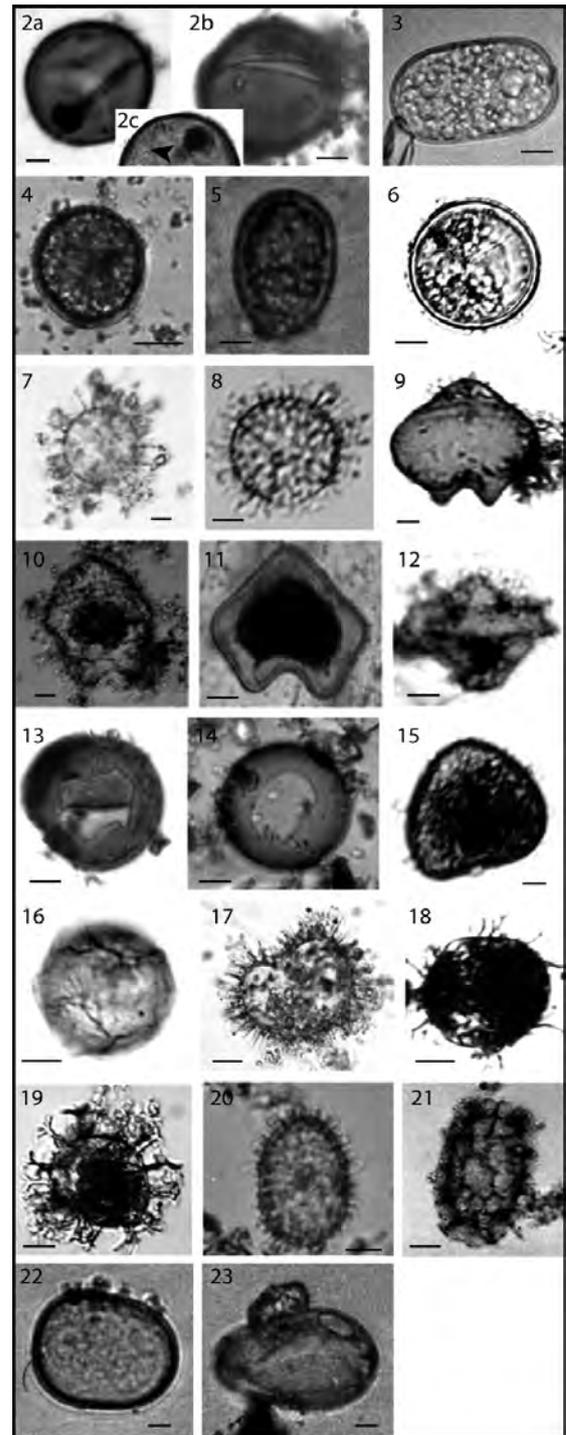
The micro-reticulate cysts of *G. catenatum* found in New Zealand are confined to North Island ports and harbours, coinciding approximately with the spread of



**Figure 1.** Map of New Zealand showing surface sediment sampling sites and distribution of six HAB species at each site. Inset: schematic representation of coastal currents, EAUC (East Auckland Current), DC (D'Urville Current), WCC (Wairarapa Coastal Current), ECC (East Cape Current), WC (Westland Current), and SC (Southland Current).

the blooms in 2000 (MacKenzie and Adamson 2000). However, during the 2000 PSP episodes, *G. catenatum* was not reported on the North Island north-east coast. Present records of *G. catenatum* cysts in ports of Opuia, Whangarei and Auckland (Fig. 1) and the recording of a small build-up of motile cells of *G. catenatum* in April 2003 in Hauraki Gulf (MacKenzie pers. comm.), implies a "spill-over" of the 2000 bloom from the north-west coast near North Cape into the East Auckland Current (Fig. 1) to 'downstream' areas on the north-east coast. Like *G. catenatum*, cysts of *Alexandrium* spp. were also found in areas where blooms were previously reported (Chang *et al.* 1997; MacKenzie *et al.* 2004).

At this point it is difficult to determine to what extent the New Zealand dinoflagellate flora is indigenous, although there is evidence of links to Australia particularly concerning *G. catenatum* and *A. minutum*. In Australia, blooms of both species were reported prior to 1987 (Hallegraeff and Lucas 1988; Hallegraeff *et al.* 1989), while in New Zealand, blooms of *A. minutum* were reported for the first time in 1993 (Chang *et al.* 1997), and of *G. catenatum* in 2000 (MacKenzie and Adamson 2000). The N.Z. strains of the former were found to be genetically and toxicologically simi-



**Figure 2-21.** Dinoflagellate cysts found in ports and harbours of New Zealand: 2a-b) *G. catenatum*; 2c) microreticulate pattern (arrowhead); 3) *A. tamarensis*; 4) *A. minutum*; 5) *A. cf. catenella*; 6) *A. cf. affine*; 7) *L. polyedrum*; 8) *P. reticulatum*; 9) *Protoperidinium leonis*; 10) *P. cf. pentagonum*; 11) *P. oblongum*; 12) *P. subinermis*; 13) *P. cf. avellana*; 14) *P. cf. punctulatum*; 15) *P. claudicans*; 16) *P. cf. americanum*; 17) *P. conicum*, 18) *Gonyaulax spinifera* (*Spiniferites mirabilis*); 19) *G. scrippsae* (*S. bulloideus*) 20) *Scrippsiella trochoidea*; 21) *Polykrikos schwartzii*. 22) and 23) Unknown spp. All scale bars 10µm.

lar to the south-eastern Australian type (de Salas *et al.* 2001), while nucleotide sequences of the later were found to be identical to isolates from Australia, Singapore, Korea, Spain and Uruguay (Holmes *et al.* 2002). However, radionuclide studies of *G. catenatum* cysts collected from sediments of the New Zealand north-west coast demonstrated the presence of this species since 1937 and from Australia around 1972 (Irwin *et al.* 2003). This rules out that *G. catenatum* was introduced by ballast waters from Australia into New Zealand.

### Acknowledgements

We thank the MAF, BNZ for making these data available (contract ZBS2000-04), the NIWA Biosecurity team for collecting the samples, Drs D. Robertson and J. M. Grieve for their constructive criticisms of this manuscript and the New Zealand Foundation of Research, Science & Technology for support (contract CO1214 to FHC).

### References

- Baldwin, R. P. (1987). N.Z. J. Mar. Freshw. Res. 21: 543-553.
- Bolch, C. J. & Hallegraeff, G. M. (2000). Bot. Mar. 33: 173-192.
- Chang, F. H., Anderson, D.M., Kulis, D.M. & Till, D. G. (1997). Toxicon 35: 39-409.
- Dale, B. (1979). In: Toxic Dinoflagellate Blooms, Taylor, L. & Seliger, H. H. (eds), Elsevier North Holland, pp. 443-452.
- Dale, B. (1983). In: Survival Strategies of the Algae, Fryxell, G.A. (ed.), Cambridge Univ. Press, Cambridge, pp. 69-136.
- De Salas, M.F., van Emmerick, M.J., Hallegraeff, G.M., Negri, A.P., Vaillancourt, R.E. & Bolch, C.J. (2001). In: Harmful Algal Blooms, Hallegraeff, G.M., Bolch, C.J. & Lewis, R.J. (eds), IOC of UNESCO, pp. 214-217.
- Guillard, R.R.L. & Ryther, J.H. (1962). Can. J. Microbiol. 8: 229-239.
- Hallegraeff, G.M. & Lucas, I. (1988). J. Plankton Res. 10: 533-541.
- Hallegraeff, G., Bolch, C., Koerbin, B. & Bryan, J. (1989). Austr. Fisher. July: 32-34.
- Hallegraeff, G.M., Stanley, S.O., Bolch, C.J. & Blackburn, S.I. (1989). In: Red Tides: Biology Environmental Science and Toxicology, Okaichi, T., Anderson, D.M. & Nemoto, T. (eds), Elsevier, pp. 77-80.
- Holmes, J.H., Bolch, C.J., Green, D.H., Cembella, A.D. & Teo, S.L.M. (2002). J. Phycol. 38: 96-106.
- Irwin, A., Hallegraeff, G.M., McMinn, A., Harrison, J. & Heijnis, H. (2003). Harmful Algae 2: 61-74.
- MacKenzie, L. & Adamson, J. (2000). In: Proc. Marine Biotoxin Science Workshop No. 14, MAF, pp. 13-25.
- MacKenzie, L., de Salas, M., Adamson, J. & Beuzenberg, V. (2004). Harmful Algae 3: 71-92.
- Matsuoka, K. & Fukuyo, Y. (2000). Technical Guide for Modern Dinoflagellate Cyst Study. WEST-PAC-HAB Office, Asian Natural Environmental Science Centre, Japan, 29 pp.

# Accumulation and transfer of the amnesic shellfish poisoning toxin, domoic acid, in the marine food web off the Portuguese coast

P.R. Costa<sup>1</sup>, S. Garrido<sup>1</sup>, R. Rosa<sup>1</sup>, M. Ferreira<sup>2</sup>, M. Sequeira<sup>2</sup>, V. Brotas<sup>3</sup> and M.A.M. Sampayo<sup>1</sup>

<sup>1</sup> IPIMAR, Av. de Brasília, 1449-006 Lisboa, Portugal, prcosta@ipimar.pt

<sup>2</sup> Instituto de Conservação da Natureza, R. Santa Marta 55, 1150-294 Lisboa, Portugal

<sup>3</sup> Instituto de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

## Abstract

In North America, hundreds of marine mammals and sea birds have died after ingestion of fish contaminated with domoic acid (DA). In European coastal waters such outbreaks have not yet been described and the transference of DA through the marine food web has only been scarcely studied. With this work we aimed to investigate the accumulation and transference of DA in the marine food web off the Portuguese coast. During 2001-2005, pelagic and benthic members of the marine fauna were collected for DA determination. The toxin was detected in sardines (*Sardina pilchardus*), 2 species of crustaceans (*Polybius henslowii* and *Necora puber*) and 6 species of cephalopods (*Octopus vulgaris*, *Eledone moschata*, *E. cirrhosa*, *Sepia officinalis*, *S. elegans* and *S. orbignyana*). Because the diet of the common dolphin (*Delphinus delphis*) in Portugal is dominated by sardines and cephalopods, tissue samples (kidney and intestine) of stranded marine mammals were analysed for DA. The analysed samples did, however, not reveal contamination in the top predator in the marine food web. The present data show that DA through interactions in the marine food web reach several groups of animals and hence pose potential risks to human health safety.

## Introduction

Upwelling events migrating from the south of Morocco to the north of Portugal mainly from April to September are responsible for the occurrence of algal blooms in Portugal (Abrantes and Moita 1999). These blooms include the diatom genus *Pseudo-nitzschia*, in which several species are known to produce the toxin called domoic acid (DA) and cause amnesic shellfish poisoning (ASP) in humans. Every year in Portugal, shellfish beds are closed to harvesting during some periods due to domoic acid contamination. The Portuguese monitoring programme as well as those from most countries of Europe only includes bivalve molluscs for routine analyses. Scientific studies on domoic acid vectors other than bivalves are scarce in Europe. On the other hand, in North America, DA contamination of the marine food web has led to DA poisoning events with massive deaths of marine mammals and birds after ingestion of contaminated fish (e.g. Scholin *et al.* 2000).

In this study, we aim to evaluate the existing knowledge and add new data to investigate potential domoic acid contamination of the marine food web off the Portuguese continental coast and answer the following questions: i) are there any filter-feeding organisms other than bivalve molluscs that can act as DA vectors? ii) can carnivorous organisms accumulate DA? iii) does DA reach the top of the marine food

web? To be able to answer these questions, we investigated DA accumulation and *Pseudo-nitzschia* ingestion in sardines, and DA accumulation in crustaceans, cephalopods and in stranded marine mammals.

## Materials and Methods

Sardines were selected as filter feeding organisms. They are planktivorous fish with high ecological and socio-economical importance. Several species of crustaceans, octopus, cuttlefish and squids were selected as carnivorous organisms. Six species of stranded marine mammals were also dissected to investigate the DA contamination at the top of the marine food web (Table 1). The selected marine organisms were captured or collected along the Portuguese west coast between 2001 and 2005 as reported in Costa *et al.* (2003, 2004, 2005a,b) and Costa and Garrido (2004). Tissues were dissected and stored at -20 °C until the DA analyses, which were carried out using LC-UV and LC-MS as reported in Costa *et al.* (2005a). Additionally immunoaffinity chromatography cartridges (Abkem Iberia, Spain) were used for DA analyses of the cephalopod matrix.

## Results and Discussion

### *Planktivorous organisms*

Domoic acid was monitored in sardine guts during 2002 and 2003. The period of toxin occurrence in both

**Table 1.** Domoic acid ( $\mu\text{g g}^{-1}$ , ww) detected in sardines (viscera), crabs (whole carapace meat), cephalopods (digestive gland) and marine mammals (kidney and intestine) (+++ >100  $\mu\text{g DA g}^{-1}$ ; ++ 10 to 100  $\mu\text{g DA g}^{-1}$ ; + up to 10  $\mu\text{g DA g}^{-1}$ , nd = not detected).

Class	Order	Species	Common Name	Life Style Strategy	Domoic Acid	Reference
Osteichthyes	Clupeiformes	<i>Sardina pilchardus</i>	Sardine	Pelagic	+++	Costa & Garrido 2004
Malacostraca	Decapoda	<i>Polybius henslowii</i>	Crab	Pelagic	+++	Costa <i>et al.</i> 2003
		<i>Necora puber</i>		Benthic	++	This study
Cephalopoda	Octopoda	<i>Octopus vulgaris</i>	Octopus	Benthic	+++	Costa <i>et al.</i> 2004
		<i>Eledone cirrhosa</i>		Benthic	+	Costa <i>et al.</i> 2005b
		<i>Eledone moschata</i>		Benthic	++	Costa <i>et al.</i> 2005b
	Sepioidea	<i>Sepia officinalis</i>	Cuttlefish	Nektobenthic	+++	Costa <i>et al.</i> 2005a
		<i>Sepia orbignyana</i>		Nektobenthic	+	This study
		<i>Sepia elegans</i>		Nektobenthic	+	This study
		<i>Rossia macrossoma</i>		Nektobenthic	nd	This study
	Teuthoidea	<i>Loligo vulgaris</i>	Squid	Nektobenthic	nd	This study
		<i>Illex coindetii</i>		Pelagic	nd	This study
		<i>Todarodes sagittatus</i>		Pelagic	nd	This study
<i>Todaropsis eblanae</i>			Pelagic	nd	This study	
Mammalia	Cetacea (Odontoceti)	<i>Delphinus delphis</i>	Marine mammal	Pelagic	nd	This study
		<i>Kogia breviceps</i>		Pelagic	nd	This study
		<i>Phocoena phocoena</i>		Pelagic	nd	This study
		<i>Stenella coeruleoalba</i>		Pelagic	nd	This study
		<i>Tursiops truncatus</i>		Pelagic	nd	This study
	Cetacea (Mysticeti)	<i>Balaenoptera acutorostata</i>		Pelagic	nd	This study

years was from May to August/September. The maximum value detected in sardine guts reached a concentration of  $128 \mu\text{g g}^{-1}$  which is within the range of values reported by Scholin *et al.* (2000) during a toxic event in the USA where hundreds of marine mammals died. In our study no DA was detected in the muscle tissue or in the sardine brain. Consequently human intoxications due to sardine consumption are unlikely to occur since sardine viscera are not usually

eaten. Nonetheless, if higher DA concentrations are reached and whole body animal are consumed human health might in such case be threatened. Since DA was not found in the animal brain neurological effects on sardines at this range of values are not expected. Identification and quantification of algal prey in sardine stomach contents permitted to establish a relationship between DA occurrence in sardines and ingested *Pseudo-nitzschia australis* (Costa and Garrido 2004).

### *Carnivorous organisms*

Crustaceans are known to be DA vectors in some regions of the world, but European studies on this subject are limited. We have chosen the swimming crab *Polybius henslowii* as a model because although it has a small commercial value, it is frequently the most abundant species on the Portuguese and Spanish continental shelf and reaches very high densities during certain seasons and areas. It may reach levels of more than 90% of the total biomass. During the summer of 2002, eleven crab samples were collected from 4 different locations and DA was detected in all of the eleven samples (max. concentration detected was 323.1  $\mu\text{g g}^{-1}$ ; Costa *et al.* 2003). In 2005, during an algal bloom including the diatom *Pseudo-nitzschia*, DA was detected in another crab species called the velvet swimming crab, *Necora puber*, which has a high commercial value. Domoic acid concentrations in pooled samples collected from Cascais reached a concentration of 29.9  $\mu\text{g g}^{-1}$  and to our knowledge this is the first report of *Necora puber* as a DA vector (Table 1). Further work is needed to identify other commercially valuable crustacean species that can also act as DA vectors.

Cephalopods are active predators of known toxin vectors such as bivalves, crabs and fish. The distribution of DA in cephalopod tissue revealed the digestive gland, a site of digestive absorption, as the main tissue for DA accumulation (up to 241.7  $\mu\text{g g}^{-1}$ ), followed by the branchial hearts (up to 114.3  $\mu\text{g g}^{-1}$ ). The branchial hearts are organs that have a pumping function, but they also have an important role in the detoxification system. Human intoxications are not expected because the toxin was not found in the cephalopods muscle, which is the main edible part. However, in some Mediterranean countries (e.g. Portugal, Spain), whole body of cuttlefish and juvenile octopus are consumed (i.e. without evisceration) and in this case, public health might be affected (Costa *et al.* 2004, 2005a,b). Domoic acid was consistently detected in several species of octopus and cuttlefish but not in squids, revealing that neritic species with benthic or near-benthic life strategies (Order Octopoda and Sepiidae) are more exposed to the toxin than pelagic and demersal ones (Order Teuthoidea) (Table 1). These differences are related to distinct feeding ecologies. The former groups are known predators of domoic acid vector prey. On the other hand, it has been suggested by Bargu *et al.* (2002) that squids by preying on contaminated krill might be exposed to DA.

### *Higher trophic levels*

Sardines followed by cephalopods are the main diet of dolphins (*Delphinus delphis*) along the Portuguese coast (Silva 1999). In this study, we examined the presence of domoic acid in the intestine and kidney tissues of 6 stranded marine mammal species (Table 1). However no domoic acid was detected in any samples analysed. The reasons could be that i) DA was rapidly excreted from the marine mammal body and therefore we could not detect it in stranded animals; ii) DA levels detected in marine mammal prey along the Portuguese coast were not as high as those found in other regions of the world where toxic events led to death of sea lions and sea birds; iii) tissue samples were only obtained from dead stranded animals and a putative DA contamination of marine mammals might not be enough to cause their death. Nonetheless, contamination of the higher trophic levels can potentially occur and we are increasing our efforts to achieve better sampling strategy and fast identification of strandings followed by a properly tissue collection procedure for marine biotoxins analysis.

On the Portuguese coast, which may serve as model for European waters, there is an evidence for the presence of DA in several organisms serving as toxin vectors that are crucial to be further studied. These combined results can be helpful for governmental agencies responsible for public health safety and for environmental agencies concerned with the marine ecosystems stability.

### References

- Abrantes, F. & Moita, M.T. (1999). *Oceanol. Acta* 22: 319-336.
- Bargu, S., Powell, C.L., Coale, S.L., Busman, M., Doucette, G.J., Silver & M.W. (2002). *Mar. Ecol. Progr. Ser.* 237: 209-216.
- Costa, P.R. & Garrido, S. (2004). *Mar. Ecol. Progr. Ser.* 284: 261-268.
- Costa, P.R., Rodrigues, S.M., Botelho, M.J. & Sampayo, M.A.M. (2003). *Toxicon* 42: 135-141.
- Costa, P.R., Rosa, R. & Sampayo, M.A.M. (2004). *Mar. Biol.* 144: 971-976.
- Costa, P.R., Rosa, R., Duarte-Silva, A., Brotas, V. & Sampayo, M.A.M. (2005a). *Aquat. Toxicol.* 74: 82-91.
- Costa, P.R., Rosa, R., Pereira, J. & Sampayo, M.A.M. (2005b). *Aquat. Living Resour.* 18: 395-400.
- Scholín, C.A. & 25 others (2000). *Nature* 403: 80-84.
- Silva, M.A. (1999). *J. Mar. Biol. Ass. U.K.* 79: 531-540.

## Domoic acid intrusion into Puget Sound, Washington, USA

F. Cox<sup>1</sup>, J. Borchert<sup>2</sup> and B. Lona<sup>3</sup>

<sup>1</sup>Washington State Department of Health, PO Box 47824, Olympia WA 98504-7824, frank.cox@doh.wa.gov

<sup>2</sup>Washington State Department of Health, PO Box 47824, Olympia WA 98504-7824, Jerry.Borchert@doh.wa.gov

<sup>3</sup>Washington State Department of Health, Public Health Laboratories, 1610 NE 150th Street, Shoreline, WA 98155, Bob.Lona@doh.wa.gov

### Abstract

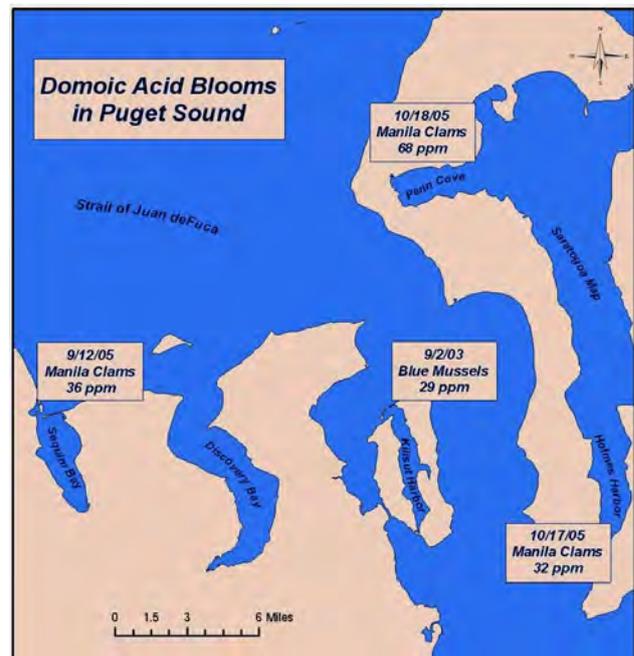
Domoic acid which causes Amnesic Shellfish Poisoning was detected in razor clams on the Pacific coast of Washington in 1991. Emergency closures of the razor clam and crab fisheries were enacted. As a result, a domoic acid monitoring program was established for the Pacific and Puget Sound fisheries. In 2003, domoic acid was detected inside Puget Sound, at Ft. Flagler State Park. The U.S. Food and Drug Administration action level of 20 ppm was exceeded and shellfish closures were initiated. Domoic acid was detected at low levels as far west as Port Angeles, as far east as East Whidbey Island and as far south as Port Ludlow. In October, 2005, domoic acid prompted new closures in Puget Sound, involving Penn Cove and Holmes Harbor, two commercial shellfish areas in the Whidbey Basin. These test results were much higher and impacted shellfish other than mussels. Dungeness crab were also tested but were below the closure level. If domoic acid continues to move into new areas inside Puget Sound, the economic and public health implications will be significant. Puget Sound could be in for some very long shellfish closures, if shellfish such as geoducks retain the toxin like razor clams do.

### History

Domoic acid (DA), which causes Amnesic Shellfish Poisoning, was detected for the first time, in razor clams (*Siliqua patula*) and Dungeness crab (*Cancer magister*) on the Pacific coast of Washington in the fall of 1991. To protect public health, emergency closures of the razor clam and crab fisheries were enacted by the Washington State Departments of Health and Fish & Wildlife (DOH) (WDFW). Since 1991, domoic acid blooms have been a recurring annual phenomenon on the Pacific coast of Washington, necessitating the establishment of a DA monitoring program for the Pacific coast razor clam and crab fisheries. Detection and quantification of domoic acid in razor clams and Dungeness crab is accomplished by use of High Performance Liquid Chromatography (Lawrence *et al.* 1989). DA, a strong neurotoxin, is produced by a number of species of diatoms belonging to the genus *Pseudo-nitzschia* that occur in Washington marine waters. The symptoms occur in two stages. Within 24 hours of consumption of toxic shellfish, gastrointestinal symptoms including vomiting, nausea, abdominal cramps and diarrhea occur. If enough toxin has been ingested, neurological symptoms, including headache, dizziness, confusion, disorientation, short term memory loss, motor weakness, seizures, coma and sometimes death, occur within 48 hours. Since 1991, a number of emergency closures for razor clam beaches have been necessary due to DA. The razor

clam resource represents about 10 million \$/year to the coastal economy. Since 1991, on average, almost 25 % of the harvest days have been lost due to DA closures, representing a significant impact to the coastal economy.

In 1990, as part of a sentinel monitoring system for PSP toxin, DOH established mussel cages at over 70 sentinel sites in Washington marine waters. How-



**Figure 1.** Puget Sound locations where DA caused the first shellfish closures.

ever, the 1991 DA coastal event prompted DOH to begin monitoring many of these sentinel sites for DA as well as PSP, even though it had not been detected in Puget Sound. Since the DA monitoring began, a number of the Puget Sound sites have sporadically recorded low levels of DA. Fortunately, the levels were well below the U.S. Food & Drug Administration action level of 20 ppm.

In September 2003, DA was detected in mussels inside Puget Sound at Ft. Flagler State Park on Kilisut Harbor (Fig.1) at levels above the FDA action level of 20 ppm. This prompted the first ever DA shellfish

closure inside Puget Sound (Trainer *et al.* in press). While the highest DA level (Fig. 2) was detected at Kilisut Harbor, it was detected at low levels as far west as Port Angeles, as far east as East Whidbey Island and as far south as Port Ludlow. During this bloom event, blue mussels were the only shellfish species to exceed the action level.

Two years later, in September and October 2005, DA prompted new closures in Puget Sound. The September bloom occurred in Sequim Bay, which had DA at levels as high as 36 ppm on Manila clams. Sequim Bay had previously been involved in the bloom of

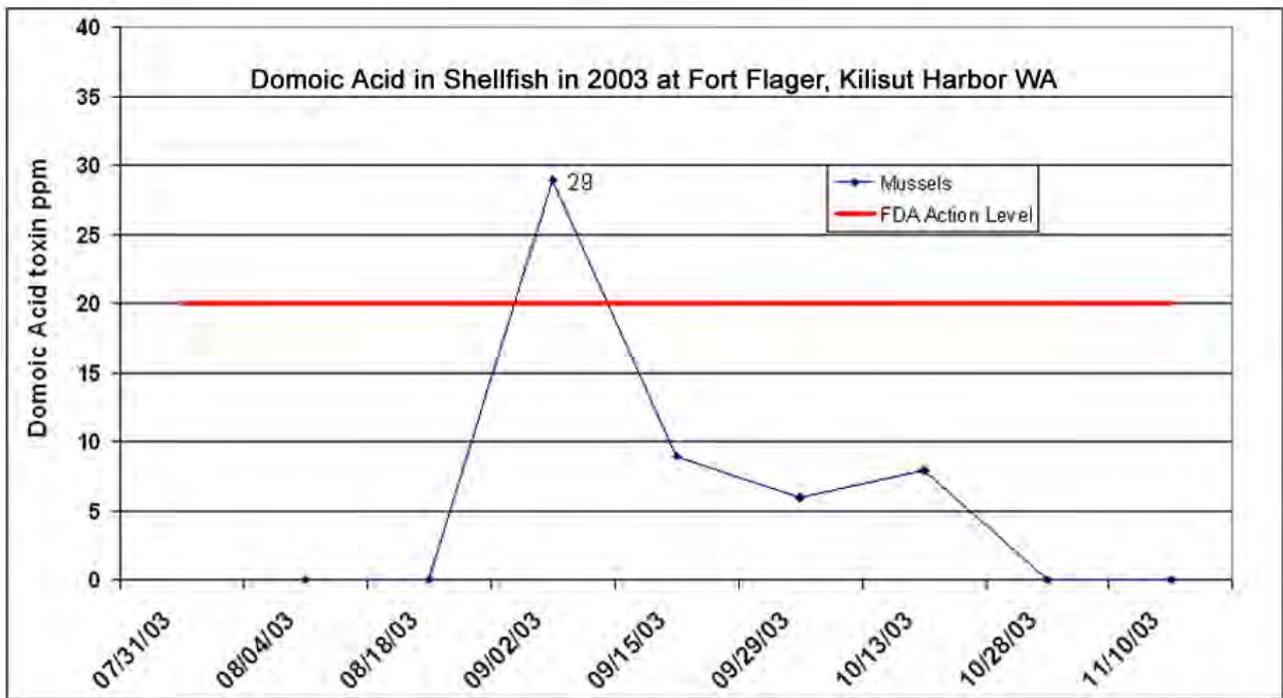


Figure 2. DA in Fort Flagler, Kilisut Harbor, shellfish in fall, 2003.

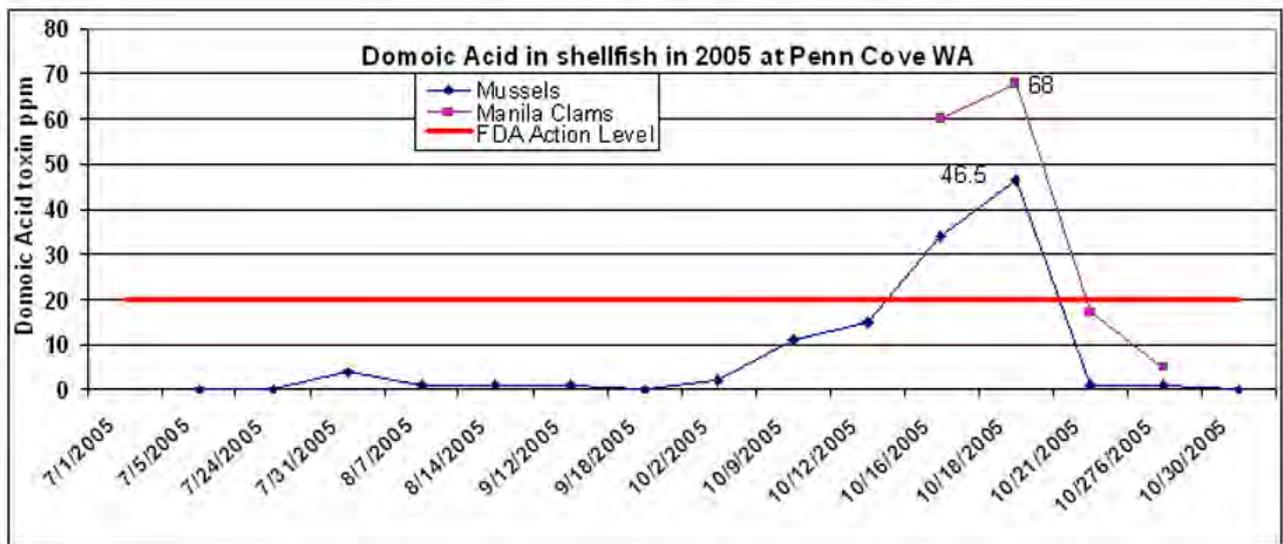


Figure 3. DA in Penn Cove shellfish in fall, 2005.



**Figure 4.** Geoduck Clams, the world's largest burrowing clam have been documented to live up to 150 years (Rice 1971). Geoducks contribute about \$9.2 million annually to Washington's economy. Extended closures of geoduck beds due to ASP would result in significant economic losses for Washington.

2003. However, in 2003, DA test results only reached 10 ppm, failing to reach closure levels. On the other hand, the October DA bloom involved new areas at Penn Cove, Saratoga Passage and Holmes Harbor (Fig. 1). These new closures had test results which were much higher than the 2003 bloom at Ft. Flagler, Kulisut Harbor and impacted shellfish other than mussels. The highest DA level was recorded in Manila clams (*Tapes philippinarium*) at 68 ppm (Fig. 3) at Penn Cove. Dungeness crab were also tested but were below the closure level.

### Continuing Issues

In 1998, DA levels in razor clams on Washington's coast reached almost 300 ppm. According to WDFW, an entire year class died of old age, before they were safe again (D. Ayres, WDFW, pers. comm.). Some razor clam beaches were closed for almost two years. Puget Sound generates a large share of Washington's annual shellfish revenue of \$73.5 million. Approximately 2 million pounds of geoduck clams (*Panopea abrupta*) (Fig. 4) are harvested per year from Puget Sound, representing about \$9.2 million in revenue to Washington. These shellfish are reported to live for 150 years or more (R. Sizemore, WDFW, pers. comm.). If they retain DA for long periods, then harvest closures could last for years. However, depuration rates for these and other Puget Sound shellfish

species are not well understood and research is needed to determine how Puget Sound shellfish metabolize DA. Puget Sound shellfish depuration rates for PSP toxins are known to vary greatly. While Blue Mussels (*Mytilus edulis*) have shown rapid PSP depuration rates, the Butter Clam (*Saxidomus giganteus*) have demonstrated a real propensity for PSP toxin retention. It is our expectation that DA depuration rates will vary just as much as the PSP depuration rates in Puget Sound shellfish.

If DA continues to move into new areas inside Puget Sound, the economic and public health implications will be significant. The Washington State Public Health Laboratory currently analyzes about 3500 PSP and 1500 DA tests per year. Continued DA movement into Puget Sound could require a large increase in DA testing, even to equal PSP in number, causing a large budgetary impact.

Additional "proactive" early warning systems for *Pseudo-nitzschia* blooms and DA in the water column need to be developed for Puget Sound. The current "reactive" system which relies on detecting DA levels in shellfish once they have become toxic is not in keeping with modern public health principles, which stresses prevention.

### Acknowledgements

The authors would like to acknowledge the Biotoxin Laboratory staff for their contribution to the biotoxin program and Nancy West for providing the map.

### References

- Lawrence, J.F, Charbonneau, C.F, Menard, C, Quilliam, M.A. & Sim, P.G. (1989). Liquid chromatographic determination of domoic acid in shellfish products using the paralytic shellfish poison extraction procedure of the Association of Official Analytical Chemists. *J Chromatogr.* 462: 349-56.
- Rice, T. (1971). *Marine Shells of the Pacific Northwest*, Ellison Industries, Inc., Edmonds, WA., 102 pp.
- Trainer, V.L., Cochlan, W.P., Erickson, A., Bill, B.D., Cox, F.H., Borchert J.A. & Trainer, V.L. In press. Recent domoic acid closures of shellfish harvest areas in Washington State inland waterways. *Harmful Algae*.

## Changes of seasonality and causative species in harmful algal blooms in the Bohai Sea area 1950-2004

BP. Di<sup>1</sup>, DanLing Tang<sup>1, 3\*</sup>, F.L He<sup>2</sup> and J. Li<sup>3</sup>

<sup>1</sup>Remote Sensing and Marine Ecology Group, LED, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China; \* Corresponding Author, lingzistdl@126.com, <sup>2</sup>Department of Renewable Resources of University of Alberta Edmonton, Alberta T6G 2H1, Canada, fhe@ualberta.ca,

<sup>3</sup>Department of Environmental Sciences and Technology, Fudan University, ShangHai, China

### Abstract

We observed changes in seasonality and causative species of harmful algal blooms in the Bohai Sea during the period 1950-2004. The months with the highest occurrences of blooms were July to August in the 1980s and 1990s and June to August in 2000-2004. Before the 1990s, the dominant causative species was *Skeletonema costatum*, but this changed to *Noctiluca scintillans* after 1992. The extension of the bloom season from two to three months starting already in June might be associated with changes in the dominant species and with increases in water temperature.

### Introduction and Method

Harmful algal blooms (HABs) may pose threats to marine ecosystems and public health and cause economic losses (Anderson 1997; Tang *et al.* 1998, 2004a, b, 2006). The Bohai Sea is a semi-enclosed sea in north-eastern China (Fig. 1), where HABs have occurred frequently and caused large economic losses in recent decades (Tang *et al.* 2006).

In the present study, we examined 161 historical HAB data sets from the period 1950-2004 obtained from the state oceanic administration of China (SOA), published papers, conference proceedings and other available sources. HABs are identified as blooms of algal species that have negative impacts on humans, marine environments, and/or coastal economies. Our results illustrate changes in seasonality and causative species of HABs in this area.

### Results and Discussion

#### *HAB occurrences and damages*

Aquaculture is important to local development. HABs have, however, caused great losses to the local economy and aquaculture industries. About 161 HABs have been observed in the Bohai area during 1950-2003 (Fig. 2). The most serious events recorded are shown in Table 1.

#### *Seasonality of HABs*

HABs occurred in spring, summer and autumn. Most occurred in summer (June to August) appearing along almost all coastal areas of the sea, while in other seasons they occurred at a relatively limited number of sites. There has, however, been an extension in the duration of the intensive bloom period from before

1980s until today as well as a shift towards an earlier start of the bloom period. The HABs appeared mainly in August in the period 1950-1979. The bloom frequency increased in the 1980s with most blooms in July and August but also some in June. In the 1990s the frequency of blooms increased further, still mostly in July-August but many in June. In 2000-2004, blooms also took place in June-August, but with a lower overall frequency (Fig. 3).

The seasonal distribution of HABs may be related to meteorology (Tang *et al.* 2004b). In the Bohai Sea, the average temperature is 25 °C in summer and below 5 °C in winter. A low water temperature (5 °C) in winter can limit algal growth. Summer is the season with high precipitation, and more nutrients enter the

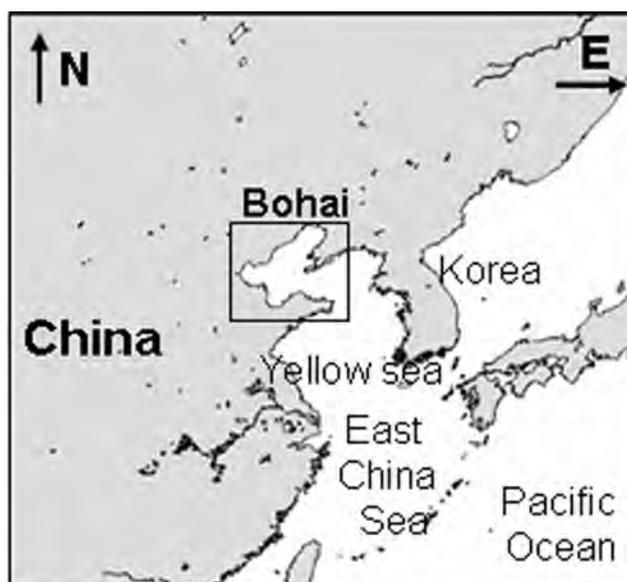
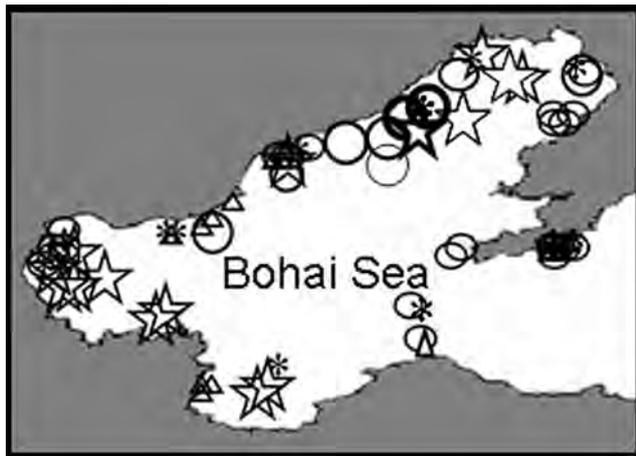


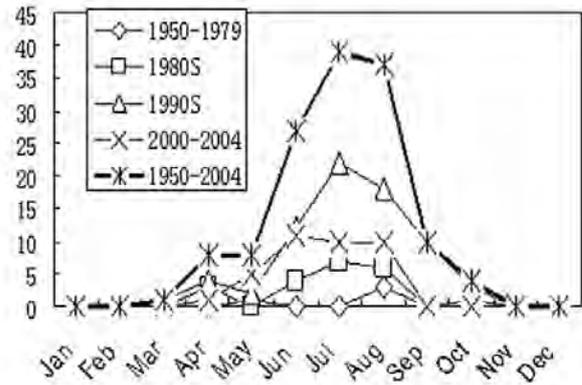
Figure 1. The location of Bohai Sea.



**Figure 2.** The spatial distributions of HAB occurrences from 1950 to 2004. Size of HAB area (km<sup>2</sup>):  $\Delta$  < 100,  $\circ$  100-1000,  $\star$  > 1000,  $*$  without precise record of size.

sea from land. The high temperature and high concentrations of nutrients in summer favour algal blooms.

The seasonal variation in HAB species may also be related to meteorology. The water temperature in the Bohai Sea increased 0.024 °C per year from 1960 to 1997 (Lin *et al.* 2001) and the warming may advance and extend the blooming season.



**Figure 3.** Monthly distribution of HABs in different periods during 1950-2004 in Bohai Sea area.

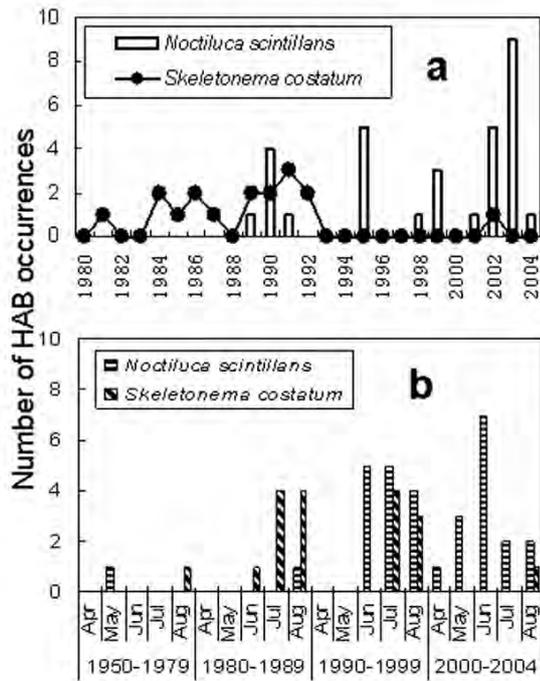
#### Causative species of HABs

*Skeletonema costatum* and *Noctiluca scintillans* are the major HAB species, *S. costatum* dominating in the 1980s, but *N. scintillans* has dominated since 1992 (Figs 3, 4). *N. scintillans* blooms increased substantially in 2004.

The shift and extension in blooming time may be due to the variation in the dominant causative species. *Skeletonema costatum* blooms in June-August with highest frequency in August, while *Noctiluca scintil-*

**Table 1.** Major HABs that have caused large economic losses and fish kills in Bohai Sea 1950 - 2004. RMB: Chinese currency (1US\$ is about 8 RMB). N: no record for algal species.

No.	Time period	Algal species	Area (Km <sup>2</sup> )	Economic losses	References
2	August –September 1989	N	Large	200 million RMB	Zhou et al, 2001
3	16 June 1990	N	10	82 million RMB	Zhang, 2004
4	8 August 1990	N	Large	20 million RMB	SOA, 1990
5	4-12 July 1991	<i>Noctiluca scintillans</i>	100	Loss	SOA, 1991
6	Sep-Oct 1998	<i>Ceratium furca</i>	5000	500 million RMB	Tang <i>et al.</i> , 2006
8	20-21 July 2000	N	180	Jellyfish deaths	SOA, 2000
9	2 August 2000	N	827	120 million RMB	SOA, 2000
10	July 2000	N	305-850	100 million Jellyfish deaths	Zhou <i>et al.</i> , 2001
11	10 August 2002	<i>Noctiluca scintillans</i>	20	5 million RMB	SOA, 2002
12	15 August 2002	<i>Skeletonema costatum</i>	30	8 million RMB	SOA, 2002
13	2002	<i>Noctiluca scintillans</i>	120	17 million RMB	SOA, 2002



**Figure 4.** HABs of *Noctiluca scintillans* and *Skeletonema costatum* in the Bohai Sea area during 1950-2004, a) HAB occurrences from 1980 to 2004, b) Monthly distribution of the two species in different periods.

*lans* blooms in April-August with highest frequency in June. The peak in the blooming time of *Noctiluca scintillans* changed from June-August in the 1990s to June during 2000-2004.

## Acknowledgements

This research was supported by the following research grants awarded to Professor Tang DanLing: (a) Chinese Academy of Science (CAS) (“One Hundred Talents Program” and KZCX3-SW-227-3); (b) Guangdong Natural Sciences Research Foundation, China (04001306 and 05102008); (c) National Natural foundation (40576053). Special thanks to L.J. Liu of Department of Environmental Sciences and Technology, Fudan University for her assistance in data collection.

## References

- Anderson, D.M. (1997). *Nature* 388: 513-514.
- Lin, C.L., Su, J.L., Xu, B.R. & Tang, Q.S. (2001). *Progr. Oceanogr.* 49: 7-19.
- SOA (State Oceanic Administration People’s Republic of China): available from <<http://www.soa.gov.cn/chichao/index.html>>
- Tang, D.L., Kawamura, H., Oh, I.S. & Baker, J. (2006). *Adv. Space Res.* 37: 681-689.
- Tang, D.L., Ni, I.H., Mülle-Karger, F.E. & Liu, Z.J. (1998). *Continental Shelf Res.* 18: 1493-1515.
- Tang, D.L., Ni, I.H., Mülle-Karger, F.E. & Oh, I.S. (2004a). *Hydrobiologia* 511: 1-15.
- Tang D.L., Kawamura, H., Doan-Nhu, H. W. & Takahashi, W. (2004b). *J. Geophys. Res. (Ocean)* 109, doi:10.1029/2003JC002045.

## Dinoflagellate dominance and blooms in Belizean mangrove embayments consistent with the predictions of Margalef's Mandala

M.A. Faust<sup>1</sup>, S.R. Kibler<sup>2</sup>, R.W. Litaker<sup>2</sup>, M.W. Vandersea<sup>2</sup>, W.C. Holland<sup>2</sup> and P.A. Tester<sup>2</sup>

<sup>1</sup>Smithsonian Institution, 10 Constitution Avenue, N.W., Washington DC 20013 USA, faustm@si.edu,

<sup>2</sup>National Ocean Service, NOAA, 101 Pivers Island Road, Beaufort, NC 28516 USA, steve.kibler@noaa.gov, wayne.litaker@noaa.gov, mark.w.vandersea@noaa.gov, chris.holland@noaa.gov pat.tester@noaa.gov

### Abstract

Margalef's Mandala predicts that shallow marine environments with low turbulence and high nutrients will be dominated by dinoflagellates. Mangrove embayments in the central lagoon off the coast of Belize provide low turbulence-high nutrient habitats. Low turbulence results because these semi-enclosed embayments are sheltered from prevailing winds and experience only limited exchange with the surrounding oligotrophic central lagoon due to the ~0.2 m tides. High nutrient concentrations occur due to the rapid recycling of organic matter retained within these habitats. Consistent with Margalef's Mandala, these systems were found to be dominated by dinoflagellates, whereas the nearby well-mixed oligotrophic waters of the central lagoon were dominated by diatoms and small cyanobacteria. Ten bloom-forming species including *Akashiwo sanguinea*, *Ceratium furca*, *Cochlodinium polykrikoides*, *Gambierdiscus* sp., *Gonyaulax polygramma*, *G. spinifera*, *Peridinium quinquecorne*, *Prorocentrum belizeanum*, *Prorocentrum* sp. and *Protoceratium reticulatum* were observed in various embayments. As many as half of these species are known toxin producers.

### Introduction

Margalef's Mandala predicts the relationship between overall nutrient supply and the kinetic energy or "turbulence" in a system. The Mandala indicates that dinoflagellates will dominate the phytoplankton taxa in systems with high nutrient supplies and low turbulence (Margalef 1978). The original prediction was based on data from reservoirs and is often true for shallow systems with significant nutrient and turbulence gradients like lagoons and estuaries. An important factor allowing the dominance by dinoflagellates is the shallow depths of these systems which permits retention of numerous species in the benthos. This allows a more consistent reintroduction of species from cysts and resting stages which can in turn be differentially selected over time. In these shallow environments, the selection for dinoflagellates therefore becomes more predictable under high nutrient supply and low turbulence conditions. Further offshore, and in certain upwelling systems, the situation is different (Smayda 2000). In these environments, benthic resupply of cells is negligible, and the distribution of species present is more stochastic. Under such conditions, the species selected by varying turbulence and nutrient regimes become less predictable (Smayda and Reynolds 2001). Despite the fact that the Mandala does not apply to all environments, it still has significant explanatory power as to when dinoflagellates are likely to dominate in shallow water systems. This study specifically focused on the shallow water embayments and fringe habitats of selected mangrove

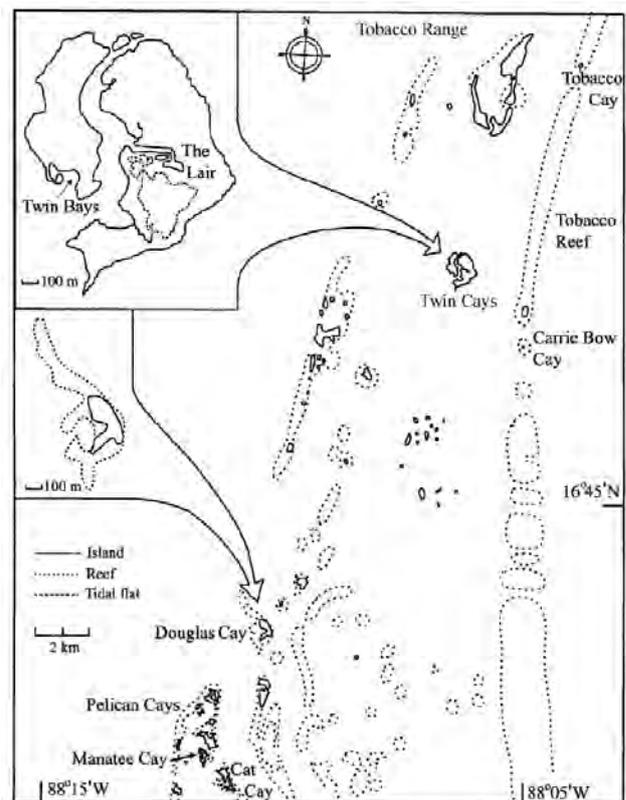


Figure 1. Central lagoon sampling sites.

cays (islands) in the central lagoon of Belize. These systems are of interest because previous observations indicated high concentrations of dinoflagellates, many of which are known toxin producers (Morton and Villareal 1998; Faust *et al.* 2005). This report presents observations on nutrients, chl *a*, and phytoplankton

community composition, gathered during five field seasons, which are consistent with Margalef's predictions.

### Site Description

The central lagoon of Belize is ~20 km wide and situated between the continent and a major reef platform (Fig. 1). This open lagoon is dominated by wind mixing and supports numerous complex coral reef and mangrove systems. Mangrove cays are common in the central and outer regions of the open lagoon, and many contain shallow embayments fringed by red mangroves (*Rhizophora mangle*). Our sampling was focused on these mangrove cay embayments, which are protected from the prevailing NE winds by dense mangrove stands. Water exchange between the embayments and the central lagoon is typically restricted due to the presence of a sill at the embayment entrance and a small (~20 cm) tidal range. Currents inside the embayments are normally <20 cm s<sup>-1</sup> (Kjerve *et al.* 1982; Kibler *et al.* 2005). This favours retention of organic material from the mangroves which is rapidly recycled, resulting in elevated levels of dissolved ammonia and other nutrients in the embayments (Rützler and Feller 1996). Direct anemometer readings show that the prevailing trade winds are reduced from 20 – 25 km h<sup>-1</sup> to <0.5 km h<sup>-1</sup> in the mangrove embayments and certain associated fringe regions. These embayments and fringe regions are all relatively shallow (~1-5 m).

### Methods

Ammonium and phosphate were determined using the methods of Holmes *et al.* (1999) and Karl and Tien (1992), respectively. For HPLC approximately 1 L water samples were gently filtered (<5 cm Hg) through 25-mm Whatman GF/F filters and stored at -80 °C in a Dewar flask until the samples could be extracted for CHEMTAX phytoplankton community analysis as described by Örnólfsson *et al.* (2003). *In situ* chl *a* measurements were made using a calibrated

**Table 1.** Mean concentrations for ammonium, phosphate and chl *a* ± 1 standard deviation measured in the lagoon, embayment/fringe waters, and in the transition zones between the two. N=66 to 4238 for Chl *a*. N averaged 33 and 32 for ammonium and phosphate, respectively, in years when they were measured.

#### Mangrove Embayment / Fringe Water

Year	NH <sub>4</sub> <sup>+</sup> (μM)		PO <sub>4</sub> <sup>-</sup> (nM)		Chl <i>a</i> (μg L <sup>-1</sup> )	
	Mean	(S.D.)	Mean	(S.D.)	Mean	(S.D.)
2002	5.5	(4.0)				
2003	0.9	(0.7)			4.8	(4.2)
2004	1.7	(2.0)	0.4	(0.5)	3.3	(7.0)
2005	0.5	(0.2)	23.3	(22.6)	3.7	(3.4)
2006	2.8	(1.8)	182.5	(260.2)	6.1	(1.9)

#### Transition Waters

2002					0.6	(0.4)
2004	0.2	(0.2)	0.02	(0.02)	1.2	(2.1)
2005	2.9	(2.1)	54.5	(36.5)	1.4	(3.3)

#### Lagoon Waters

2002	0.4	(0.4)				
2003	0.2	(0.05)	0.01	(0.01)	0.2	(0.2)
2004	0.09	(0.1)	30.6	(13.2)	0.8	(0.5)
2005	0.3	(0.1)			0.4	(0.8)
2006	0.9	(0.5)			0.3	(0.1)

YSI sensor. Samples were preserved in Utermöhl's solution until counting using a settling chamber (Guillard 1973). Cells were also preserved in a final concentration of 2 % glutaraldehyde and processed for LM and SEM. The embayment sites sampled included Cat Cay, Douglas Cay, Manatee Cay, Tobacco Range, The Lair, and Twin Bays (Fig. 1). Transition sites included the entrances to Douglas Cay, The Lair, and Twin Bays. Open lagoon sites included areas outside of Douglas Cay, Twin Bays, and in open water near the Carrie Bow Cay field station.

### Results

Chl *a*, nutrients and cell counts were higher in the embayments relative to the oligotrophic central lagoon

**Table 2.** Cell counts from the mangrove embayment/fringe sites, the open lagoon sites and the transitional areas between the two taken over the past 5 field seasons. Data from a 2005 *Prorocentrum mexicanum* bloom in Twin Bays was included as an example of the species composition during a typical bloom situation.

	Mean diatoms cells L <sup>-1</sup>	S.D.	n	Mean Dinoflagellate cells L <sup>-1</sup>	S.D.	n
<b>Mangrove Embayment/Fringe Sites</b>	<b>10,749</b>	<b>23,884</b>	<b>63</b>	<b>35,307</b>	<b>99,868</b>	<b>63</b>
<b>Transition Sites</b>	<b>7,857</b>	<b>11,772</b>	<b>18</b>	<b>3,286</b>	<b>5,125</b>	<b>18</b>
<b>Lagoon Sites</b>	<b>589</b>	<b>791</b>	<b>7</b>	<b>262</b>	<b>274</b>	<b>7</b>
<b>TB 2005 - Dinoflagellate Bloom</b>	<b>2,758</b>	<b>3,196</b>	<b>33</b>	<b>76,168</b>	<b>22,870</b>	<b>33</b>

(Table 1). Based on direct cell counts, dinoflagellates were the dominant taxa within the embayment and fringe regions (Table 2). Diatoms were the next most abundant taxa, followed by a small number of cyanobacteria and other taxa. The dinoflagellate abundances were highly variable in time and space due to the occurrence of frequent small blooms, often lasting only a few days. Additionally, the dinoflagellates were observed to vertically migrate over the course of the day adding to the spatial and temporal variability. A number of common bloom-forming dinoflagellates were noted, and some of the species are known to be toxic (Table 3). The open lagoon sites were dominated by diatoms and small cyanobacteria. CHEMTAX analyses done using standard ratios, consistently and grossly underestimated dinoflagellate relative to diatom abundances (Mackey *et al.* 1996; data not shown).

**Table 3.** Location and density of dinoflagellate blooms observed since 1991.

Locality	Year	Species	Concentrations (Cells L <sup>-1</sup> )
The Lair	1991	<i>Cochlodinium polykrikoides</i>	3.0 x 10 <sup>3</sup>
The Lair	1991	<i>Prorocentrum belizeanum</i>	1.3 x 10 <sup>4</sup>
Douglas Cay	1995	<i>Gonyaulax polygramma</i>	3.6 x 10 <sup>6</sup>
Manatee Cay	1996	<i>Ceratium furca</i>	10.7 x 10 <sup>4</sup>
Cat Cay	1997	<i>Akashiwo sanguinea</i>	4.5 x 10 <sup>3</sup>
Manatee Cay	1997	<i>Gonyaulax polygramma</i>	1.8 x 10 <sup>6</sup>
Douglas Cay	1997	<i>Gonyaulax spinifera</i>	8.3 x 10 <sup>3</sup>
Tobacco Range	2000	<i>Protoceratium reticulatum</i>	5.3 x 10 <sup>3</sup>
Douglas Cay	2002	<i>Peridinium quinquecorne</i>	1.1 x 10 <sup>3</sup>
Twin Bays	2005	<i>Prorocentrum mexicanum</i>	7.6 x 10 <sup>4</sup>
Douglas Cay	2005	<i>Gambierdiscus</i> sp.	0.4 x 10 <sup>3</sup>
Douglas Cay	2006	<i>Gambierdiscus</i> sp.	1.1 x 10 <sup>4</sup>

## Discussion

The sheltered nutrient-enriched mangrove embayments examined in this study were consistently dominated by dinoflagellates. These embayments had higher ambient residual nutrient concentrations and much greater standing biomass, consistent with their higher nutrient status compared to the oligotrophic lagoon. These observations agree well with those from semi-enclosed coastal lagoons in the Mediterranean which are also dominated by dinoflagellates, many of which are harmful (Lopez-Flores *et al.* 2006). The observed dominance of dinoflagellates, in the low turbulence, high nutrient embayments, and of diatoms and cyanobacteria in the well-mixed oligotrophic lagoon, was consistent with the predictions of Margalef's Mandala. Interestingly, the specific dinoflagellate species which dominated in the embayments at any given time, as well as the actual biomass of cells

present in a sample, was highly variable, both spatially and temporally. This variability was due to both diel vertical migration and to a rapid succession of dinoflagellate blooms. Another significant observation is that the CHEMTAX results did not accurately reflect the dominance of the dinoflagellates. One possible explanation is that many of the species present do not contain peridinin, the pigment most frequently used to identify the presence of dinoflagellates.

## Acknowledgements

We thank Dr. Klaus Rützler, NMNH, Smithsonian Institution for supporting long term research in Belize. Special thanks to Michael Carpenter for logistic support. This investigation was supported by grants from the Caribbean Coral Reef Ecosystem Program (CCRE) NMNH, and the National Ocean Service, NOAA. CCRE Publication No. 775.

## References

- Faust, M.A., Litaker, R.W., Vandersea, M.W., Kibler, S.R. & Tester, P.A. (2005). *Atoll Res. Bull.* 534: 103-134.
- Lopez-Flores, R., Garcés, E., Boix, D., Badosa, A., Brucet, S., Maso, M. & Quintana, X.D. (2006). *Harmful Algae* 5: 637-648.
- Guillard, R.R.R. (1973). In: *Handbook of Phycological Methods*, Stein, J. (ed.), Cambridge University Press, New York, pp. 289-311.
- Holmes, R.M., Aminot, A., Kerouel, R., Hooker, B.A. & Peterson, B.J. (1999). *Can. J. Fish. Aquat. Sci.* 56: 1801-1808.
- Karl, D.A. & Tien, G. (1992). *Limnol. Oceanogr.* 37: 105-116.
- Kibler, S.R., Faust, M.A., Vandersea, M.W., Varnam, S.M., Litaker, R.W. & Tester, P.A. (2005). *Atoll Res. Bull.* 535: 133-156.
- Kjerfve, B., Rützler, K. & Kierspe, G.H. (1982). In: *The Atlantic Barrier Reef Ecosystem at Carrie Bow Cay, Belize, I: Structure and Communities*, Rützler, K. & Macintyre, I.G. (eds), Smithsonian Institution Press, Washington, D.C., pp. 47-51.
- Örnólfsson, E. B. & Pickney, J.L. (2003). *J. Phycol.* 39: 449-457.
- Mackey D.J., Higgins, H.W., Mackey, M.D. & Holdsworth, D. (1996). *Mar. Ecol. Progr. Ser.* 144: 265-283.
- Margalef, R. (1978). *Oceanol. Acta* 1: 493-509.
- Morton, S.L. & Villareal, T.A. (1998). *Bull. Mar. Sci.* 63: 1-4.
- Rützler, K. & Feller, C.I. (1996). *Sci. Am.* 274: 94-99.
- Smayda, T.J. (2000). *South Afr. J. Mar. Sci.* 22: 219-253.
- Smayda, T.J. & Reynolds, C.S. (2001). *J. Plankton Res.* 23: 447-461.

## Summer *Alexandrium catenella* bloom and the impact on fish farming in the XI Aysén region, Chile

C. Fuentes<sup>1</sup>, A. Clement<sup>2</sup> and A. Aguilera<sup>2</sup>

<sup>1</sup>Lillo # 169, Castro, palchiloe@telsur.cl, <sup>2</sup>P.O Box 1036, Puerto Varas, alexcle@telsur.cl, Chile

### Abstract

During the summer of 2005 and 2006 a bloom of *Alexandrium catenella*, a well-known PSP (Paralytic Shellfish Poisoning) producer was detected in the northern fjords of the XI Aysén region. The bloom caused seven human intoxications and one fatality. Unusual behaviour of farmed salmon, coincident with high *A. catenella* abundance in surface waters of fish farms (>356 cells/mL) preceded fish gill damage and an extensive fish kill. Cells were mainly distributed in the upper surface layer and cell concentrations diminished with depth. At one site located in the Guaitecas Archipelago (44° SL) maximum *A. catenella* concentration was 961 cells/mL, representing 86 % of the total phytoplankton community. The bloom began in December 2005 and reached maximum abundance during the 2nd and 3rd week of January. Presence of this “ichthyotoxic” dinoflagellate was correlated with sea temperatures higher than 12.0 °C.

### Introduction

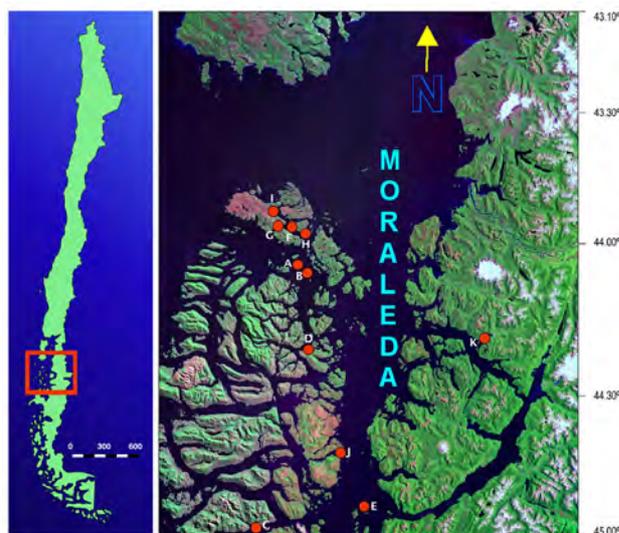
Mortalities of farmed fish associated with PSP outbreaks have been reported in Canada and the Faroe Islands (Mortensen 1985; Cembella *et al.* 2002). In Chile, the first record of farmed salmon kills due to a red tide was registered in 1983 (Lembeye and Campodónico 1984). In recent years the spatial distribution of *Alexandrium catenella* in southern Chile has progressed northwards; 45° 47' to 42° 10' LS (Clément *et al.* 2002) invading an area that contains a significant fraction of fish farms with a yearly production > 200,000 tons. During summer and early fall 2002 an extensive bloom of *A. catenella* was associated with >50 human intoxications, 3 fatalities, and losses of over 1800 metric tons of farmed salmon. The highest abundance of *A. catenella* observed during the event was 789 cells/mL in Quellón, Chiloé Archipelago (Fuentes *et al.* 2004).

We describe here an extensive bloom of *A. catenella* in the northern section of Aysén region. Its presumed impacts included seven human intoxications, one fatality and significant losses due to an extensive fish kill. The main objectives of this paper are: to describe the temporal and spatial distribution of *A. catenella* abundance and to relate this outbreak with impacts on farmed fish. Although a conclusive cause of the fish mortalities was not proven, we report the coincidence between the bloom of *Alexandrium catenella* and the extensive salmon mortalities.

### Materials and Methods

Due to unusual fish behaviour and an increase in salmon mortalities in the reported area, a programme of phytoplankton sampling was implemented.

Samples (500 ml SW) were taken from December 2005 to the end of February 2006 from 11 different salmon farms in the Aysén region (Fig. 1).



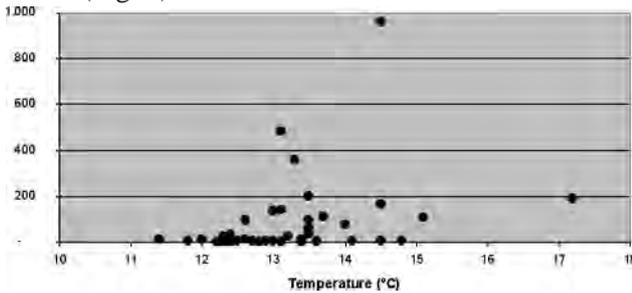
**Figure 1.** Map of monitoring station during the *Alexandrium catenella* bloom 2006.

The samples were collected every two days in fish farms, either by a scuba diver or a bottle sampler, from the upper surface of the water column. Temperature (°C), oxygen (mg/L) and salinity were measured with a YSI 85 probe. Live samples (24 h since collection) were analyzed using an inverted Nikon microscope TS100, (20x magnification) and a modified Sedgewick-rafter method for quantification. Phytoplankton abundance was expressed in cells/mL. Site managers recorded fish behaviour in some cases during the outbreak. The bivalve *Aulacomya ater* was collected at the end of January (Site E, 2m deep) for saxitoxin analyses (AOAC mouse bioassay). Aerial

observations were conducted covering an area larger than 500 km<sup>2</sup>. Total losses at one site (K) are described to illustrate the economic impact on this aquaculture activity.

**Results**

During mid-December 2005, the first record of *A. catenella* (3 cells/mL) was obtained in the north area of the XI region, at sites F, H, and I (Fig. 1). The highest abundances of this “ichthyotoxic” alga were detected when sea surface temperature (SST) was higher than 12 °C (Fig. 2).



**Figure 2.** *Alexandrium catenella* cell densities (cells/ml) at different temperatures from surface samples, in different salmon farming site, XI Aysén.

The first week of January 2006 *Alexandrium catenella* was detected in the majority of the monitoring stations. The highest concentration of 961 cells/mL was seen at site K, where fish losses were significant. We also detected three clear peaks, or maximum concentrations of *A. catenella*. The highest (961 cells/mL) was measured twenty two days after the first detection, a second pulse between the 2nd and 3rd week of January (356 cells/mL), diminishing to 10<sup>7</sup> cells/mL the second week on February (Fig. 4).

Our records suggest that the outbreak affected an area from 43° 10' S to 45° 00' S in the XI region, more

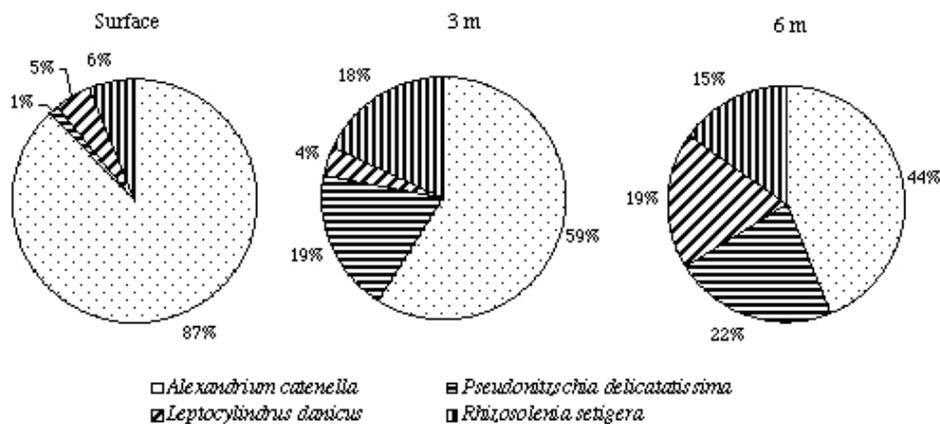
than 200 km in the inland sea, the local and aerial observations, in addition to the spatial phytoplankton data show “patches” of *A. catenella* and brown-red water discoloration. The oceanographic conditions of this area showed different patterns. West of Moraleda channel was characterized by a stable water column with SST from 9.0 °C in winter to 15 °C in summer, a salinity range of 26 -34; both parameters were influenced by oceanic condition. To the east of Moraleda fjord and bay waters were characterized by a heterogeneous water column, with important freshwater runoff that mixed at the surface. SST varied from 4 °C in July to 20 °C in February and salinities 10–32 through the year.

The measured variables at site K are presented in Table 1.

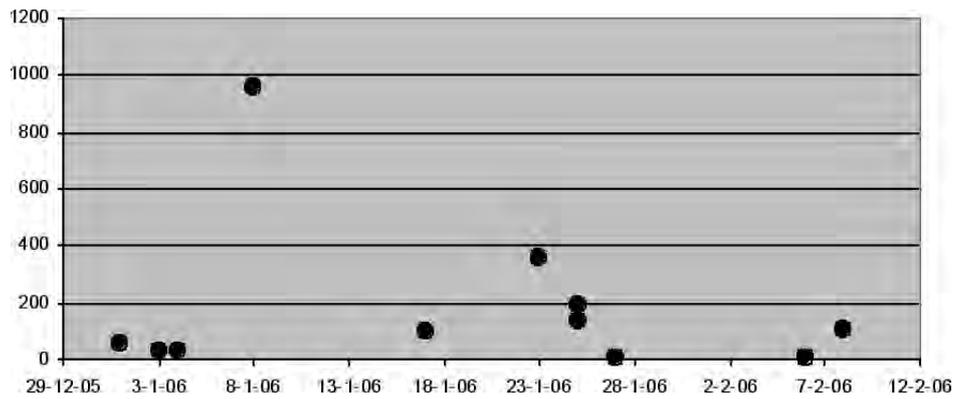
**Table 1.** Oceanographic conditions and concentration of *Alexandrium catenella* at site K.

Depth (m)	1	3	6
Temperature (°C)	14.5	13.1	13.5
Salinity (ppt)	15.0	22.0	28.0
Oxygen (mg/L)	6.2	8.3	8.6
<i>Alexandrium catenella</i> (cells/mL)	961	481	198

Phytoplankton composition is shown in Fig. 3, communities at all depths at site K were dominated by *A. catenella*. In this area the phytoplankton community before and after the *A. catenella* bloom was dominated by diatoms, principally *Leptocylindrus danicus*, *Rhizosolenia setigera* and *Pseudo-nitzschia delicatissima* at all stations monitored. The maximum toxin concentration measured during the outbreak was 9215 µg stx eq/ 100 g of tissue. Dead fish showed



**Figure 3.** Phytoplankton composition and percentage at different depths at site K (n = total phytoplankton): sampling was at midday.



**Figure 4.** Maximum concentration of *Alexandrium catenella* at different sites in the XI Aysén region, during the summer bloom.

gill irritation and excessive mucus (reported *in situ* by site manager).

At site K over 90 % of total biomass of *Salmo salar* was lost within a few days. Average fish weight was 4.35 kg, average number of fish per cage was 21,000, and total number of cages affected was 20, with a total of 1,827 tons lost. Economical losses at site K were estimated assuming a market price for *Salmo salar* of 5.7 USD/kg, the total losses amounted to 9.2 million USD.

## Discussion

This red tide was less intense in temporal and spatial scale than the 2002 episode because it affected a smaller area. This is consistent as a “Inland Sea Scale” according to Clement *et al.* (2004). In the most recent case the bloom began in December 2005 and lasted until February 2006 and the affected area was less than 200 km. In comparison, the 2002 bloom began in December 2001 in the north occidental area of Aysén region (45 °S) and reached the mid part of the X region (42 °S) in May 2002, covering more than 500 km longitudinally.

The origin of the bloom was in the same area where we identified *Alexandrium catenella* in 2002 and 2004 (east coast of north occidental XI region). This was also reported by Molinet *et al.* 2003, who suggested that the origin was associated with a resting cyst bank and regional wind patterns.

We have detected two different areas where *Alexandrium catenella* proliferates, one of them is located in the the Melinka area (west of Moraleda Channel) with maximum concentration of 356 cell/mL and marginal fish loss. A second area has been located east of Moraleda channel, this area showed significant fish losses and the highest cell densities of *A. catenella* (> 700 cell/mL) were recorded from at least three different sampling sites. This coincided with a highly strat-

ified water column. The parameters were similar to those found in the 2002 event (Clément *et al.* 2002).

The concentration of 961 cell/mL is the highest record in Chile, this value is higher that reported by Guzman *et al.* (Magallanes region 1972; 600 cells / mL) and that reported by Fuentes *et al.* for Chiloé Archipelago (789 cells/mL) in the 2002 outbreak.

Aquaculture fish kills associated with ichthyotoxic algae require more understanding. Usually wild fish mortality is due to intoxication via the food chain. In our study, cultivated salmon was fed “artificial food”, but their mortality could be explained by the presence of soluble toxins in the water, or direct exposure to toxic *Alexandrium* cells. Cembella *et al.* (2002), mention saxitoxin as a probable cause of dead caged fish in Nova Scotia.

Due to the records of oxygen (> 6.2 mg/L), anoxia should be discarded as a probable cause of mortality.

The presence of siliceous and sharp microalgae such as *Leptocylindrus danicus* and *Rhizosolenia setigera* also could be discarded because cell concentrations were below the problematic levels of 3500 cells/ml for *Leptocylindrus* and 500 cells/ml for *Rhizosolenia* during the *Alexandrium* proliferation.

Our hypothesis is that when *Alexandrium catenella* is present in high cell concentrations (more than 700 cells/ml) and represents >80% of the phytoplankton community, fish kills occur. The last three episodes: Nova Scotia 2000, Chiloé Archipelago 2002, and this report support this hypothesis.

## Acknowledgements

We thank Ximena Rojas & Nathalie Fuica from INTESAL for phytoplankton information, Dr. Benjamin Suarez-Isla for reviewing this work. Servicio Salud Aysén for VPM data. To PLANCTON ANDINO team for the success in this work, and especially to IOC-

HAB programme and ISSHA for travel support to the 12th International Conference on Harmful Algae.

### References

- Cembella, A.D., Quilliam, M.A., Lewis, N.I., Bauder, A.G., Dell'aversano, C., Thomas, K., Jellet, J. & Cusack, R.R. (2002). *Harmful Algae* 1: 313-325.
- Clément, A. (1988). *Biota*: 79-84.
- Clément, A., Aguilera, A. & Fuentes, C. (2002). Resumen, XXII Congreso de Ciencias del Mar, Valdivia, UACH, Chile.
- Clément, A., Aguilera, A., Fuentes, C., Grünewald, A., Dressmann, S. & Rojas, X. (2004). Oral presentation, GEOHAB, Viña del Mar, Chile.
- Fuentes, C., Aguilera, A., Arriagada, G., Caniggia, M., Clément, A., Contreras, V., Córdova, M., Dressmann, S., Fonseca, M., Gárate, C.G., López, A., Silva, L. & Suárez, B. (2004). Poster, GEOHAB, Viña del Mar, Chile.
- Guzmán, L., Pacheco, H., Pizarro, G. & Alarcón, C., (2002) In: *Floraciones Algales Nocivas en el Cono Sur Americano*, E. Sar, M. Ferrario & B. Reguera (eds), pp. 237-256.
- Lembeye, G. & Campodonico, I. (1984). *Bot. Mar.* 27: 491-493.
- Mortensen, A.M. (1985). In: *Toxic Dinoflagellates*, D.M. Anderson, A.W. White & D.G. Baden (eds), Elsevier, pp. 165-170.
- Molinet, C., Lafon, A., Lembeye, G. & Moreno, C. (2003). *Revista Chilena de Historia Natural*: 681-698.

## Comparison of the accumulation of lipophilic marine biotoxins in passive samplers, transplanted mussels and indigenous mussels on the west coast of Ireland

E. Fux, R. Bire and P. Hess

Biotoxins Chemistry - Marine Institute, Rinville, Galway, Ireland, elie.fux@marine.ie, ronel.bire@marine.ie, philipp.hess@marine.ie

### Abstract

The use of polymeric resin as passive sampling for an early warning system was recently published and referred to as Solid Phase Adsorption Toxin Tracking (SPATT). This technique involves the immersion of a polymeric resin in the seawater and was used during the summer 2005 on the west coast of Ireland in shellfish production areas. Live mussels were placed next to the SPATT disks to compare toxins accumulated in shellfish with those accumulated in the resin. Furthermore, all data obtained by LC-MS were compared to the toxin levels in indigenous mussels. The SPATT disks showed the ability to adsorb a wide range of lipophilic toxins (AZAs, OA, DTX2, PTX2, and YTX) even when no major toxic events occurred. The relocated mussels that were placed near the SPATT disks accumulated toxins (OA, DTX2, OA esters, DTX2 esters, AZA1 and AZA2) to a greater extent than the indigenous mussels but only during the occurrence of toxin-producing algae. The SPATT disks allow the detection of toxins in the water but did not provide an early warning seven days prior to the toxic event as they accumulated toxins at the same time as the relocated mussels. The use of passive sampling had major advantages over current monitoring methods which use shellfish, as it enables the quantification of parent toxins rather than their metabolites and offered high sensitivity.

### Introduction

The use of passive sampling has shown major advantages for the monitoring of organic pollutants in the aqueous environment. The ability to obtain a long term integration of an analyte, including episodic contaminations, without the need of energy and avoiding biotransformation make passive sampling an attractive tool to obtain temporally and spatially integrated levels of contaminants. In 2004, MacKenzie developed the Solid Phase Adsorption Toxin Tracking (SPATT) bags, passive samplers designed for the detection of lipophilic marine toxins (MacKenzie *et al.* 2004).

### Materials and Methods

#### Sampling

Three locations on the west coast of Ireland were selected according to their history of contamination in the last years. Field sampling during the summer 2005 was set up in three sites in Killary Harbour (Killary inner [53°36'0.0001"N, 9°45'19.9008"W]; Killary middle [53°36'14.0039"N, 9°48'10.0080"W]; Killary outer [53°36'52.9919"N, 9°49'59.0160"W]), one site in Bantry [51°41'35.1600"N, 9°28'41.9880"W] and one site in Bruckless [54°36'47.9879"N, 8°23'31.9920"W]. The SPATTs and the mussels were replaced on a weekly basis.

A 15-m polypropylene rope was used to deploy the SPATT disks. Three SPATTs were attached

to PVC tubes going through the rope at three different depths: surface, 5 and 10 m. Nets containing 300 g of uncontaminated mussels (*Mytilus edulis*) were placed at the same depth as the SPATT.

#### SPATT handling

HP20 DIAION resin was weighed ( $3.00 \pm 0.05$  g) and methanol (100 mL) was subsequently added. The resin was activated by a 40-min shaking step in a multi-tube vortexer prior to a filtration on 95- $\mu$ m mesh ( $\approx 21 \times 12$  mm). The resin was wrapped with the mesh and clipped in an embroidery frame (diameter 8.8 cm) allowing exposure on both sides of the frame. Methanol residues were removed by a 10-min sonication step in 500 mL water. The SPATT disks were stored in MilliQ water at 6 °C until deployment.

#### SPATT extraction-method development

Extraction efficiencies were assessed using naturally contaminated resins. Following several attempts to improve the existing extraction method, it was found that a slow elution gave the best recovery. This was assessed by packing the naturally contaminated resin in a preparative glass column, which was connected to a pump set at 1 mL min<sup>-1</sup> flow with methanol as mobile phase. The elution was carried out over 60 min and 5 mL fractions were taken and analysed by LC-MS.

### Extraction method

SPATTs were rinsed twice in 500 mL of MilliQ water and vigorously shaken to remove salts. The contaminated resins were removed from the mesh and inserted into empty SPE glass cartridges placed on a manifold. Vacuum was applied in order to remove the remaining water. The Killary-middle results presented here were the first set of SPATT that were analysed and in order to ensure that the limit of detection was achieved, all extracts were concentrated by a factor 5 after elution with 25 mL of methanol at ca 1 mL min<sup>-1</sup>. It was found that the concentration step could be avoided, as the toxins of interest were quantifiable in a 25-mL solution. Therefore, the following procedure was applied for the remaining samples. A 23-mL methanol portion was used to elute the resin at ca 1 mL min<sup>-1</sup> flow rate. The extracts were transferred into 25 mL volumetric flasks and an additional 2 mL was used for rinsing and to complete up to the mark. Extract aliquots were taken from the volumetric flask, inserted into LC vials and injected in a LC-MS system.

### Mussel extraction

At receipt, the transplanted mussels were steam cooked and stored frozen until extraction while the indigenous mussels were analysed fresh as part of the national shellfish safety monitoring programme. The extraction procedure consisted of a double methanolic extraction previously described by Hess *et al.* (2003).

### Instrumentation

Two systems were used for quantitative LC-MS analysis, using a binary mobile phase with A (100 % aqueous) and B (95 % acetonitrile) both containing 2 mM ammonium formate and 50 mM formic acid. The indigenous mussel samples were analysed using 2695 Waters HPLC coupled to a Micromass Quattro Ultima (triple quadrupole) equipped with a z-spray ESI source. The Quattro Ultima was operated in multiple reaction-monitoring (MRM) mode, analyzing two fragment ions per compound. Monitored transitions were reported elsewhere (Hess *et al.* 2003). A C8 BDS Hypersil (50 x 2 mm, 3 µm particle size, guard column, 10 x 2 mm, 3 µm) was used with a gradient elution, starting with 30 % B at time zero linearly rising to 90 % B at 8 min. Then, 90 % B was held for 0.5 min, decreased to 30 % B over 0.5 min which was held again for 3 min until the next run.

The SPATT disks and the relocated mussels were analysed using a 2795 Waters HPLC equipped with a C18 ACE column (30 mm x 2.1 mm) coupled to a Micromass Q-TOF Ultima (quadrupole-time-of-flight

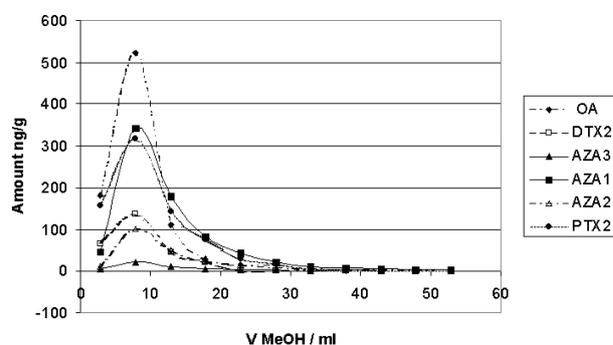
hybrid) also equipped with a z-spray ESI source. The Q-TOF was used in TOF-MS-MS mode, where the molecular ion is isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum is obtained in the TOF. AZAs and PTX2 were analysed in positive ionisation mode with an isocratic run of 60 % B for 7 min and OA, DTX2 and YTX were analysed in negative ionisation mode with an isocratic run of 55 % B for 6.5 min.

## Results and Discussion

### Extraction method development

The elution profile obtained by eluting 3 g of naturally contaminated resin is shown in Figure 1.

**Figure 1.** Elution profile of OA, DTX2, AZA1, AZA2,



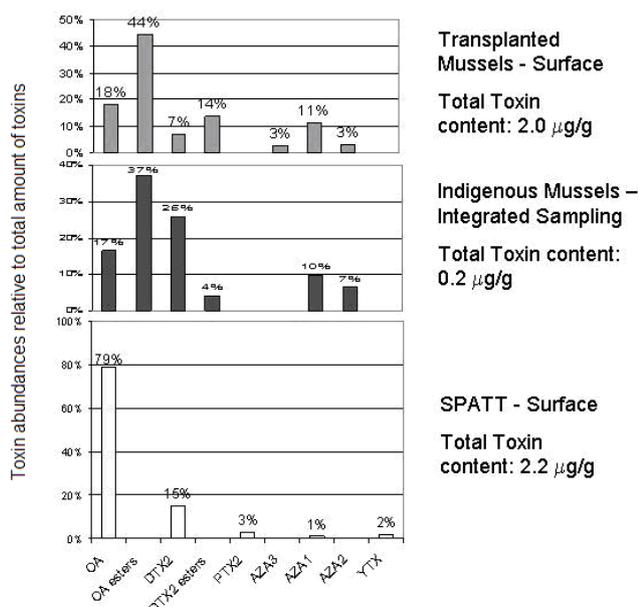
and PTX2 using 3 g of naturally contaminated HP20 resin from Bruckless at the beginning of September eluted at 1 mL min<sup>-1</sup> of methanol.

An elution with 23 mL yielded recoveries greater than 92 % of all the toxins considered (calculated relatively to 60 mL). This was considered to be sufficient for our application. Thus, in these conditions, one analyst may prepare 18 SPATT samples per day for LC-MS analysis.

### Field Trials

As shown in Figure 2, PTX2, AZA1 and YTX were found at quantifiable levels on the SPATT disks. This is the first study reporting quantifiable levels of YTX in Ireland. The toxin is believed to be produced by *Lingulodinium polyedrum* as this organism was reported as one of the YTX producers (Paz *et al.* 2004) and is occasionally found along the west coast of Ireland in very low amounts. No esters of OA and DTX2 were found in the SPATT after hydrolysis despite high amounts found in relocated and transplanted mussels. PTX2 was not detected in relocated and transplanted mussels as it readily opens to produce PTX2 seco-acids by rapid bioconversion (Suzuki *et al.* 2001).

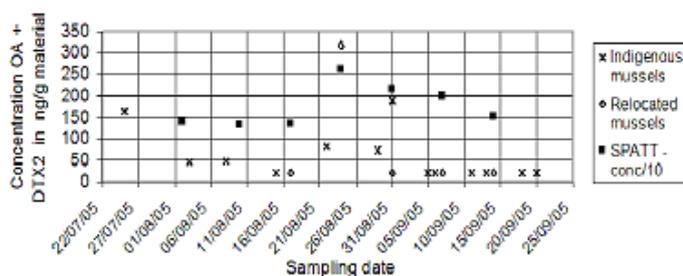
**Figure 2.** Toxin profile in SPATT disks and transplanted



mussels retrieved from Killary on 24 August and in indigenous mussels retrieved on 29 August.

AZA1 accounted for 1 % of the total toxin content and no AZA2 was detected in the SPATT while 10 % of AZA1 and 7 % of AZA2 were found in indigenous mussels and 11 % and 3 % in relocated mussels. However, AZA2 accumulation was observed in SPATTs from other locations such as Bruckless (Fig. 1). Water samples were taken on a weekly basis in Killary-middle during the study period using a long tube. Known toxin-producing dinoflagellates were only found at one occasion, on 22 August 2005 where  $t$  reached  $160 \text{ cells L}^{-1}$ . This explains the increase obtained in both SPATT disks and transplanted mussels on 24 August (Fig. 3).

**Figure 3.** Concentration of OA and DTX2 found in SPATT,



transplanted and indigenous mussels in Killary middle from 25 July until 20 September.

During the three weeks prior to the toxic event, the SPATT disks indicated a constant level of OA and no DTX2. The presence of *D. acuta* led to an increase of OA and to the appearance of DTX2 confirming a previous study (James *et al.* 1997). The amounts of toxins that accumulated on SPATT disks were signifi-

cantly different through the water column and usually accumulated several micrograms of toxins in all sampling stations (data not shown). The transplanted mussels accumulated quantifiable levels of OA and DTX2 only once during the study despite the presence of toxins in the water. This suggests that the mussels were only able to accumulate the toxins when feeding on toxic plankton. Indigenous mussels did not accumulate toxins to a great extent even after the toxic event. Two reasons could explain the large difference observed between the amount of toxins accumulated in the relocated mussels and in the indigenous mussels: i) since the relocated mussels were cooked when returned in the lab, the loss of water resulted in a concentration step of the lipophilic toxins (Hess *et al.* 2005); and ii) the relocated mussels were purchased after storage in clean water tanks that did not contain food and spent 1-2 d in a starvation period. Therefore, once back in the water, the bivalves fed intensively which would result in more accumulation.

SPATT monitoring at one week frequencies did not yield early warning in our conditions as the concentration of toxins in the SPATT increased at the same time as in the mussels.

## Conclusions

The extraction procedure presented here shows a significant improvement compared to the existing method in terms of solvent consumption and time. It was found that OA was present at relatively high levels in the water despite the absence of toxic plankton. However, shellfish did not accumulate toxins directly from the water. This is the first study reporting YTX in Irish water.

## References

- Hess, P., Nguyen, L., Aasen, J., Keogh, M., Kilcoyne, J., McCarron, P. & Aune, T. (2005). *Toxicon* 46: 62-71.
- James, K.J., Bishop, A.G., Gillman, M., Kelly, S.S., Roden, C., Draisci, R., Lucentini, L., Giannetti, L. & Boria, P. (1997). *J. Chromatog. A* 777: 213-221.
- MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P. & Selwood, A. (2004). *Toxicon* 44: 901-918.
- Paz, B., Riobo, P., Fernández, L.M., Fraga, S. & Franco, J.M. (2004). *Toxicon* 44: 251-258.
- Suzuki, T., Mackenzie, L., Stirling, D. & Adamson, J. (2001). *Toxicon* 39: 507-514.

## Quantification of epibenthic communities, including toxic dinoflagellates, in different green macroalgal substrates in Ria de Aveiro (Portugal)

M.F. Hinzmann, S.C. Craveiro and A.J. Calado

Departamento de Biologia, Universidade de Aveiro, P-3810-193 Aveiro, Portugal

mhinzmnn@bio.ua.pt, scraveiro@bio.ua.pt and acalado@bio.ua.pt

### Abstract

The coastal lagoon 'Ria de Aveiro' is a complex system of channels, marshes and puddles that supports the growth of a variety of macrophytes. In sheltered areas extensive macroalgal masses containing mixtures of green algae can be found. Three main types can be distinguished: those predominantly made up of species of *Ulva* (including *Enteromorpha*), entangled filaments of *Cladophora*, and elongate masses of *Chaetomorpha linum*. High densities of microscopic algae are attached to, or dwelling close to the macroalgal surfaces, forming diverse epibenthic communities. Attached diatoms make up the greatest abundance of epibenthic organisms, but a variety of dinoflagellates, including toxin-producing species, are regularly present. The composition of the communities within the green algal masses was examined in two sheltered localities, using a quantification method that involved the forced detachment of the organisms from a known mass of macroalgae. Different macroalgal substrates did not correlate with different macroalgal communities. However, some microalgal species were more abundant at higher salinities. *Prorocentrum lima* and *P. cassubicum* were present, and sometimes abundant, in the site with lower salinity during the whole period of study (winter and spring).

### Introduction

Ria de Aveiro (NW Portugal) is a shallow, tidal, bar-built estuary, 45 km long and 10 km wide in its broadest portion, covering nearly 47 km<sup>2</sup>. It contains three main channels radiating from the mouth, each with several branches, islands, mud-flats, natural and man-made puddles, ponds and salt pans (many of them no longer in use). As an important area for aquaculture and fisheries, Ria de Aveiro has a history of toxic outbreaks that have adversely affected the shellfish industry (e.g. Vale and Sampayo 2003). Plankton-

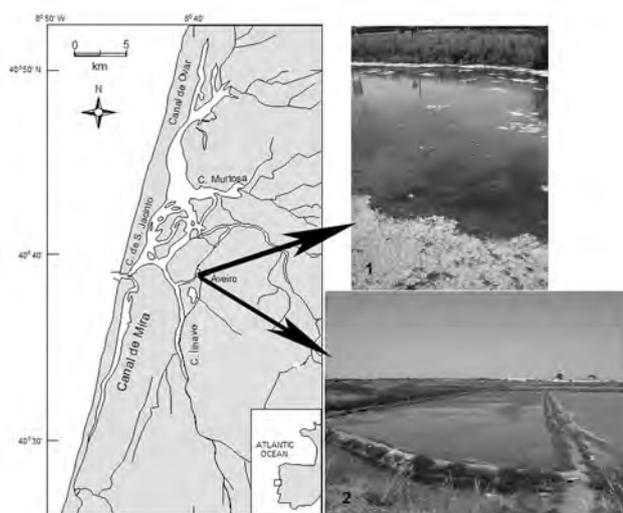
ic dinoflagellate and diatom species are usually the problem-causing agents. Monitoring of phytoplankton in the main channels has been conducted for many years. However, the sheltered and highly variable microhabitats that are rich in macrophytes and known to harbour a diverse community of epibenthic organisms (Bomber *et al.* 1989), including potentially toxic dinoflagellates (Vila *et al.* 2001; Gayoso *et al.* 2004), are usually not included in monitoring programs.

In the present work, a 6-month field survey was conducted at two sites. The objective was to study the relative abundance of epibenthic species attached or surrounding green macroalgal masses within the microhabitats. Special attention was devoted to dinoflagellate communities.

### Material and Methods

Macroalgae were collected from a small artificial pond (Site 1) and from an accessory canal to a salt pan (Site 2; Fig. 1) fortnightly from December 2005 to July 2006. The samples were placed (1-20 g fresh weight) into separate plastic bags. Approximately 250 ml of ambient water was collected from each site. Water temperature and salinity were measured *in situ*.

In the laboratory, the macroalgae were processed using an adaptation of the method described in Foden *et al.* (2005). To each sample, 40 ml of filtered, ambient water was added, and then shaken vigorously for 1 min. The resulting suspension was collected and fixed



**Figure 1.** Map of Ria de Aveiro showing the main channels. Inset: map of Iberian Peninsula. Sampling sites: 1 – artificial pond; 2 – accessory canal to a salt pan.

with Lugol's solution. Fresh and dry weight (FW and DW) of the macroalgae were measured.

A sub-sample of the fixed suspension was placed in a 5-10 ml settling chamber for species quantification using an inverted microscope. Cell counts were expressed in cells/g DW of macroalgae.

Sub-samples were collected on polycarbonate membrane filters (5 µm pore), dehydrated with a graded ethanol series and critical point dried. They were observed with Jeol JSM 5400 and 6335F scanning electron microscopes.

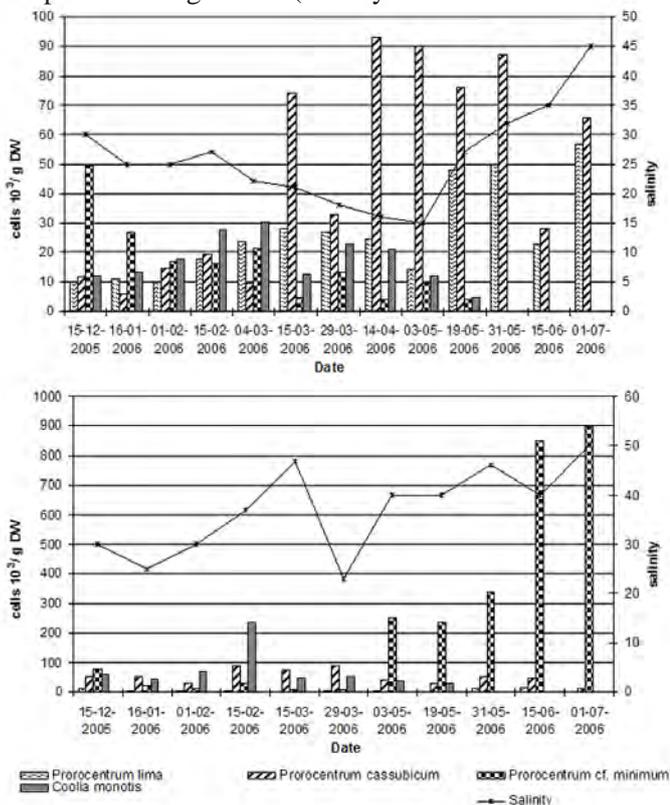
The data obtained were statistically analysed with the programme Primer v.6β.

## Results

Water temperature was similar at both sites and varied from 5.8 °C in December to 25.8 °C in June. Salinity was 15-45 psu at site 1. It was always higher in site 2, between 23 and 50 psu (Fig. 2).

At site 1 three different species of macroalgae were regularly collected: *Cladophora* sp., *Ulva* (*Enteromorpha*) sp. and *Chaetomorpha linum* (O.F. Müller) Kützing. At site 2 only *Cladophora* sp. was present.

Diatoms were always the dominant group of epibenthic organisms (usually ~ 80 % the number of

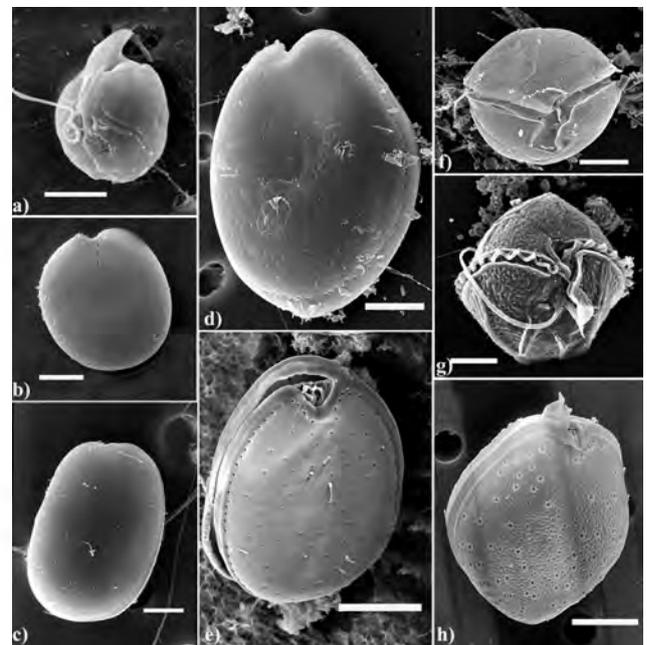


**Figure 2.** Abundance of potentially toxic dinoflagellates and variation of salinity during the sampling period in site 1 (top) and site 2 (bottom). Note the different vertical scales.

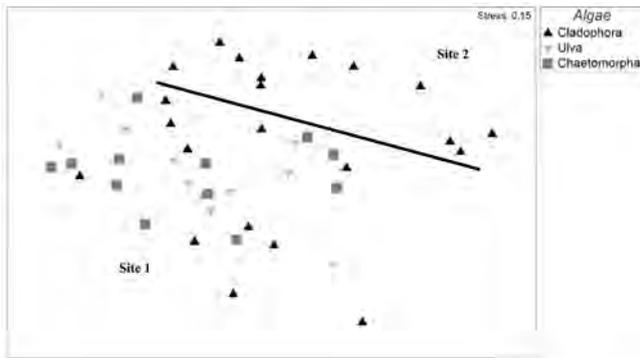
cells). Dinoflagellates (6-10 %) and small flagellates (mostly Cryptophyceae and Prasinophyceae; 5-20 %) were second in importance. General species composition was similar at the two sites, and for all the substrates at site 1.

The most common dinoflagellates were *Prorocentrum lima* (Ehrenberg) F. Stein, *Prorocentrum* sp. (undescribed species), *P. cassubicum* (Wołoszyska) J.D. Dodge, *P. micans* Ehrenberg, *P. cf. minimum* (Pavillard) J. Schiller, *Scrippsiella* sp., *Bysmatrum subsalsum* (Ostenfeld) M. Faust & Steidinger, *Coolia monotis* Meunier, *Amphidinium species* (including *A. massartii* Biecheler), *Gyrodinium* sp. and *Oxyrrhis marina* Dujardin (Fig. 3). *Kryptoperidinium foliaceum* (F. Stein) Er. Lindemann and *Polykrikos schwartzii* Buetschli were occasionally found.

A multi-dimensional scaling (MDS) ordination (Clarke and Gorley 2001) of dinoflagellate abundance data revealed that different macroalgal substrates were not a determinant for dinoflagellate community composition (Fig. 4), although the abundance of *P. lima* was usually higher on *Cladophora* sp. than on *Ulva* sp. and *Chaetomorpha linum*. In general, the communities from site 1 were distinct from the community found on *Cladophora* at site 2 (Fig. 4), reflecting differences in the relative abundance of dinoflagellate species. As an example, *Prorocentrum lima* and *P. mi-*



**Figure 3.** Some of the most common epibenthic dinoflagellates (SEM): a) *Amphidinium massartii*; b) *Prorocentrum cf. minimum*; c) *P. cassubicum*; d) *Prorocentrum* sp.; e) *P. lima*; f) *Coolia monotis*; g) *Bysmatrum subsalsum*; h) *P. micans*; scale bar, 5 µm in a), b) and c), 10 µm in all the others.



**Figure 4.** Multi-Dimensional Scaling (MDS) ordination of all the samples based on square root transformation and Bray–Curtis similarity of the abundance of dinoflagellate cells.

*cans* were usually more abundant at site 1, while *Prorocentrum* sp. and *P. cf. minimum* were more abundant at site 2. However, dinoflagellate abundance was usually higher at site 2, and the highest numbers were found in July ( $3 \times 10^7$  cells/g DW of *Cladophora*), mostly due to *P. cf. minimum*, *Amphidinium* spp. and *Scrippsiella* sp.

Two potentially toxic species appeared in higher numbers in site 1: *Prorocentrum cassubicum* (maximum of  $\sim 90 \times 10^3$  cells/g DW macroalgae in April and May) and *P. lima* (maximum of  $\sim 55 \times 10^3$  cells/g DW macroalgae in July) (Fig. 2). At site 2, *P. cf. minimum* and *Coolia monotis* were more abundant, with values of  $\sim 900 \times 10^3$  cells/g DW macroalgae in June and July for the former and  $\sim 200 \times 10^3$  cells/g DW macroalgae in February for the latter (Fig. 2).

Some species followed opposing tendencies throughout winter and spring: numbers of *Prorocentrum lima* and *P. cassubicum* increased during the spring, whereas *Coolia monotis* decreased (Fig. 2). *Bysmatrum subsalsum* was abundant at both sites May–July and reached  $\sim 250 \times 10^3$  cells/g DW macroalgae in late May at site 2. Although *P. micans* was never abundant at site 2, at site 1 it peaked with  $\sim 200 \times 10^3$  cells/g DW macroalgae in February–March and sharply decreased afterwards. *Prorocentrum cf. minimum* showed a different tendency at the two sites: the population declined at site 1 in May but bloomed at site 2 from May to July (Fig. 2).

## Discussion

This study represents the first published report of *Amphidinium massartii*, *Bysmatrum subsalsum* and *Prorocentrum cassubicum* in Portuguese waters. The sustained presence of the seldom reported *Prorocentrum cassubicum* at both sites is noteworthy because of its potential role in diarrhetic shellfish poisoning events

(see Faust 1995). The occurrence of *P. cassubicum* in communities containing *P. lima* has previously been noted (Maranda *et al.* 2000; Koray 2004).

The similarity of the epibenthic dinoflagellate communities found on different green algae concurs with some previous observations that dinoflagellates are not species-specific, but are influenced by the general characteristics of the substrate (Bomber *et al.* 1989; Vila *et al.* 2001). The somewhat greater abundance of *P. lima* associated with *Cladophora* suggests this species benefits from the conditions provided by *Cladophora* filaments (higher surface area, or perhaps lower shading effects or some nutritional benefits) as opposed to larger, more laminar substrates (see Vila *et al.* 2001; Gayoso *et al.* 2004).

The preliminary observations compiled during this winter-spring survey imply that the efforts to find abundant populations of potentially toxic dinoflagellates in Ria de Aveiro should be directed to areas rich in thin filamentous algae, including not only green algae but also red algae, e.g. Ceramiales, and epiphytic filamentous brown algae, e.g. Ectocarpales and Sphaecelariales. Data on the occurrence and abundance of epibenthic dinoflagellate communities need also to be extended to the summer-autumn period when higher numbers of species like *P. lima* may be expected.

## References

- Bomber, J.W., Rubio, M.G. & Norris, D.R. (1989). *Phycologia* 28: 360–368.
- Clarke, K.R. & Gorley, R.N. (2001). PRIMER v5: User Manual/Tutorial. PRIMER-E, Plymouth.
- Faust, M.A. (1995). In: Harmful Marine Algal Blooms, Lassus, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou, C. (eds), Lavoisier, Intercept Ltd., pp. 847–854.
- Foden, J., Purdie, D.A., Morris, S. & Nascimento, S. (2005). *Harmful Algae* 4: 1063–1074.
- Gayoso, A.M., Fulco, V.K. & Muglia, C.I. (2004). In: Harmful Algae, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), UNESCO, Florida, pp. 338–340.
- Koray, T. (2004) In: Harmful Algae, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), UNESCO, Florida, pp. 335–337.
- Maranda, L., Keller, M. D., Hurst, J.W., Bean, L.L., McGowan, J.D. & Hargraves, P.E. (2000). *J. Shellfish Res.* 19: 1003–1006.
- Vale, P. & Sampayo, M.A.M. (2003). *Toxicon* 41: 187–197.
- Vila, M., Garcés, E. & Masó, M. (2001). *Aquat. Microb. Ecol.* 26: 51–60.

## *Pseudo-nitzschia* and ASP in the northern Adriatic Sea

G. Honsell<sup>1</sup>, C. Dell'Aversano<sup>2</sup>, F. Vuerich<sup>1</sup>, S. Sosa<sup>3</sup>, L. Tartaglione<sup>2</sup> and A. Tubaro<sup>3</sup>

<sup>1</sup>Department of Applied Biology in Plant Protection, University of Udine, via Cotonificio 108, I-33100 Udine, Italy, giorgio.honsell@uniud.it; <sup>2</sup>Department of Chemistry of Natural Products, University of Naples, via D. Montesano 49, I-80131 Naples, Italy; <sup>3</sup> Department of Materials and Natural Resources, University of Trieste, via A. Valerio 6, I-34127 Trieste, Italy, tubaro@units.it

### Abstract

The recent finding of domoic acid in the northern Adriatic Sea and the lack of taxonomic information on *Pseudo-nitzschia* in this area prompted a one-year survey on *Pseudo-nitzschia* species and ASP toxins in the Gulf of Trieste. *Pseudo-nitzschia* species were identified by transmission electron microscopy. Domoic acid was quantified in natural phytoplankton samples, seawater and mussels (*Mytilus galloprovincialis*) by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) and enzyme-linked immunosorbent assay (ELISA). *Pseudo-nitzschia* species were never abundant (maximum concentration 55,280 cells/l in October 2005). The most frequent species were *P. calliantha* Lundholm, Moestrup et Hasle, mainly present in autumn, and *P. decipiens* Lundholm et Moestrup in summer and early autumn. The concentration of domoic acid in natural phytoplankton extracts, seawater, and mussels was always below the detection limit of Biosense ASP ELISA (50 pg/ml) and HILIC-MS (3 ng/ml). The presence of these *Pseudo-nitzschia* species in the Gulf of Trieste in low numbers do not allow us to demonstrate that they are involved in ASP toxins production in the Adriatic Sea.

### Introduction

The occurrence of domoic acid (DA) in mussels (*Mytilus galloprovincialis*) in the northern Adriatic Sea has been recently demonstrated (Ciminiello *et al.* 2005), indicating the presence of ASP-producing species in this part of the Adriatic. The presence of *Pseudo-nitzschia* in northern Adriatic waters has been known for many years; they are the dominant diatom species in the western part of the basin during some periods of the year, where they play an important role in phytoplankton dynamics and form large blooms (Revelante & Gilmartin 1977; Totti *et al.* 2000; Bernardi Aubry *et al.* 2004). Taxonomic information on the species present is generally lacking.

For this reason, a one-year survey on *Pseudo-nitzschia*, based on qualitative and quantitative phytoplankton analysis, taxonomic identification by transmission electron microscopy (TEM) and ASP toxin analysis in mussels, phytoplankton and seawater has been carried out in the Gulf of Trieste, Northern Adriatic Sea in 2005-2006.

### Material and Methods

#### Sampling

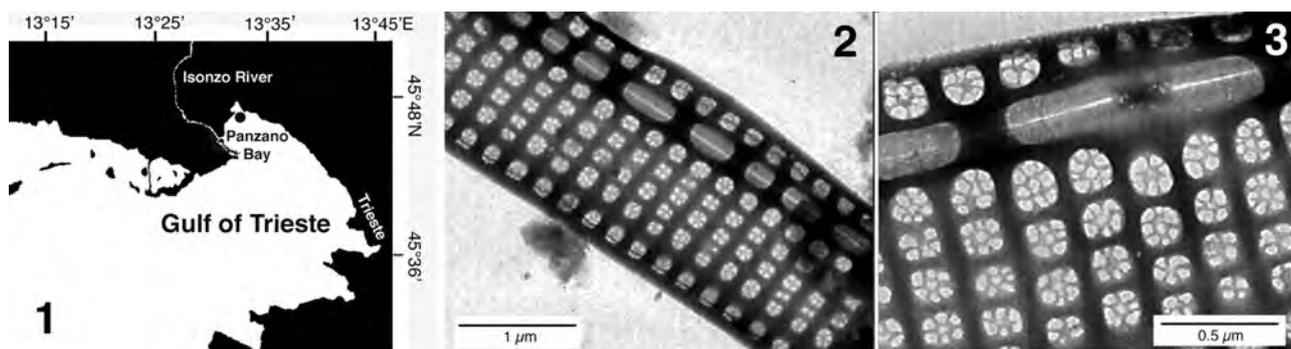
Thirty-six phytoplankton and *Mytilus galloprovincialis* samples were collected in a mussel farm (Panzano, Gulf of Trieste, Italy) February 2005-February 2006, with the same frequency throughout the year (Fig.1). Mussel flesh (5 kg) was homogenized and stored at -80 °C until extraction. Phytoplankton (2 l) was col-

lected by a net (mouth diameter 16 cm, length 94 cm, mesh 25 µm), towed vertically from bottom to surface. Part of each sample (1.75 l) was analyzed for DA, while the rest (0.25 l) was fixed in 1.6 % formaldehyde for algal identification and counting, to determine the phytoplankton composition of the sample used for toxin analysis. The concentration of algae in seawater was obtained by phytoplankton qualitative and quantitative analysis of surface bottle samples fixed in 1.6% formaldehyde.

*Quantification of domoic acid by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS)*

**Mussel extraction.** Mussel flesh (20 g) was extracted with 100 ml 50 % aqueous methanol. After centrifugation at 4,500 rpm for 20 min, the supernatant was dried and dissolved in 20 ml 50 % aqueous methanol.

**Phytoplankton and filtered seawater extraction.** Net seawater (1.5 l) was filtered (0.45 µm filters; Millipore, Bedford, USA) and the phytoplankton on the filter was extracted three times with 10 ml of 50 % aqueous methanol. After centrifugation at 3,000 rpm for 20 min, pooled supernatants were dried and dissolved with 5 ml of 50 % aqueous methanol. Filtered seawater was passed through a C18 Sep-Pak plus cartridge (Waters, Milford, USA), conditioned with methanol, 50 % aqueous methanol and water (5 ml each) and eluted with 50 % aqueous methanol (10 ml)



**Figure 1.** Map of the Gulf of Trieste showing the sampling site in the Panzano Bay. **Figure 2, 3.** *Pseudo-nitzschia calliantha*. Fig. 2. Valve view. Fig. 3. Central part of valve showing central nodule and poroids.

and methanol (50 ml). The eluate was dried and dissolved with 5 ml of 50 % aqueous methanol.

**HILIC-MS analysis.** Extract separation was carried out on a 250 x 2 mm i.d. column packed with TSK-GEL Amide-80, followed by isocratic elution with 71.25 % aqueous acetonitrile, containing 2 mM ammonium formate and 3.6 mM formic acid (pH 3.5; flow rate: 200 µl/min; injected volume: 5 µl). MS detection was carried out in positive ion mode and acquisition in multiple reaction monitoring (API 2000 triple-quadrupole mass spectrometer; Applied Biosystems, Foster City, USA). Transition 312/266 (m/z value of precursor and fragment ion) was monitored to quantify DA. The detection limit of HILIC-MS is 3 ng DA/ml.

#### *Quantification of DA by ELISA*

**Mussel extraction.** Mussels (4 g) were extracted with 16 ml 50 % aqueous methanol. After centrifugation at 3,000 rpm for 10 min, the supernatant was diluted 1:20 and subsequently 1:200 with phosphate-buffered saline containing 10 % methanol and 0.05 % Tween 20 (sample buffer).

**Phytoplankton and filtered seawater extraction.** Net seawater (0.1 l) was filtered through 0.45-µm filter (Millipore, Bedford, USA). The filter was extracted with 10 ml 20 % aqueous methanol and centrifuged at 3,000 rpm for 20 min. The supernatant was diluted 1:10 and subsequently 1:100 with sample buffer. The filtered seawater was diluted 1:30 with sample buffer.

**ELISA.** A commercial competitive ELISA kit (Bio-sense, Bergen, Norway) was used. In each well, samples (50 µl) were incubated with anti-domoic acid antibody conjugated to horseradish peroxidase (50 µl) at 25 °C for 1 h. After washing, 100 µl of tetramethylbenzidine and peroxidase substrate were added. After 15 min in the dark, and addition of 100 µl of 0.3 M sulphuric acid, absorbance was read (450 nm). DA was quantified interpolating absorbance values from

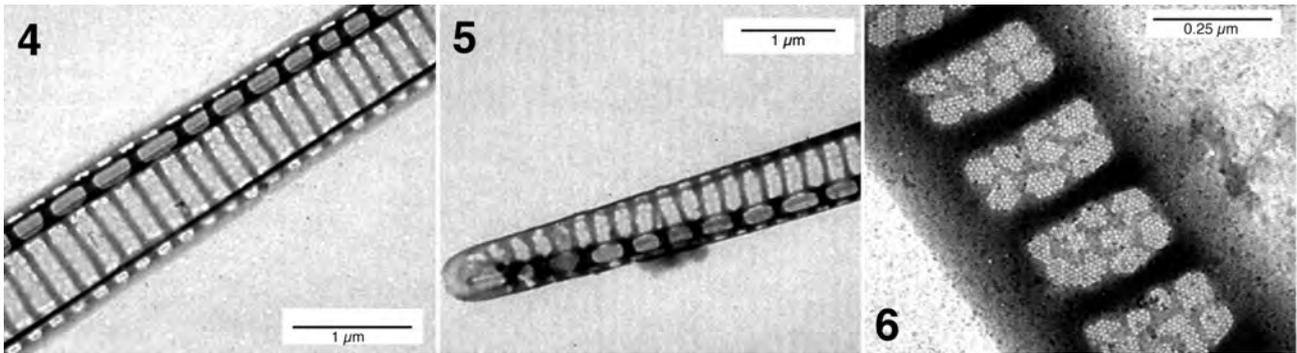
the calibration curve of DA standard solutions. The detection limit of the assay is 50 pg DA/ml.

#### *Phytoplankton analysis*

Microalgae in 1 ml of phytoplankton net samples were examined by an inverted light microscope (Leitz Diavert, Wetzlar, Germany), using phase contrast at 500 x magnification. *Pseudo-nitzschia* species were counted over the whole sedimentation chamber to determine their concentration in the samples used for toxin extraction and analysis. 50 ml of each seawater surface bottle sample were settled in a sedimentation chamber for qualitative and quantitative analysis and *Pseudo-nitzschia* species were counted. *Pseudo-nitzschia* species were identified by transmission electron microscopy. Net samples were cleaned using hydrogen peroxide (30 % w/v) and a few drops of concentrated hydrochloric acid and observed in a Philips EM 208 (Philips, Eindhoven, Netherlands) transmission electron microscope.

#### **Results and Discussion**

The genus *Pseudo-nitzschia* was not common in the phytoplankton of the Gulf of Trieste during the sampling period, and its percentage never exceeded 10.8 % of total diatoms in surface bottle samples, and 9 % in net samples. The maximum abundance was in autumn (4 October 2005: 55,280 cells/l) with low numbers in spring (May) and summer (August), never exceeding 20,000 cells/l. The most abundant species identified by TEM were *Pseudo-nitzschia calliantha* Lundholm, Moestrup et Hasle (Figs 2-3), and *P. decipiens* Lundholm et Moestrup (Figs 4-6). Other taxa, belonging to *P. seriata* group were observed in spring and late autumn but their low numbers did not allow for preparation of specimens for TEM identification. *Pseudo-nitzschia calliantha* was recognized by the poroid hymen being divided in sectors arranged in a circle and resembling a flower (Fig. 3). The cell size was rather small, and valve width did not exceed 1.3 µm. The main morphological features (valve shape,



**Figure 4-6.** *Pseudo-nitzschia decipiens*. Fig. 4. Valve showing striae with two rows of poroids. Fig. 5. Tip of valve. Fig. 6. Detail of girdle band showing hymen perforations arranged in hexagonal pattern.

central nodule, interstria and fibula spacing, poroid shape and arrangement) fitted well with the original description. It was mainly found in autumn and reached its maximum concentration in October. *Pseudo-nitzschia decipiens* was identified because of the presence of two rows of poroids (Fig. 4) and distinguished from *P. delicatissima* by the higher density of striae (44 striae/10 µm). Cells were very narrow (valve width 1 µm or less) but corresponded well to the species description in the biseriatae, higher density of striae, and hexagonal pattern of perforations in the poroids (Fig.6). It was found in summer and early autumn with a maximum concentration in September. This is the first report of *P. decipiens* in the Adriatic Sea after the taxonomic revision of the *P. delicatissima* complex (Lundholm *et al.* 2006). The presence of *P. calliantha* in the Adriatic Sea (Gulf of Trieste and Kastela Bay) was also reported in the original description of this species (Lundholm *et al.* 2003).

These results confirm that *P. calliantha* and the *P. delicatissima* complex, including *P. decipiens*, are the dominant *Pseudo-nitzschia* species in the Adriatic Sea, as they were recognized also in the southern Adriatic Sea (Caroppo *et al.* 2005).

The concentration of DA in phytoplankton extracts, seawater, and mussels during this monitoring period was always below the detection limit of Biosense ASP ELISA (50 pg/ml) and HILIC-MS (3 ng/ml). Some clones of *P. calliantha* and *P. delicatissima*, from which *P. decipiens* has been only recently split, are known to produce low amounts of DA, such as 0.007-0.221 pg/cell (Martin *et al.* 1990; Lundholm *et al.* 1997) and 0.005-0.12 pg/cell (Smith, 1991; Rhodes *et al.* 1998), respectively. However, the low presence of these *Pseudo-nitzschia* species in the Gulf of Trieste and the lack of DA in mussels and phytoplankton do not prove that *Pseudo-nitzschia* species will not present a problem in the Adriatic Sea in the future. Further studies and a more complete survey of the *Pseudo-nitzschia* species in the area will be neces-

sary to determine whether DA acid in the Adriatic Sea is produced by these species.

### Acknowledgements

This work was supported by Regione Friuli Venezia Giulia – Direzione Centrale Risorse Agricole, Naturali, Forestali e Montagna – Servizio Pesca e Acquacoltura.

### References

- Bernardi Aubry, F., Berton, A., Bastianini, M., Socal, G. & Acri, F. (2004). *Cont. Shelf Res.* 24: 97-115.
- Caroppo, C., Congestri, R., Bracchini, L. & Albertano, P. (2005). *J. Plankt. Res.* 27: 763-774.
- Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Quilliam, M.A., Tubaro, A. & Poletti, R. (2005). *Rapid Commun. Mass Spectrom.* 19: 2030-2038.
- Lundholm, N., Skov, J., Pocklington, R.G. & Moestrup, Ø. (1997). *Phycologia* 36: 381-388.
- Lundholm, N., Moestrup, Ø., Hasle, G.R. & Hoef-Emden, K. (2003). *J. Phycol.* 39: 797-813.
- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. & Miller, P. (2006). *J. Phycol.* 42: 464-481.
- Martin, J.L., Haya, K., Burrige, L.E. & Wildish, D.J. (1990). *Mar. Ecol. Progr. Ser.* 67: 177-182.
- Revelante, N. & Gilmartin, M. (1977). *Hydrobiologia* 56: 229-240.
- Rhodes, L., Scholin, C., Garthwaite, I., Haywood, A. & Thomas, A. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T. (eds), Xunta de Galicia and IOC, UNESCO, Santiago de Compostela, pp. 274-277.
- Smith, J.C., Pauley, K., Cormier, P., Angus, R., Odense, P., O'Neil, D., Quilliam, M.A. & Worms J. 1991. *Can. Techn. Rept. Fish. Aquat. Sci.*: 1799.
- Totti, C., Civitarese, G., Acri, F., Barletta, D., Candelari, G., Paschini, E. & Solazzi, A. (2000). *J. Plankton Res.* 22: 1735-1756.

## Alexandrium cysts in Puget Sound, Washington, USA

R.A. Horner<sup>1</sup>, C.L. Greengrove<sup>2</sup>, J.R. Postel<sup>1</sup>, J.E. Gawel<sup>2</sup>, K.S. Davies-Vollum<sup>2</sup>, A. Cox<sup>3</sup>, S. Hoffer<sup>2</sup>, K. Sorensen<sup>2</sup>, J. Hubert<sup>2</sup>, J. Neville<sup>2</sup> and B.W. Frost<sup>1</sup>

<sup>1</sup>School of Oceanography, Box 357940, University of Washington, Seattle, WA 98195-7940; rita@ocean.washington.edu; <sup>2</sup>Interdisciplinary Arts and Sciences, Box 358436, University of Washington, Tacoma, WA 98402; cgreen@u.washington.edu; <sup>3</sup>Huxley College of the Environment, Western Washington University, Bellingham, WA 98225; anniecox1@hotmail.com

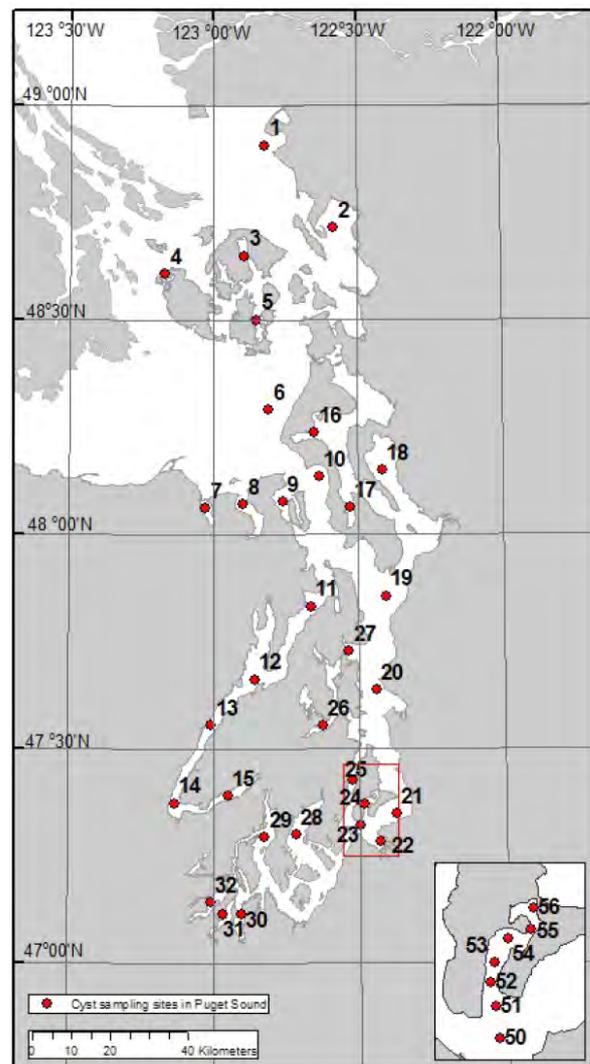
### Abstract

A survey of 32 sites in March 2005 showed cysts of *Alexandrium catenella* in surface sediments throughout Puget Sound, Washington, USA. Cysts were most abundant in the northern and central areas. Sequim Bay in the north with 200 cysts/ml sediment and Quartermaster Harbor in the central Sound with 12,000 cysts/ml sediment had the highest abundances. In other areas where cysts were found, numbers ranged from <10 to about 100 cysts/ml sediment. Other sediment parameters, including grain size, total organic carbon (TOC) content, and heavy metal (Cu, Cd, Zn, As, and Pb) concentrations, were measured to evaluate possible correlations with cyst abundance. At some sites, cyst concentrations and <sup>210</sup>Pb were also measured in sediment cores to assess how long the risk of PSP had been present. Initial results indicate that there is no correlation between sediment grain size, TOC, or metal concentration with cyst abundance and that in some areas of Puget Sound the potential for PSP outbreaks has been present since the 1950s.

### Introduction

Paralytic Shellfish Poisoning (PSP) is often present in shellfish in Puget Sound, but little is known about the distribution and biology of either the motile cells or cysts of the major causative species, *Alexandrium catenella*. In 1981 a cyst survey in southern Puget Sound showed motile cells, cysts, or low levels of PSP throughout the area (L. Nishitani pers. comm.). Motile cells and concurrent PSP in shellfish are often present at irregular intervals during spring and summer, but occurrence, bloom intensity and PSP levels are extremely variable. For example, in 1997, PSP occurred in blue mussels (*Mytilus edulis*) in November-December with toxin levels of 6,800 µg/100 g, while in 1998 toxin levels in blue mussels reached 11,000 µg/100 g in August and October-November. These outbreaks occurred throughout Puget Sound and resulted in harvest closures in new areas, especially in southern Puget Sound. In 2006, an *Alexandrium catenella* bloom was present in central Puget Sound from mid-August to mid-September with toxin levels in blue mussels reaching 17,000 µg/100 g (F. Cox, Washington Department of Health). To better prepare for and manage these PSP outbreaks, it is important to know which areas of the Sound are most susceptible. Our study shows where cysts are most abundant in surface sediments and suggests areas where future blooms might be expected.

**Figure 1.** Sampling sites in Puget Sound, Washington USA. Insert shows sampling sites in Quartermaster Harbour.

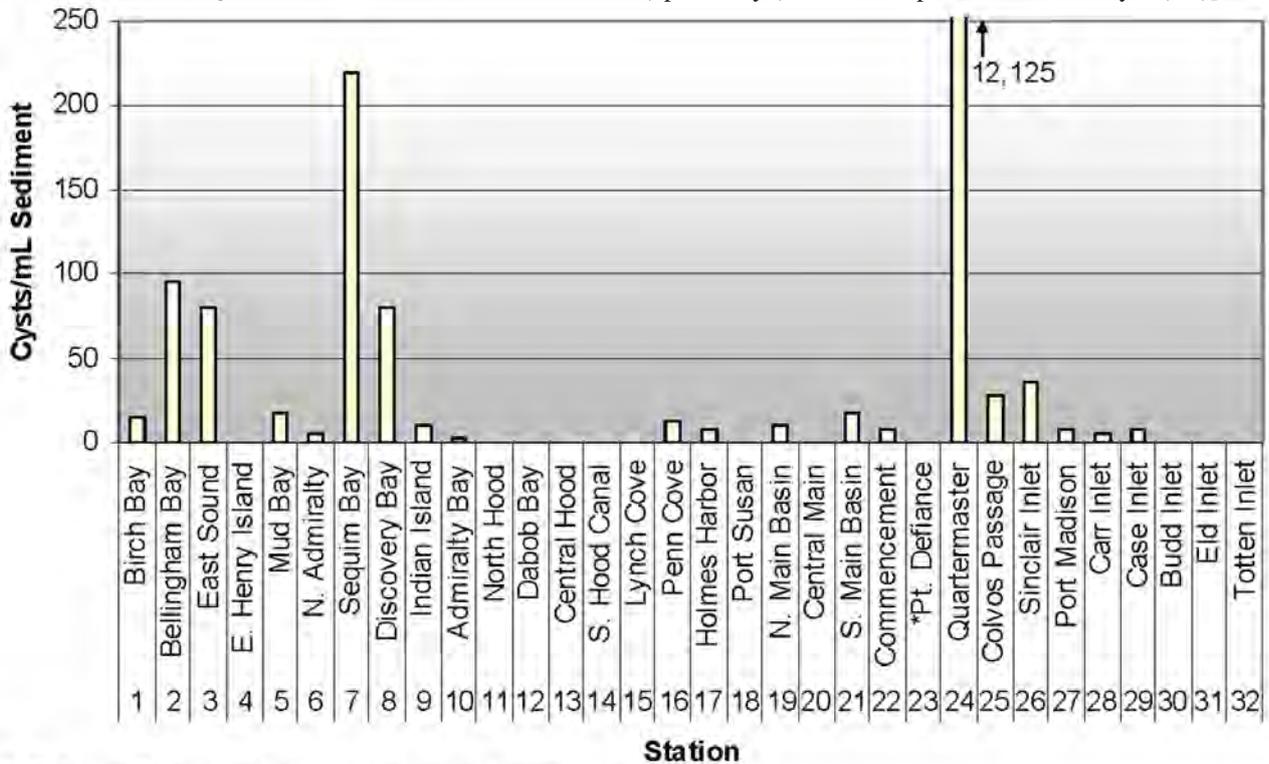


**Methods**

Sediment was collected with a Soutar box corer and/or Van Veen sampler at 32 sites in Puget Sound in 2005 and at an additional 5 sites in Quartersmaster Harbor in 2006. The sediment was subsampled using 7.6-cm diameter polycarbonate tubing, and down-core cyst concentrations were determined. <sup>210</sup>Pb activity in subsamples was measured to estimate sediment age, accumulation rates, and mixing parameters. Cyst abundance, grain size, TOC, and heavy metal concentrations were determined from the upper 2 cm of sediment. Cyst counts were done using epifluorescence microscopy after staining with primulin (Yamaguchi *et al.* 1995). <sup>210</sup>Pb activity was determined using the methods of Nittrouer *et al.* (1979), and approximate dates were calculated following Appleby and Oldfield (1978). Sediment grain size was analyzed using a Coulter LS 200 Particle Analyzer, and TOC was determined by loss on ignition using a muffle furnace. Metal concentrations were measured using standard methods (EPA 2001).

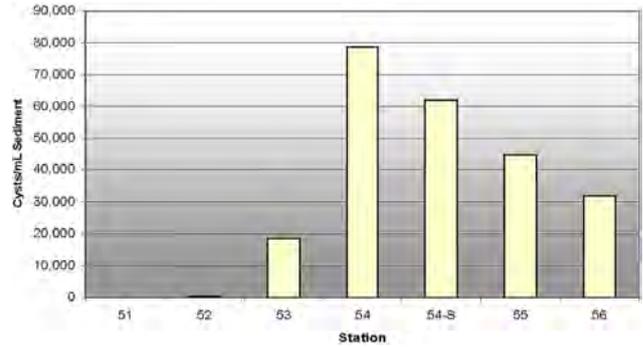
**Results**

Surface cyst counts were highest at Station 24 in Quartersmaster Harbor at 12,000 cysts/ml sediment - two orders of magnitude higher than at any other station in 2005 (Figs 1, 2). Within Quartersmaster

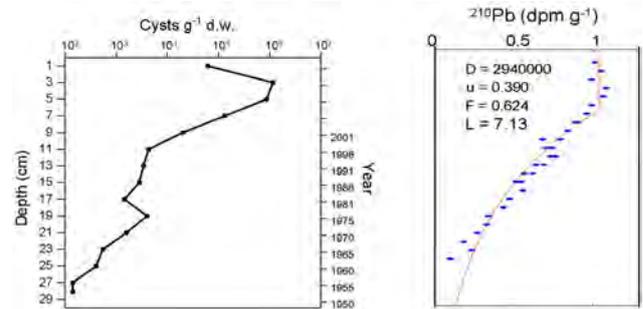


\* Pt. Defiance (23) had no data because no sample was obtained.

**Figure 2.** Surface cyst concentrations at all stations during the 2005 general survey.



**Figure 3.** Surface cyst concentration in Quartersmaster Harbour during the 2006 intensive survey. Stations 54 and 54-S are from two separate Van Veen samples and show variability in cyst numbers over a very small area.



**Figure 4.** Cyst concentrations and <sup>210</sup>Pb activity down core in Quartersmaster Harbour [D = bioturbation coefficient (cm<sup>2</sup> y<sup>-1</sup>, U = sedimentation rate (cm y<sup>-1</sup>), F = <sup>210</sup>Pb flux (dpm cm<sup>2</sup> y<sup>-1</sup>), and L = depth of the mixed layer (cm)].

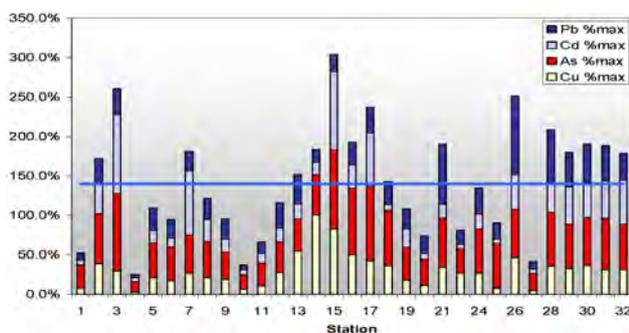
Harbor in 2006, highest numbers were at Station 54, dropping off toward the mouth and outside the bay (Fig. 3). Cysts were found in the deepest samples in the core, which  $^{210}\text{Pb}$  analysis indicates to be about 1955 (Fig. 4). Highest cyst concentrations were found in the upper 5 cm.

Largest grain size, predominately silt and sand, and lowest TOC occurred in areas with strongest currents, while smallest grain size and highest TOC were generally found in more protected sites (Fig. 1, Table 1). Higher metal concentrations were found in finer grain sediments (Fig. 5).

## Discussion

Based on data from the Washington Department of Health (WDOH, data not shown) highest cyst abundance in surface sediments was found in areas where PSP often occurs. Cyst abundance in surface sediments was highest in Quartermaster Harbor where the presence of cysts in the deepest part of the core suggests that there has been a potential for PSP for at least 50 years. However, PSP monitoring did not begin in southern Puget Sound until after 1978 when a massive bloom of *Alexandrium catenella* in northern Puget Sound apparently led to the southerly movement of PSP (Trainer *et al.* 2003). Thus it is not known whether PSP toxins were present earlier or if they were present at such low levels that there was no health risk. The first closure in the southern Sound occurred in 1988.

Dating of cores by  $^{210}\text{Pb}$  starts at the bottom of the mixed layer, about 7 cm in Quartermaster Harbor, and represents the present day (Fig. 4). Below the biologically mixed layer, cysts are entered into the permanent sediment record and are moved downward at the local sedimentation rate. Therefore cysts cannot be younger than the date assigned, in this case about 50 years old, but they could be older since a cyst could be retained in the mixed layer for several years



**Figure 5.** Cumulative toxic metal concentrations in surface sediments at all stations normalized to the maximum value found in 2005 (line).

and not be immediately transported to the bottom of the mixed layer.

We have not measured toxicity in cysts in Quartermaster Harbor, but this shallow, protected bay has been called a possible breeding bay for PSP (Nishitani and Chew 1984). Further, WDOH PSP data indicate relatively frequent outbreaks and harvest closures suggesting the potential for cysts to be toxic.

Our results indicate that for both down-core and surface sediments no correlation exists between cyst abundance and sediment characteristics. Cyst presence does not appear to be influenced by grain size, organic carbon content or metal concentrations in the surface and near-surface sediment.

**Table 1.** Grain size (volume %) and TOC content (%) by station. Samples collected with Soutar box corer except \*collected with Van Veen sampler.

Station Number	Clay (%)	Silt (%)	Sand (%)	TOC (%)
1	2.2	12	88	1.4
2	16	74	10	7.0
3	7	73	20	10.4
4*	0.5	3	96.5	0.6
5	9.5	62	28.5	7.5
6	9	59	32	4.8
7	7	68	25	8.9
8	8	71	21	6.6
9	9	57	34	6.3
10*	2	6	92	1.3
11	5	27	68	2.3
12	10	71	19	7.2
13	13	75	12	8.7
14	11	75.5	13.5	7.9
15	6	68.5	25.5	11.2
16	13	77.5	9.5	7.1
17	8	70	22	9.2
18	25.5	71	3.5	5.6
19	8	46	46	3.3
20*	6	38	56	3.6
21	14	68	18	8.2
22*	16	68	16	5.2
23	--	--	--	1.8
24*	6	50	44	4.5
25	1	4	95	8.6
26	8	59	33	7.6
27	2	9	89	2.1
28	8	73	19	7.6
29	9	74	17	8.8
30	6.5	78.5	15	8.6
31	7	68	25	8.9
32	6	68	26	8.7

### Acknowledgements

This project is funded by the NOAA ECOHAB Program (NA04NOS4780273, publication #217). Thanks to the crew of the R/V Barnes, R. McQuin and N. Hix, for providing logistical support during cruises, M. Holmes for allowing us to piggy-back on R/V Thompson student cruises and for showing us how to operate the piston corer. D. Shull provided advice on the cyst counting method and  $^{210}\text{Pb}$  data analyses and J. Coyle helped with the maps.

### References

- Appleby, P.G. & Oldfield, F. (1978). *Catena* 5: 1-8.
- EPA. (2001). *Methods 200.7 - - Revision 5.0*. EPA-821-R-01-010.
- Nishitani, L. & Chew, K.K. (1984). *Aquaculture* 39: 317-329.
- Nittrouer, C.A., Sternberg, R.W., Carpenter, R. & Bennett, J.T. (1979). *Mar. Geol.* 31: 297-316.
- Trainer, V.L., Eberhardt, B.L., Wekell, J.C., Adams, N.G., Hanson, L., Cox, F. & Dowell, J. (2003). *J. Shellfish Res.* 22: 213-223.
- Yamaguchi, M., Itakura, S, Imai, I. & Ishida, Y. (1995). *Phycologia* 34: 207-214.

## Paralytic Shellfish Poisoning (PSP) toxins in *Alexandrium catenella* and *A. tamarense* isolated from southern coastal and offshore waters of Korea

Y.-S. Kim, C.-H. Kim

Department of Aquaculture, Pukyong National University, Busan 608-737, Korea, chkpkn@hanmail.net

### Abstract

Thirty-two isolates of *Alexandrium catenella* and *A. tamarense* from southern coastal and offshore waters in Korea were analyzed for PSP toxins. In seventeen coastal isolates, the major toxins were C2, GTX1,4 and neoSTX, and the minor toxins were C1 and GTX3. In contrast, in fifteen offshore isolates, the major toxins were C2 and GTX4, and the minor toxins were C1, GTX1,3,5, and neoSTX. Among them, eight isolates contained GTX5, which was not detected in any coastal isolates. In addition, there was a clear difference in the proportion of carbamate to N-sulfocarbamoyl toxins between coastal and offshore isolates, which were 69:31 and 44:56, respectively. Cluster analysis based on toxin composition and profiles divided the Korean *Alexandrium* isolates into three distinct groups, characterized by higher proportions of C2, neoSTX, and GTX4, respectively.

### Introduction

Two PSP incidents that resulted in human deaths were reported from Korea in 1986 and 1996. Chang *et al.* (1997) first reported a correlation between the occurrence of *A. tamarense* (as *Protogonyaulax tamarensis*) and shellfish intoxication, and Kim (1995) demonstrated that the causative microorganisms were *A. tamarense* in Jinhae Bay, Korea. A number of studies have been performed over the last two decades to verify the toxin production of PSP causative organisms. However, these studies have only been performed in coastal areas, such as Jinhae Bay. In this study, we established toxic *Alexandrium catenella* and *A. tamarense* isolates from the southern coastal and offshore waters of Korea, and analyzed the PSP toxins to elucidate any differences in toxin production related to locality.

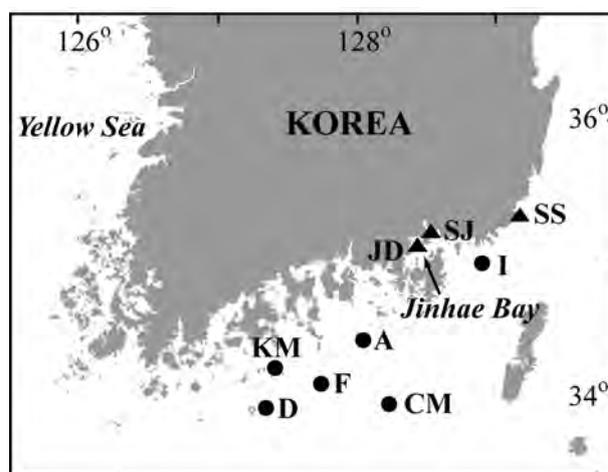
### Material and Methods

Thirty-two axenic cultures of *Alexandrium* were established. Phytoplankton samples from coastal waters were collected by towing a 20- $\mu$ m mesh plankton net. Sediment samples from southern offshore waters were collected by a piston core sampler (Fig. 1). Germinated and vegetative cells were picked out with a micropipette and successively transferred into tissue culture wells containing sterile f/2 medium. After growing the cells at 17 °C and 14:10 L:D (50  $\mu$ mol photons  $m^{-2}s^{-1}$ ), the cultures were purified by washing with a micropipette. Cultured cells in mid-exponential growth phase were harvested by centrifugation (2000 $\times$ g for 5 min) in the early dark period. Pelleted cells were suspended in 0.05 N acetic acid and disrupted by sonication on ice. The supernatant was

passed through a membrane filter (10,000 MW cut-off, Ultrafree C3GC, Millipore) by centrifugation at 8,000 $\times$ g for 5 min. Archived samples stored at -20 °C were analyzed using the three-step isocratic elution method with HPLC-FD (Hewlett Packard 1100 System), recommended by Oshima (1995). A Wakosil 5C8 column (4.6 $\times$ 250 mm, Wako Pure Chemical) was used to separate individual toxins.

### Results

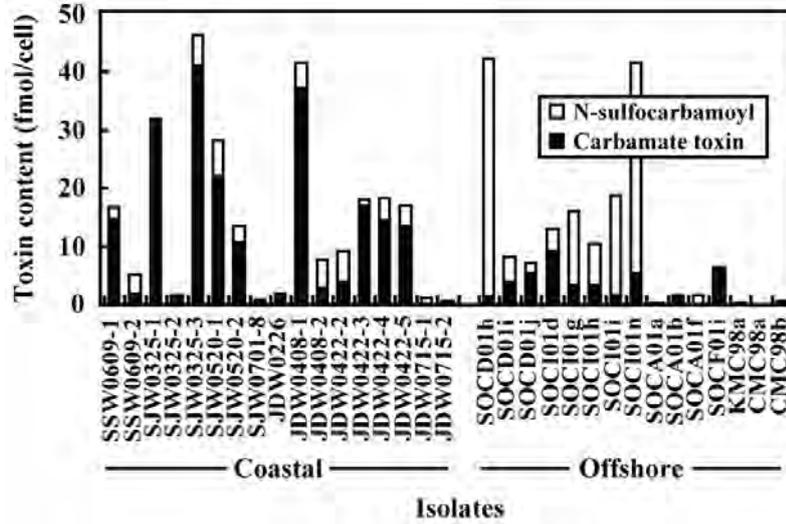
Thirty-two isolates of *Alexandrium catenella* and *A. tamarense* contained toxins in the range of 0.04~46.38 fmol/cell. The toxin composition depended on the origin of the isolates. Toxins C1, 2, GTX1, 3, 4, and neoSTX were present in most isolates. The concentration of GTX4 and neoSTX showed a tendency to be higher in coastal isolates than in offshore ones, whereas the concentration of C2 was higher in the lat-



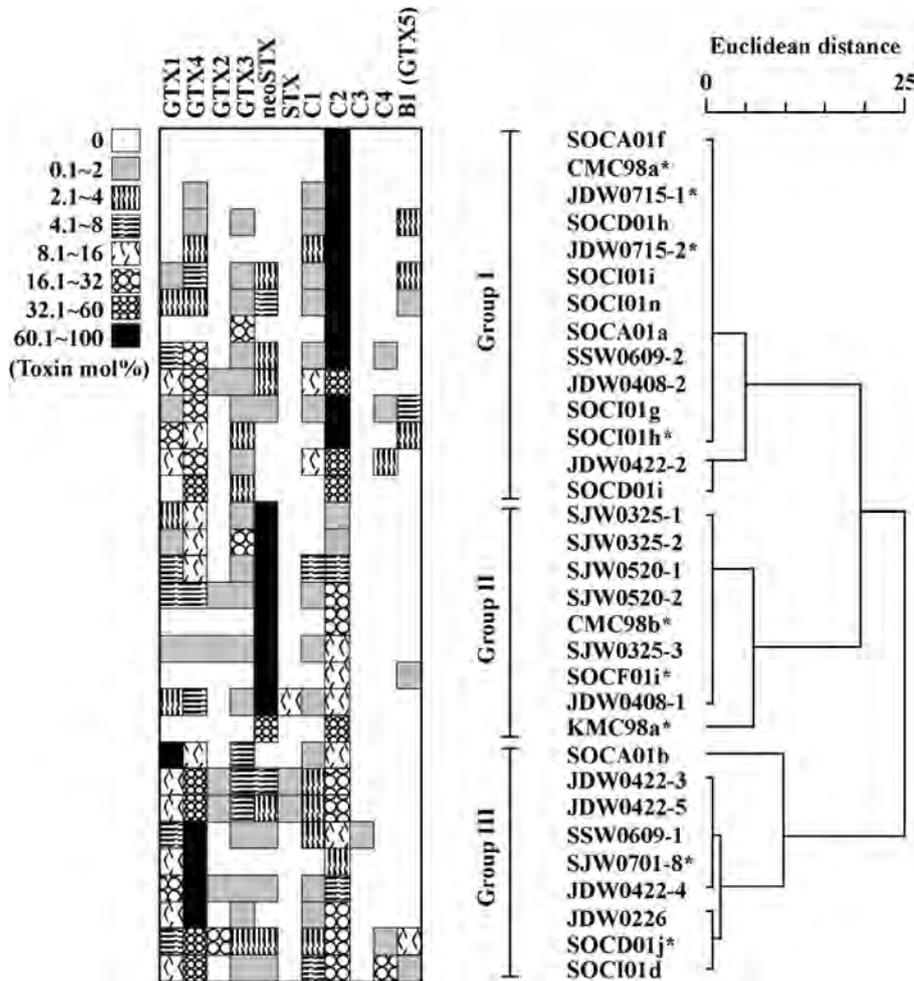
**Figure 1.** Map showing the sampling stations of the *Alexandrium* regional isolates for toxin analysis in Korean coastal (▲) and offshore (●) waters.

ter than the former. Most isolates of *A. catenella* had fewer toxin components and lower toxin concentrations than those of *A. tamarensis*. However, there was no identifiable relationship between toxin composition and species identity.

Fifteen isolates from Jinhae Bay (JD and SJ) shared similar toxin composition. However, six isolates were distinctive because of a relatively high concentration of neoSTX (66.71–85.57 mol %), and three isolates were distinctive because of the presence



**Figure 2.** The proportion of carbamate to N-sulfocarbamoyl toxins of *Alexandrium* isolates from coastal and offshore waters of Korea.



**Figure 3.** Clustering matrix and dendrogram showing Euclidean distances for *Alexandrium* isolates based on PSP toxin composition (% molar) and toxin profiles. \* Isolates represent *A. catenella*, the other isolates are *A. tamarensis*.

of STX. The major toxins in offshore isolates were C2 and GTX4. The minor ones were C1, GTX1, 3, 5 and neoSTX. Among them, GTX5 (= B1) was present only in eight isolates. Three *A. catenella* strains isolated from offshore had a high concentration of neoSTX (mol %), but the total toxin content was lower than in *A. tamarense*.

One notable distinction between coastal and offshore isolates was the average proportion of carbamate to N-sulfocarbamoyl toxins, which was 69-31 in the former and 44-56 in the latter (Fig. 2).

Cluster analysis based on toxin components and toxin concentration (mol %) indicated the presence of at least three distinct groups in Korea. Group I contained C2 as the major toxin, GTX1, 3, 4, 5, and neoSTX as the minor. Group II contained neoSTX as the major toxin, and C2 as the minor. Group III contained GTX1, 4 as the major toxins. Groups II and III were clearly distinguished from Group I by a higher proportion of carbamate toxins than N-sulfocarbamoyl toxin (Fig. 3).

## Discussion

The PSP toxin composition of single *Alexandrium* isolates is generally considered stable throughout the broad range of physico-chemical conditions. Therefore, PSP toxin composition has been used as a practical biomarker to differentiate among *Alexandrium* isolates from different geographical locations (Kim *et al.* 1993; Park *et al.* 2004).

We found that *A. tamarense* and *A. catenella* from coastal areas contained carbamate toxins as the major toxin component, and were clearly distinguishable from those from offshore areas, which contained N-sulfocarbamoyl toxins as the major toxin component. This variation can be attributed to differences in environmental regimes. A perpetual front is located between the two areas and prevents direct influx of the Tsushima Current into coastal areas. Thus, the environmental differences between the two areas may result in distinctive geographical populations. Similarly, Park *et al.* (2004) reported that *Gymnodinium catenatum* strains in the Yellow Sea at a higher latitude compared to the south inshore of Korea included isolates of higher toxicity, predominantly producing carbamate toxins. Furthermore, it was suggested that high toxicity of the northern isolates was due to the production of the highly potent carbamate toxins, and the regional trend in toxicity could result from latitudinal differences in environmental parameters and their influence on the establishment of the genotypically different blooms (Anderson *et al.* 1994).

From the cluster analysis, isolates from the Southern offshore belonged largely to Group I, while isolates from the south eastern sea including Jinhae Bay belonged mostly to Group II-III. Moreover, there is a strong possibility that a variety of toxin components in regional populations as showed in Jinhae bay isolates would result from the genetic exchange in sexual reproduction showing phenotypically different toxin profiles (Sako *et al.* 1992; Ishida *et al.* 1993). Therefore, this might be explained by heterogeneity with a genetic trait as well as with the advantage of environmental separation (Cembella *et al.* 1987; Kim 1995; Park *et al.* 2004) rather than by the influence of the regional environments, for example elevated nutrients (Group III are dominant in Jinhae Bay) and compositional changes observed in culture studies (Hwang and Lu 2000).

## Acknowledgements

The authors wish to thank Prof. Y. Oshima (Tohoku University) for providing PSP standard solutions. This work was supported by grant (No. R02-2000-00226) from the Basic Research Program of the Korea Science & Engineering Foundation.

## References

- Anderson, D.M., Kulis, D.M., Doucette, G.J., Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R. & Anderson, R.J. (1987). *Biochem. Syst. Ecol.* 15: 171-186.
- Chang, D.S., Shin, I.S., Pyeon, J.H. & Park, Y.H. (1987). *Bull. Korean Fish. Soc.* 20: 293-299.
- Gallagher, J.C. & Balech, E. (1994). *Mar. Biol.* 120: 467-478.
- Hwang, D.F. & Lu, Y.H. (2000). *Toxicon* 38: 1491-1503.
- Ishida, Y., Kim, C.H., Sako, Y., Hirooka, N. & Uchida, A. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J. & Shimizu, Y. (eds), Elsevier, Amsterdam, pp. 881-887.
- Kim, C.H. (1995). *J. Korean Fish. Soc.* 28: 364-372.
- Kim, C.H., Sako, Y. & Ishida, Y. (1993). *Nippon Suisan Gakkaishi* 59: 641-646.
- Oshima, Y. (1995). In: *Manual on Harmful Marine Microalgae*, Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D. (eds), UNESCO, Paris, pp. 81-94.
- Park, T.-G., Kim, C.-H. & Oshima, Y. (2004). *Phycol. Res.* 52: 300-305.
- Sako, Y., Kim, C.H. & Ishida, Y. (1992). *Biosci. Biotechnol. Biochem.* 56: 692-694.

## Status of potentially harmful algae in the lower Chesapeake Bay estuarine system

H.G. Marshall<sup>1</sup>, L. Burchardt<sup>2</sup>, T.A. Egerton<sup>1</sup> and M. Lane<sup>1</sup>

<sup>1</sup>Department of Biological Science, Old Dominion University, Norfolk, VA, 23529-0266, U.S.A., hmarshal@odu.edu; <sup>2</sup>Department of Hydrobiology, Adam Mickiewicz University, 61-614 Poznan, Poland, burchard@amu.edu.pl

### Abstract

Through several monitoring programmes and independent cruises, 37 potentially harmful or toxin-producing algal species have been identified within the Chesapeake Bay and its tidal estuaries. Most common are 13 dinoflagellates and 16 cyanobacteria, with long-term trends (1985-2005) identifying increased cyanobacteria biomass and abundance in these waters.

### Introduction

Water samples analyzed in this study came from the lower (southern) Chesapeake Bay and 3 tidal rivers (Rappahannock, James, and York Rivers) through the Chesapeake Bay Monitoring Programme with a 20-year data base (1985-2005). Additional sample analysis came from independent cruises and collections in the Virginia Harmful Algae Monitoring Programme (1997-2006). Water samples were examined with light microscopy and supplemented with SEM and PCR analysis. Cell cultures were established for fish toxic bioassays (Marshall *et al.* 2000). The seasonal Kendall test was applied to identify monotonic long-term trends, and the Van Belle and Hughes test to confirm the homogeneity of trends.

### Potential harmful of toxin producing species

The following 37 taxa were identified in either the Bay or its tidal tributaries:

#### Diatoms (5):

*Amphora coffeaeformis* (Agardh) Kützing, *Pseudo-nitzschia multiseriis* (Hasle) Hasle, *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle, *Pseudo-nitzschia pungens* (Grunow) Hasle, and *Pseudo-nitzschia seriata* (Cleve) Peragallo

#### Dinoflagellates (13):

*Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup, *Cochlodinium polykrikoides* Margalef, *Dinophysis acuminata* Claparède et Lachmann, *Dinophysis acuta* Ehrenberg, *Dinophysis caudata* Saville-Kent, *Dinophysis fortii* Pavillard, *Dinophysis norvegica* Claparède et Lachmann, *Gyrodinium aureolum* Hulburt, *Karlodinium veneficum* (Leadbeater et Dodge) J.

Larsen, *Lingulodinium polyedrum* (Stein) Dodge, *Pfiesteria piscicida* Steidinger et Burkholder, *Pfiesteria shumwayae* Glasgow et Burkholder, *Prorocentrum minimum* (Pavillard) Schiller

#### Raphidophytes (3):

*Chattonella subsalsa* Biecheler, *Chattonella verruculosa* Hara et Chihara, *Heterosigma akashiwo* (Hada) Hada

#### Cyanobacteria (16):

*Anabaena affinis* Lemmermann, *Anabaena circinalis* Rabenhorst, *Anabaena flos-aquae* (Lyng.) Brebisson, *Anabaena recta* Komárek et Kovacik, *Anabaena solitaria* Klebahn, *Anabaena spiroides* Klebahn, *Aphanizomenon flos-aquae* (L.) Ralfs, *Aphanizomenon issatschenkoi* (Ussac.) Proschkina-Laurenko, *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya et Subba Raju, *Microcystis aeruginosa* Kützing, *Microcystis firma* (Breb. et Lemm.) Schmidle, *Microcystis viridis* (A. Braun) Lemmermann, *Planktothrix agardhii* (Gomont) Anagnostidis et Komárek, *Planktothrix limnetica*, (Lemm.) Komárek et Anagnostidis, *Planktothrix limnetica* f. *acicularis* (Nyg.) Poljanski, and *Snowella lacustris* (Chodat) Komárek et Hindak. Cyanobacteria were most common in the tidal freshwater and oligo-mesohaline regions of the tributaries. The more widely distributed dinoflagellates in the bay included *A. sanguinea*, *C. polykrikoides*, *K. veneficum*, and *P. minimum*. Taxa from the Atlantic coastal waters (e.g. *Dinophysis* spp.) have entered the bay in sub-pycnocline waters which have transported them to northern bay regions as potential bloom producers (Marshall *et al.* 2004). *G. aureolum* was noted in the U.S. Naval Base, Norfolk, VA (Marshall 1995), with its presence associated with ballast water discharge.

In June 2006, Dr. Y. Tang (Old Dominion University) confirmed its presence (SEM, PCR) during a bloom in a Chesapeake Bay tributary. *L. polyedrum* was identified only as a dinocyst from surface sediment in the lower Bay. The occasional presence of exotic species has also resulted in high cell concentrations, e.g. in 1992, a *Noctiluca scintillans* bloom lasted 3 days (Marshall 1995).

### Significant and more recent bloom events in southern Chesapeake Bay

1. *Ceratium furca*: July-August 1992. An extensive bloom covering 272.2 km<sup>2</sup>, 10<sup>3</sup> cells/ml, extended over the western and central lower Chesapeake Bay regions (Marshall 1995). Non-toxin producer.

2. *Cochlodinium polykrikoides*: a) bloom covered ca. 215.7 km<sup>2</sup>, in west-central Chesapeake Bay, at 10<sup>3</sup> cells/ml, August-September 1992; b) August-September 2005, a bloom mainly in rivers and inlets of the western shore, 10<sup>3</sup> cells/ml (Marshall 1995). This is a well-established species in the region, and a common bloom producer.

3. *Pfiesteria piscicida*: September 1997. Extensive blooms in several MD rivers, associated with fish kills. A resident species with *Pfiesteria shumwayae*.

4. *Pseudo-nitzschia cuspidata* (Hasle) Hasle: January 1999. A sub-pycnocline bloom localized in the lower Potomac River, high concentrations persisted for several weeks. A rare non-toxin producer bloomer in the bay.

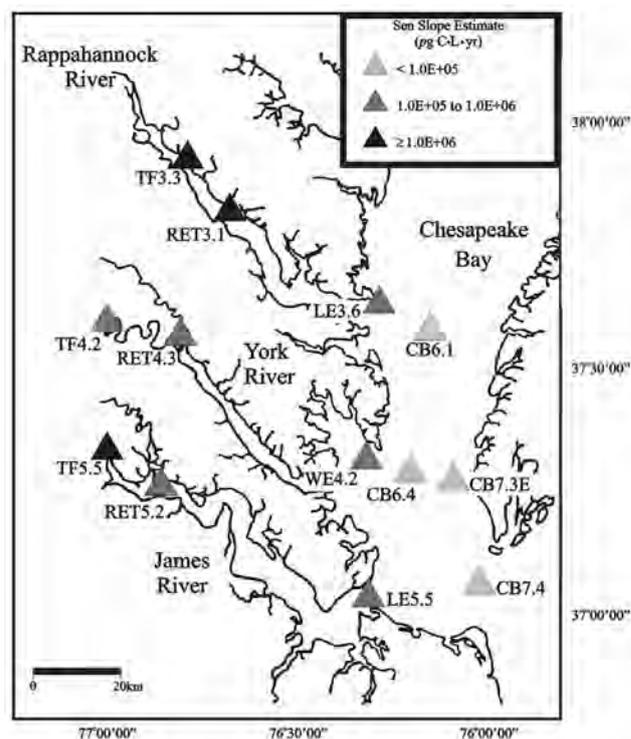
5. *Dinophysis acuminata*: February-April 2002, concentrations ca. 194 cells/ml in several Virginia Potomac River inlets, with trace levels of okadaic acid. Blooms occurred over temperatures of 4.4 -20.7 °C, salinities 10.6-21.3 (Marshall *et al.* 2004).

6. *Microcystis aeruginosa*: June-August 2004. In the Potomac River and its inlets, at several million cells/ml, in salinities 5.2-7.5, and temperatures 25.1-28.5 °C (Marshall *et al.* 2005). Microcystins were detected throughout this period.

7. *Karlodinium veneficum*: June 2006, Potomac River, streams and inlets along Virginia shoreline. Associated with fish kills, reduced oxygen, at ca. 40,000 cells/ml. A common bay species.

### Long-term phytoplankton trends

The 20 year data set of the monitoring programme in Virginia indicated significant ( $p=0.01$ ) long-term trends of increasing cyanobacterial abundance and biomass in the tidal regions of three major tributaries

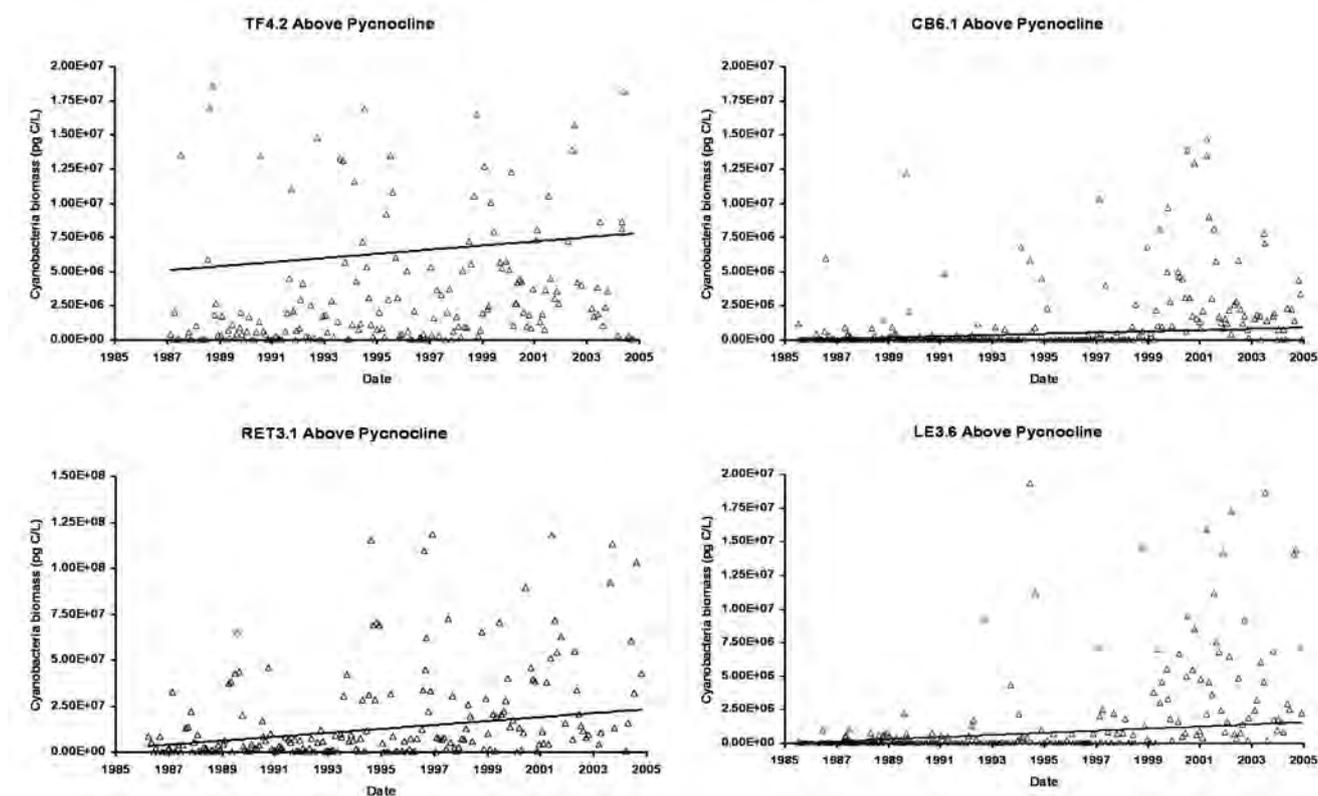


**Figure 1.** Long term trends in cyanobacterial biomass in Chesapeake Bay and major tributaries (1985-2005). Trends are Sen slope estimates expressed in pg C/L/yr.

ies of Chesapeake Bay (James, York, Rappahannock Rivers), plus stations in Chesapeake Bay (Figs 1, 2). These trends were located at the established monitoring stations, with no additional sites added, or an expansion of the sampling protocol. The greatest trends were in tidal river locations, with the lowest trends in Chesapeake Bay. Within these rivers over the monitoring period, 16 potentially toxic cyanobacteria were identified, the most prominent being *Microcystis aeruginosa* (Marshall *et al.* 2005). Colonial and filamentous cyanobacteria were represented in this group.

### Summary

A current listing is provided of 37 potentially harmful and toxin producing phytoplankton taxa for the Chesapeake Bay estuarine complex. It includes 3 raphidophytes, 5 diatoms, 13 dinoflagellates, and 16 cyanobacteria. The list will likely grow following more intense examination of these waters, and introduction of new species. Algal blooms of non-toxin producing species also continue to be frequent and widely distributed in both the bay and its tidal tributaries (Marshall 1995; Marshall *et al.* 2005). Their impact to en-



**Figure 2.** Long term trends in Chesapeake Bay and tributary stations (1985-2005), based on the Sen slope estimate and monthly mean values of cyanobacterial biomass (pg C/L) in tributary stations A) TF4.2 and B) RET3.1, and C-D) in Chesapeake Bay, CB6.1, LE3.6.

environmental conditions and biota will vary according to their composition, areal coverage, and the duration of the bloom. The common dinoflagellate bloom producers include *Heterocapsa rotundata* (Lohmann) G. Hansen, *Heterocapsa triquetra* (Ehrenb.) Stein, and *Scrippsiella trochoidea* (Stein) Loeblich, in addition to several diatoms and the sporadic appearance of bloom-producing species from other categories. There is evidence of entry to the bay of potentially toxic species from off shore Atlantic coastal waters, with others entering from regional river systems, and ballast water discharge. Several present concerns include the occurrence of *Dinophysis acuminata* in regional shellfish harvesting locations, the ongoing trends for increasing biomass and abundance of cyanobacteria within these waters, and the potential for other harmful species to become established and represent a serious threat to the bay's ecosystem.

## Acknowledgements

Components of this study were supported by the Virginia Department of Health, the Virginia Department of Environmental Quality, and USEPA.

## References

- Marshall, H.G. (1995). In: Harmful Marine Algal Blooms, Lassus, P., Arzul, G., Erard-Le Denn, E., Gentien, P., Marcaillou-Le Baut, C. (eds.), Lavoisier, Ltd, pp. 615-620.
- Marshall, H.G., Burchardt, L. & Lacouture, R. (2005). *J. Plankton Res.* 27: 1083-1102.
- Marshall, H.G., Gordon, A., Seaborn, D., Dyer, B., Dunstan, W. & Seaborn, A. (2000). *J. Exp. Mar. Biol. Ecol.* 255: 51-74.
- Marshall, H.G., Egerton, T., Stem, T., Hicks, J. & Kokocinski, M. (2004). In: Harmful Algae 2002, Steidinger, K., Landsberg J.H., Tomas, C.R. & Vargo, G.A. (eds), UNESCO, pp. 364-366.

## ***Alexandrium fundyense* - red tides, PSP shellfish toxicity, salmon mortalities and human illnesses in 2003-04 – before and after**

J.L. Martin, M.M. LeGresley, A. Hanke and F.H. Page

Fisheries and Oceans Canada, Biological Station, 531 Brandy Cove Rd., St. Andrews, NB Canada E5B 2L9,  
MartinJL@mar.dfo-mpo.gc.ca

### **Abstract**

The Bay of Fundy has a long history of *Alexandrium fundyense* blooms and annual shellfish harvesting area closures due to unsafe levels of PSP toxins. Fisheries have been affected where bloom-associated herring mortalities occurred in 1976 and 1979 and the salmon farming industry suffered mortalities in 2003 and 2004. Human illnesses have also been documented through the years.

Data on PSP toxin concentrations in shellfish have been collected since the 1940s and provide an important perspective on *A. fundyense* inter-annual and seasonal patterns. Toxicity data and regular phytoplankton monitoring indicate consecutive years of greater toxicities, the last major period being the late 1970s and early 1980s.

Highest cell densities since the early 1980s were observed in 2003 ( $8.8 \times 10^5$  cells L<sup>-1</sup>) in the Grand Manan Island area and in 2004 in Bliss Harbour (>3 million cells L<sup>-1</sup>) and both bloom events resulted in farmed salmon mortalities. Events surrounding the 2003-04 blooms are presented.

### **Introduction**

Wild herring (*Clupea harengus harengus*) mortalities occurred in the Bay of Fundy in the late 1970s. *Alexandrium fundyense* and its toxins were implicated as probable causes of death when the paralytic shellfish (PS) toxins were transferred to the herring via herbivorous zooplankton (White 1980).

The first aquaculture site for Atlantic salmon (*Salmo salar*) in the southwest New Brunswick region of the Bay of Fundy was established in 1978 in an inshore area. The number of sites has since expanded to more than 90 with some cages located in more exposed areas where higher concentrations of *A. fundyense* and its overwintering resting cysts have been observed (White and Lewis 1982; Martin and White 1988; Martin and Wildish 1994). Prior to 2003, the last outstanding bloom event of *A. fundyense* was in 1980 when more than 18 million cells L<sup>-1</sup> were observed (Martin and White 1988).

Massive concentrations were again observed in 2003 and 2004, resulting in greater than “normal” levels of shellfish toxicity, farmed salmon mortalities and human illnesses from PSP. As a result of the 2003 salmon mortalities, a project was funded to investigate the feasibility and usefulness of early warning approaches for predicting harmful phytoplankton blooms at salmon farms in southwestern New Brunswick. Components of this project included: training of farm personnel on the sampling, identification, and counting of harmful phytoplankton species; im-

plementation of daily phytoplankton monitoring by workers at selected salmon farms; and retrospective analyses of existing weekly *A. fundyense* monitoring data since 1988.

### **Materials and Methods**

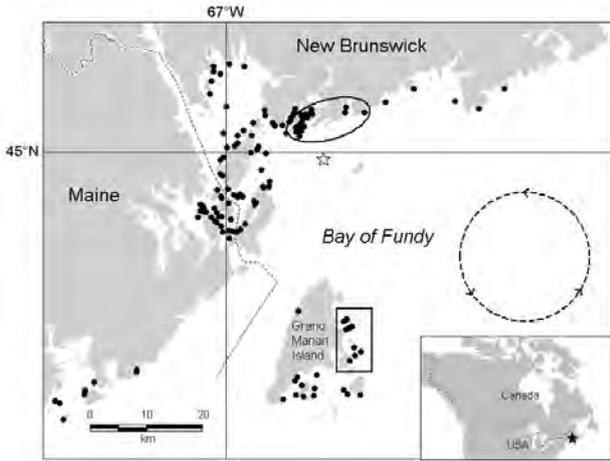
Samples (280) were obtained in 2003 from industry personnel in the Grand Manan region of the Bay of Fundy and analysed (239) according to methods outlined by Martin *et al.* (2006a). In 2004, 57 of 193 samples were analysed.

Industry personnel were instructed in: the collection, identification and enumeration of key HAB species including *A. fundyense* using a Sedgewick-Rafter counting chamber and compound microscope (Guillard and Sieracki 2005). Industry analysed >250 samples.

Shellfish samples are routinely collected and analysed for PS toxins using the mouse bioassay according to approved guidelines used by the CFIA (AOAC 1990).

### **Results and Discussion**

Salmon mortalities occurred in 2003 and 2004 when *A. fundyense* concentrations reached as high as  $8.8 \times 10^5$  cells L<sup>-1</sup> and  $3.0 \times 10^6$  cells L<sup>-1</sup>, respectively. Both events occurred in waters influenced by the central Bay of Fundy gyre (Fig. 1) where high concentrations of *A. fundyense* vegetative cells and cysts reside (White and Lewis 1982; Martin and White 1988; Mar-



**Figure 1.** Locations of salmon farms in the Bay of Fundy (solid circles); phytoplankton monitoring (•), and farms affected by *A. fundyense* in 2003 (rectangle) and 2004 (oval). The circle shows the Bay of Fundy gyre.

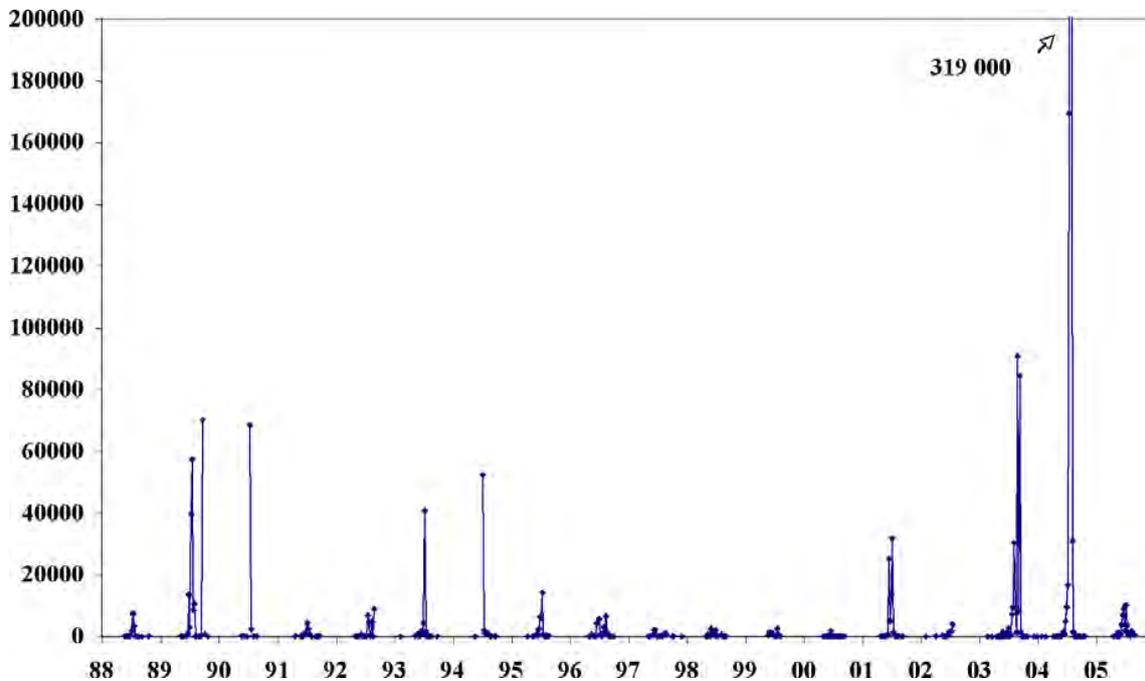
tin and Wildish 1994). Samples from 8 September to the end of the month 2003 from affected aquaculture sites in the Grand Manan region had cell concentrations of  $2.9-8.8 \times 10^5$  cells  $L^{-1}$ . Results from surface and depths (to 2.8 m) indicated that the bloom was patchy but persisted through the month of September (Martin *et al.* 2006b). Although greatest cell densities were observed in surface waters, samples collected at a depth of 2.8 m had concentrations greater than  $1.0 \times 10^5$  cells  $L^{-1}$ . Observations when high concentrations of *A. fundyense* were in surface waters were that fish tended to concentrate at depth. Fish were not fed when

*A. fundyense* concentrations exceeded  $1.0 \times 10^5$  cells  $L^{-1}$  as there was concern that the fish would rise to the surface to feed exposing them to the higher densities.

During the 2004 event that affected salmon (Fig. 1), daily samples were collected by industry from two areas and showed that *A. fundyense* concentrations exceeded  $1.0 \times 10^5$  cells  $L^{-1}$  at one location for more than 25 days from late July through mid-August (Fig. 2). The highest concentration observed from those sites was greater than 1 million cells  $L^{-1}$  in mid August. Industry counts at an adjacent affected site exceeded 3 million cells  $L^{-1}$ . As in 2003, the bloom was patchy and tended to concentrate in surface waters.

The suggested mode of action for the mortalities is through the gills. As a follow-up to the field work and mortalities in 2003-04, threshold experiments were conducted where salmon (150-380 g) were exposed in the laboratory to varying concentrations of *A. fundyense* (Burrige *et al.*, this volume).

Further evidence of the abnormally high concentrations of *A. fundyense* cells during 2003-04 was seen in the levels of PS toxins in wild shellfish which were higher than normal during the 2 years. PS toxins detected in blue mussels (*Mytilus edulis*) from Grand Manan Island were  $>4,000 \mu g$  STX equiv.  $100 g^{-1}$  tissue on 15 September 2003. On 2 August 2004, a tourist became ill after collecting and consuming blue mussels ( $18,080 \mu g$  STX equiv.  $100 g^{-1}$  cooked tissue) from a salmon site experiencing mortalities.



**Figure 2.** *A. fundyense* concentrations (cells• $L^{-1}$ ) from 2 affected sites along the coast of southwest New Brunswick from mid-July through early August 2004.

Results from regular monitoring of *A. fundyense* cell densities since 1988 suggest great inter-annual variability with years of high concentrations (greater than 50,000 cells L<sup>-1</sup>). There can be periods with fewer cells such as 1996-2001 and 2005-06 – years prior to and immediately following massive blooms (Page *et al.* 2006, Martin unpubl.). These data suggest that the extremely high concentrations observed in 2003-04 are periodic events although, as White (1987) suggested, there are documented periods of higher toxicities (mid 1940s, early 1960s and late 1970s). This suggests that the salmon industry could be subjected to high concentrations again in the future, but not necessarily every year.

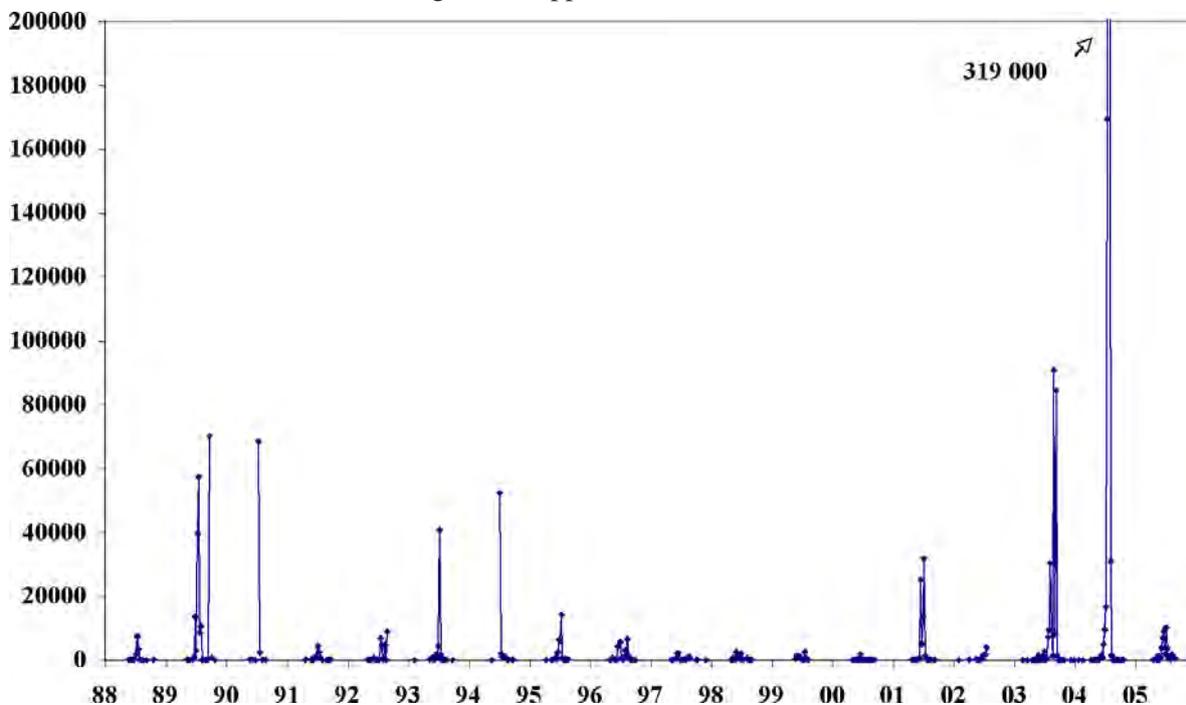
### Acknowledgements

Funding for part of this work was provided by the Aquaculture Collaborative Research and Development Programme (ACRDP). We thank the Canadian Food Inspection Agency for providing shellfish toxicity data; the staff from Cooke Aquaculture, Stolt Sea Farm Inc., Heritage Salmon and AquaFish for providing phytoplankton samples; and Blythe Chang for providing the map.

### References

AOAC (1990). Official Methods of Analysis, 15th ed., Sec 959.08. Association of Official Analytical Chemists, Hellrich K. (ed.), Arlington, VA, pp.

- 881-882.  
 BurrIDGE, L.E., Martin, J.L., Lyons, M.C., LeGresley, M.M. & Chang, B.D. (this volume).  
 Guillard, R.R.L., Sieracki, M.S. (2005). In: Algal Culturing Techniques, R. Andersen, (ed.), Elsevier Academic Press, Oxford, pp. 239-252.  
 Martin, J.L. & White, A.W. (1988). Can. J. Fish. Aquat. Sci. 45: 1968-1975.  
 Martin, J.L. & Wildish, D.J. (1994). Proc. Fourth Canad. Workshop Harmful Marine Algae, Forbes, R. (ed.), Can. Tech. Rept. Fish. Aquat. Sci. 2016: pp. 22-24.  
 Martin, J.L., LeGresley, M.M. & Strain, P.M. (2006a). Can. Tech. Rept. Fish. Aquat. Sci. 2629. iv + 88 p.  
 Martin, J.L., LeGresley, M.M., Haya, K., Sephton, D.H., BurrIDGE, L.E., Page, F.H., & Chang, B.D. (2006b). In: Harmful Algae 2004, Pitcher, G.C., Probyn, T.A. & Verheye, H.M. (eds), African J. Mar. Sci. pp. 431-434.  
 Page, F.H., Martin, J.L., Hanke, A., & LeGresley, M.M. (2006). In: Harmful Algae 2004, Pitcher, G.C., Probyn, T.A., & Verheye, H.M. (eds), African J. Mar. Sci. pp. 203-208.  
 White, A.W. (1980). Can. J. Fish. Aquat. Sci. 37: 2262-2265.  
 White, A.W. & Lewis, C.M. (1982). Can. J. Fish. Aquat. Sci. 39: 1185-1194.  
 White, A.W. (1987). Rapp. Réun. Cons. Int. Explor. Mer 187: 38-46.



**Figure 2.** *A. fundyense* concentrations (cells·L<sup>-1</sup>) from 2 affected sites along the coast of southwest New Brunswick from mid-July through early August 2004.

## Red tide due to the dinoflagellate *Karenia mikimotoi* in Hiroshima Bay 2002: environmental features during the red tide and associated fisheries damages to finfish and shellfish aquaculture

Y. Matsuyama

2-17-5, Maruishi, Hatsukaichi, Hiroshima 7390452 Japan, yukihiko@affrc.go.jp

### Abstract

A large-scale red tide due to the harmful dinoflagellate *Karenia mikimotoi* occurred in Hiroshima Bay, western part of Seto Inland Sea, Japan in 2002. Hydrographic and biological investigations were conducted at a monitoring station from the initial outbreak to cessation of the red tide. A visible bloom ( $>10^6$  cells/l) occurred from 6 through 22 July at this station, where the maximum cell density was  $1.35 \times 10^7$  cells/L and  $2.74 \times 10^8$  cells/L in a nearby harbour. Water temperature and salinity during the red tide ranged from 23.5 to 26.1 °C and 27.5 to 31.3 psu, respectively. Finfish and oyster aquaculture in the bay was devastated with an estimated damage to finfish farming of 950,000 USD. Further, average mortality in the Pacific oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis* were 46 % and 65 %, respectively. Massive kills of shellfish were probably caused by *K. mikimotoi* because the mortalities occurred before the most anoxic water (2.0 mg/l in bottom) had developed in the farming ground.

### Introduction

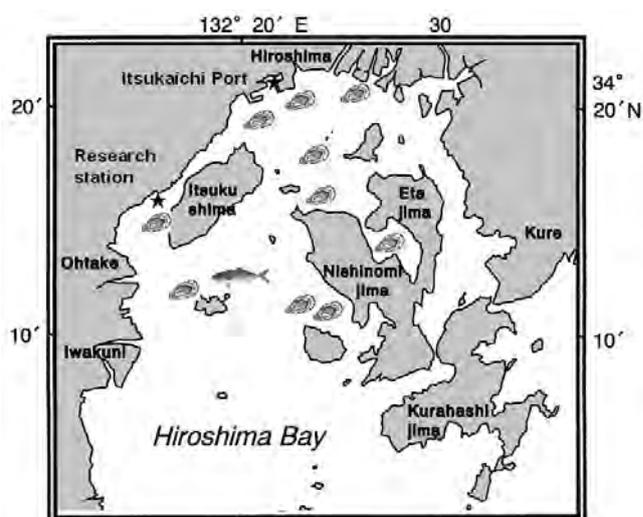
The dinoflagellate *Karenia mikimotoi* (Miyake et Kominami ex Oda) G. Hansen and Moestrup is one of the species associated with massive finfish and shellfish mortalities worldwide (Honjo 1994; Gentien 1998; Yamasaki *et al.* 2004). A red tide due to *K. mikimotoi* occurred for the first time in Ago Bay, Japan, in 1903 associated with a massive kill of cultured pearl oyster *Pinctada fucata* (Nishikawa 1903). Although a number of bloom episodes caused by *K. mikimotoi* have been reported in previous studies (Nishikawa 1903; Shiokawa and Irie 1966; Tangen 1977; Gentien 1998; Matsuyama *et al.* 1999), the ecophysiological mechanism underlying the bloom occurrence and the toxic effect on marine animals still remains unclear. In 2002, a large-scale red tide due to *K. mikimotoi* occurred in Hiroshima Bay, western part of Seto Inland Sea, Japan. Hydrographic and biological investigations were conducted at a research station sampling site from the initial outbreak to the cessation of the red tide.

### Materials and Methods

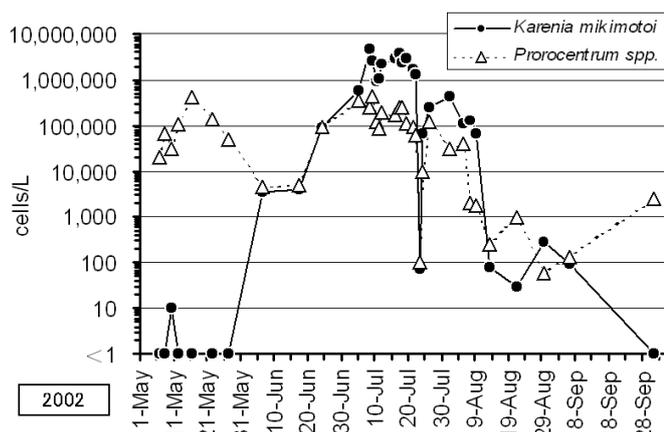
Seawater sampling was carried out at the research site (6 m water depth, Fig. 1) in western Hiroshima Bay from April to November 2002. Water-column samples were collected within 2 h of high tide with a pressure-resistant tube (inner diameter 38 mm x length 5 m). An integrated sample of this seawater was collected to represent the entire water column. *K. mikimotoi*

in these samples were counted in a Sedgwick-Rafter chamber containing 0.05 to 1 ml fresh or concentrated samples. If the concentration of *K. mikimotoi* was below 1 cell/ml, 200-400 ml of seawater was concentrated to 2 ml on a cellulose-acetate membrane filter (47 mm diameter, pore size 3 µm; Advantec) by gravity.

Damage to finfish and oyster fisheries are cited from official reports from Hiroshima Prefecture. Further, monitoring on natural populations of the mussel *Mytilus galloprovincialis* and Pacific oyster *Crassostrea gigas* etc. around the research station was conducted throughout the bloom period.



**Figure 1.** Location of red tide observations and seawater sampling (□) in Hiroshima Bay. Major farming grounds for finfish and oyster are shown.



**Figure 2.** Cell counts (cells/L) of *Karenia mikimotoi* and *Prorocentrum* spp. (*P. dentatum*, *P. minimum*, *P. triestinum*) at the research station sampling site.

## Results and Discussion

### Hydrographic characteristics of the 2002 bloom

In Hiroshima Bay, blooms of *Karenia mikimotoi* typically occur in August to early September (Matsuyama *et al.* 1998). They normally succeed mixed blooms of small diatoms, *Prorocentrum* and *Heterosigma akashiwo* which are more characteristic of the June / July rainy season. According to previous studies, the diatom and small flagellate blooms of early summer coincide with the rainy season, while subsequent late-summer *K. mikimotoi* blooms coincide with bottom anoxia and concurrent nutrient release into the upper layers (Ikeda and Matsuno 1990; Koizumi *et al.* 1994). Figure 2 shows temporal changes of *K. mikimotoi* at the research station site in 2002. *K. mikimotoi* appeared in early June and increased from the middle of June through early July. The dinoflagellate community changed from a dominance of *Prorocentrum* to *K. mikimotoi* within a short period. No anoxia in the bottom water was observed during this period. Precipitation in the rainy season (June to July) of 2002 was less than 60 % of the last 30 year average. In addition, prolonged high irradiance and a rapid increase in water temperature were observed during the initial outbreak of red tide. The hydrographic conditions might have allowed the early proliferation of the *K. mikimotoi* bloom. The high cell density ( $>10^6$  cells/L, Fig. 2) lasted 8 - 22 July 2002. In mid July irradiance markedly decreased with prolonged cloudy weather. Associated with this decrease, *K. mikimotoi* swarmed at the sea surface and the coexisting predominant small diatom population declined probably due to shading effect and/or nutrient starvation. Maximum cell density was  $2.74 \times 10^8$  cells/L at Itsukaichi port (Fig. 1). In late July, the bloom of *K. mikimotoi* had been suppressed

due to grazers such as the heterotrophic dinoflagellate *Polykrikos kofoidii* (max. 64,000 cells/L) and the tintinnid ciliates *Metacyclis* cf. *corbula* (15,500 cells/L). These grazers appeared to regenerate nutrients, and a subsequent massive diatom bloom ( $>6 \times 10^7$  cells/L) was observed. This bloom subsided in late August.

### Fisheries damage to finfish

The major finfish farming ground affected by *K. mikimotoi* bloom was the Atada Shima area shown in Fig. 1. More than 300,000 mortalities associated with the bloom were recorded. They comprised mainly commercially important finfish species (*Pagrus major*, *Sebastes inermis*, *Paralichthys olivaceus*, *Takifugu rubripes*, *Thamnaconus modestus* etc.). Dead finfish showed marked gill damage. Official reports from Hiroshima Prefecture summarized the total amount of losses to cultured finfish at 115.4 million Japanese yen (ca. 950,000 USD). Previous studies revealed that *K. mikimotoi* produces lipophilic phycotoxins (Yasumoto *et al.* 1990; Seki *et al.* 1995; Sola *et al.* 1999; Satake *et al.* 2002). Recently, Yamasaki *et al.* (2004) found that *K. mikimotoi* generates reactive oxygen species (ROS), indicating its potential for fish killing. However, further investigations are necessary to clarify the fish-killing mechanism mediated by *K. mikimotoi*.

### Fisheries damage to oyster culture

Hiroshima Bay is the most important oyster farming ground in Japan (Korringa 1976). The oyster industry was also affected by the bloom of *K. mikimotoi*. Mortalities ranged from 55 % to 80 % in market-sized products, with a total estimated loss of 1.8 billion Japanese yen. In addition, oyster aquaculturists suffered from a marked settlement failure of the oyster spat during the red tide. Also, oyster spat collected during the course of the bloom showed lower growth and survival rates. These sub-lethal effects were not included in the official fisheries damage reports. Previous reports (Nishikawa 1903; Shiokawa *et al.* 1966) suggested that massive death of shellfish may be caused by hypoxia since *K. mikimotoi* blooms were closely associated with anoxic conditions. During the 2002 *K. mikimotoi* bloom, the lowest hypoxia ( $<2.0$  mg/L, 23 July) was suspected to induce the massive death of oysters. However, our investigation revealed that minimum oxygen concentration during the bloom period was not so low (more than 3 mg/L at the bottom) and thus probably not so harmful for the oyster until 22 July. Oxygen concentration at the surface was almost saturated due to *K. mikimotoi* photosynthesis, however, surface populations of mussels were killed by *K. mikimotoi* before 21 July (Fig. 3). As shown

by previous research, *K. mikimotoi* has a detrimental effect on the physiology of shellfish species (Tangen 1977; Widdows *et al.* 1979; Matsuyama *et al.* 1999, 2001). The massive death of shellfish observed during this event was probably caused by cytotoxicity of *K. mikimotoi*, compounded by a co-occurring low level hypoxia.

#### *Other remarks*

Other interesting phenomena that occurred during the 2002 bloom included the mass occurrence of sea foam near the research station. This phenomenon was probably due to the agitation of ferry propellers that frequently ply between the mainland and Itsuku-Shima (Miyajima) Island. Catastrophic mortalities of the grazing gastropod *Chlorostoma turbinatum* were found in the blooming area but sea urchins (*Hemicentrotus pulcherrimus*) survived. Several subtropical coral reefs reared at our institute turned white (they lost the zooxanthellae) when *K. mikimotoi* cells contaminated the running seawater.

#### **Acknowledgements**

Thanks due to Dr. Usuki H., for providing important information during the investigation.

#### **References**

- Gentien, P. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (eds), Springer-Verlag, Berlin, Heidelberg, pp. 155-173.
- Honjo, T. (1994). *Rev. Fish. Sci.* 2: 225-253.
- Ikeda, T. & Matsuno, S. (1990). *Bull. Yamaguchi Pref. Fish. Exp. Stat.* 18: 37-49.
- Koizumi Y., Takashima K., Kamizono M., Etho T., Baba T., Hiyama S., Ikeda T., Iwao T., Hinoshita Y., Uchima M., Yanuma T., Uchida T. & Honjo T. (1994). *J. Oceanography* 3: 99-110.
- Korringa K. (1976). In: *Farming the Cupped Oyster of the Genus Crassostrea*, Korringa, K. (ed.), Elsevier, Amsterdam, pp. 153-182.
- Matsuyama, Y., Uchida, T. & Honjo T. (1999). *Fish. Sci.* 65: 248-253.
- Matsuyama, Y., Usuki, H., Uchida, T. & Kotani Y. (2001). In: *Harmful Algal Blooms 2000*, Hallegraeff, G.M., Blackburn, S.I., Bolch C.J. & Lewis R.J., (eds), IOC of UNESCO, Paris, pp. 411-414.
- Nishikawa, T. (1903). *Zool. Mag.*, 15: 347-353.
- Seki, T., Satake, M., Mackenzie, L., Kaspar, H.F. & Yasumoto, T. (1995). *Tetrahedr. Lett.* 36: 7093-7096.
- Shiokawa, T. & Irie, H. (1966). *Bull. Fac. Fish. Nagasaki Uni.* 21: 115-129.
- Sola, F., Masoni A., Fossat, B., Porthé-Nibelle, J., Gentien, P. & Bodennec, G. (1999). *J. Appl. Toxicol.*, 19: 279-284.
- Tangen, K. (1977). *Sarsia* 62: 123-133.
- Widdows, J., Moore, M.N., Lowe, D.M. & Salkeld, P. N. (1979). *J. Mar. Biol. Ass. U.K.* 59: 522-524.
- Yamasaki, Y., Kim, D. I., Matsuyama, Y., Oda, T. & Honjo, T. (2004). *J. Biosci. Bioeng.* 97: 212-215.
- Yasumoto, T., Underdal, B., Aune, T., Hormazabal, V., Skulberg, O.M. & Oshima Y. (1990). In: *Toxic Marine Phytoplankton*, Granéli, E., Sundström, B., Edler, L. & Anderson, D.M. (eds), Elsevier, New York, pp. 436-440.

## Blooms of *Pyrodinium bahamense* var. *compressum* along the Pacific Coast of Central America and southern México

E. Meave del Castillo<sup>1</sup>, R. Rodríguez S.<sup>2</sup> and M. Vargas M.<sup>3</sup>

<sup>1</sup>UAM-Iztapalapa, México, D.F., México, mem@xanum.uam.mx, <sup>2</sup>CETMAR, Puerto Madero, Chiapas, México, aurora43@prodigy.net.mx, <sup>3</sup>University of Costa Rica., <sup>3</sup>vmontero@cariari.ucr.ac.cr

### Abstract

The first well documented toxic episode caused by *Pyrodinium bahamense* in the Americas was in Guatemala in July 1987, when 26 people died after consuming clams. In Costa Rica and the Gulf of Tehuantepec (México), the first serious bloom took place in October 1989, resulting in 99 sick people and 3 fatalities, and a toxin level of 811 µg STXeq·100 g<sup>-1</sup>. In 1995-1996, the same species was recorded in México, reaching as far west as the coast of Michoacán and killing marine organisms at a toxin level of 6,337 µg STXeq·100 g<sup>-1</sup>. In Costa Rica, succeeding blooms took place in 1999-2001, covering the entire Pacific coast with both morphotypes var. *compressum* and var. *bahamense*, which occurred together. The same bloom (2001) was observed in Acapulco, México, with 7,309 µg STXeq·100 g<sup>-1</sup>. In Chiapas, México, cysts were recorded in the water column from January 2001, the first vegetative cells appearing in March 2001. Exponential growth was observed until the formation of the bloom 6 months later. Formation of cysts was also observed in the water column as the bloom advanced. The last records were made in November-December 2005 in the Gulf of Papagayo, Costa Rica, with densities of 3.5·10<sup>6</sup> cells l<sup>-1</sup>, and in Chiapas, México, albeit with moderate densities of 950 cells·l<sup>-1</sup> and a toxin level of 200 µg STXeq·100 g<sup>-1</sup>.

### Introduction

*Pyrodinium bahamense* Plate var. *compressum* is considered highly dangerous because of its high toxicity, which causes PSP (Corrales & Maclean 1995). *Pyrodinium* is a monotypic genus with two varieties: var. *bahamense* and var. *compressum* (Böhm) Steidinger, Tester et Taylor (Steidinger, Tester and Taylor 1980). Var. *compressum*, although similar to var. *bahamense* in form and composition of thecal plates, is anterior-posteriorly compressed, forms chains, has larger pores in the theca and is toxic. It is exclusively distributed in the Pacific and Indian Oceans. Although only var. *compressum* had been reported to be toxic, toxicity was recently found in var. *bahamense* from areas off the coast of Florida (Landsberg *et al.* 2006). For by this reason it was proposed that there may be no varietal distinction between var. *bahamense* and var. *compressum*.

The first known bloom of *P. bahamense* var. *compressum* in Central America took place in 1942 (Orellana *et al.* 1998), but the first well documented record of this species dates from July 1987, off the coast of Guatemala. The HAB covered a surface of 160 km<sup>2</sup> (4 km wide by 40 km long), causing the death of 26 people and sickening 187 following consumption of clam (*Amphicaena kindermanni*) (Rosales-Loessener 1989).

In México, HABs of *P. bahamense* var. *compressum* have occurred at 3–5 yr intervals (winter 1989, November 1992, November 1995 to February 1996, January 2001 to February 2002, and the beginning of 2006), and in all cases the first sightings of the species were made off the coast of Chiapas state, in the Gulf of Tehuantepec. In that region winter upwellings are frequent (November to February) due to the action of strong northern winds (called Tehuanos) coming from the Gulf of México and blowing across the country with great speed through the Isthmus of Tehuantepec. The highest layers of water are pushed away from the coast and replaced by deeper layers. In México, HABs of *Pyrodinium* are associated with such upwellings, but the species may sporadically remain in the water column for several months. The highest densities have been observed during the rainy season (August) ca. 7 months after the upwelling.

The synchrony in the occurrence of different *Pyrodinium* blooms in Central America, from Costa Rica to México, suggests that they represent a regional event.

In Costa Rica, *Pyrodinium* HABs began in 1989 and persisted until early 1990, the vector being the shellfish *Spondylus calcifer* (Mata *et al.* 1989).

The first recorded HAB of *Pyrodinium* in México took place in November 1989, in the Gulf of Tehuantepec. The bloom extended from the La Paz River

in El Salvador as far as Huatulco Bay in Oaxaca State, México. Densities reached values as high as  $1.7 \cdot 10^6$  cells  $l^{-1}$ , a relative abundance of 98.5 % and saxitoxin values of  $811 \mu\text{g STXeq} \cdot 100 \text{ g}^{-1}$ . The bloom caused the poisoning of 99 people and 3 deaths from consumption of the shellfish *Ostrea iridescens* and *Chromytilus palliopunctatus* (Cortés-Altamirano *et al.* 1993).

From November 1995 to February 1996 a *Pyrodinium* bloom reached the coasts of Michoacán and Guerrero states in México, causing 6 deaths and many affected people, with a maximum toxicity of  $6,337 \mu\text{g STXeq} \cdot 100 \text{ g}^{-1}$  in *Ostrea iridescens* in Tecpan, Guerrero (Orellana *et al.* 1998).

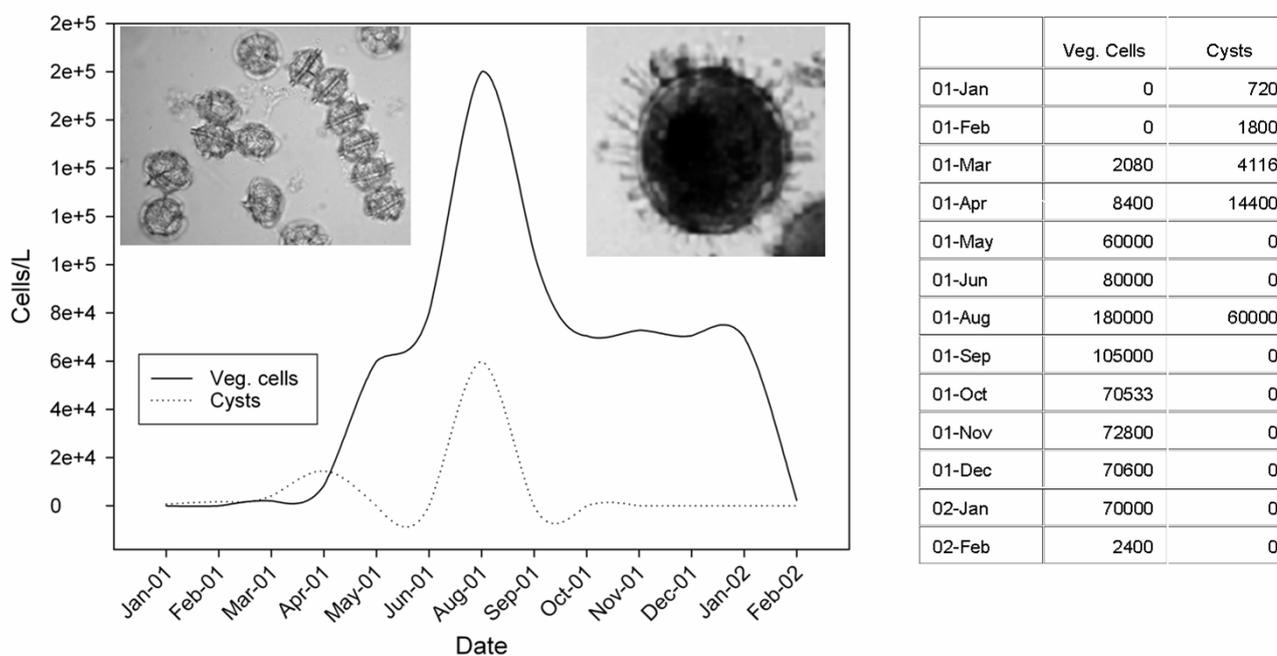
In Costa Rica, the largest HAB took place between December 1999 and early 2002, and covered the entire Pacific coast, alternating with *Gymnodinium catenatum* and other HAB species such as *Trichodesmium erythraeum* (Ehrenberg) Gomont, and *Cochlodinium polykrikoides* Margalef. It affected 70 people. Saxitoxin values were very high, above  $2000 \mu\text{g STX} \cdot \text{ml}^{-1}$  in August-September 2001 (Vargas and Freer 2003).

An aspect that has raised the discussion about the varieties of *Pyrodinium bahamense* was the finding that along the Pacific coast of Costa Rica during the 2002 HAB, the bloom was initially dominated by var. *compressum*, but two months later the variety *bahamense* took over, and by December the latter was dominant. At that time (September – December 2000)

and in the same samples, morphotypes of both varieties *bahamense* and *compressum* were present, as shown in Figure 1 (Vargas and Freer 2003).

From November 2001 to August 2002 HABs coming from Chiapas, México, reached the coast of Guerrero with cell concentrations of  $3.5 \cdot 10^6$  cells  $l^{-1}$ , and  $7,309 \mu\text{g STXeq} \cdot 100 \text{ g}^{-1}$  in shellfish in the port of Acapulco (Ramírez-Camarena *et al.* 2004). The HABs began off the coast of Chiapas and moved west (Guerrero and Michoacán) with patches 1,357 km long. They resulted in 101 poisonings, 6 casualties, 150 days of shellfish closures and 48 tons of dead fish (Ramírez-Camarena *et al.* 2004).

Since 2001, *P. bahamense* has been monitored by researchers from the Centro Tecnológico del Mar (CETMAR No. 24) located in Puerto Madero, Chiapas, México, including the study of the behaviour of cysts and vegetative cells in the water column. During the 2001 HABs, cysts appeared in the water column in January, 3 months before the appearance of the first vegetative cells (March). Afterwards, an exponential growth of vegetative cells was observed along with cysts, but the number of vegetative cells was three times larger than that of cysts, the bloom taking place about 6 months later. Cyst formation was also observed in the water column as the bloom advanced (Fig. 1). Several environmental factors, such as temperature ( $31\text{--}32 \text{ }^\circ\text{C}$ ), salinity (35 psu), and concentrations of phosphates ( $24 \mu\text{M}$ ), ammonium ( $1.85 \mu\text{M}$ ), and nitrates ( $17 \mu\text{M}$ ) reached their highest val-



**Figure 1.** Densities of vegetative cells and cysts in the water column at Puerto Madero, Chiapas, México January 2001-February 2002.

ues during the peaks of vegetative cell and new cysts (Table 1).

The most recent record of *Pyrodinium* was in November-December 2005 in the Gulf of Papagayo, CR, with densities of  $3.5 \cdot 10^5$  cells  $l^{-1}$ . The same event was observed in Puerto Madero, Chiapas, México, from December 2005 to March 2006 (Fig. 2), although with a moderate intensity, 950 cells  $l^{-1}$  and  $200 \mu g$  STXeq  $100 g^{-1}$ .

The PSP toxins affect not only humans; mortality of fish and crustaceans is also common and there has been an increase in the mortality of marine turtles (from jellyfish ingestion) since the 1950s both in Asia and México (Maclean 1973; Orellana *et al.* 1998), and again in 2002 (Ramírez-Camarena *et al.* 2004). Most recently mortality of turtles occurred in January 2006 on the Pacific coast of Central America, particularly in El Salvador (La Prensa Gráfica newspaper, El Salvador).

The presence of different morphotypes of *P. bahamense* on the Pacific coasts of Central America and México needs to be investigated further, together with their irregular presence in the water, which may be related to ENSO events. The different morphotypes may be the different stages of the species' life cycle depicted by Usup & Azanza (1998), namely planozy-

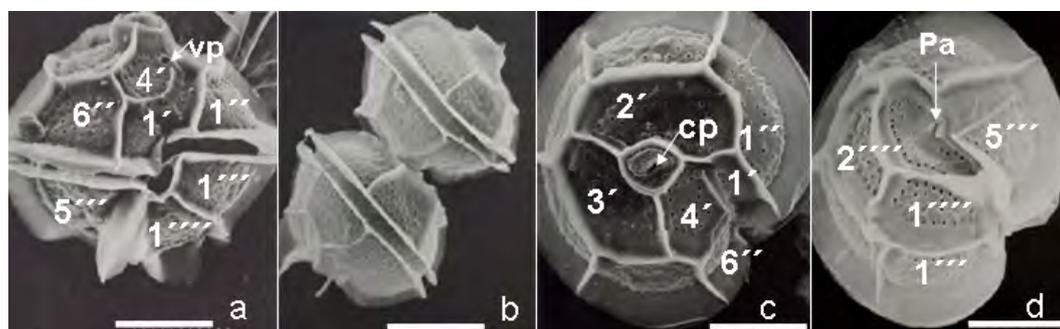
gotes or planomeiocytes. Balech (1985) and Landsberg *et al.* (2006) discarded the existence of varieties in this species; following study of theca patterns in numerous cells of both varieties, Balech was unable to find a consistent and robust character separating the two varieties. The theca varied according to thickness and age of the cell. Based on Maclean's (1979; in Balech 1985) findings on changes in the toxicity of *P. bahamense* from more or less low in open waters to high in enclosed water bodies, Balech concluded that toxin production may interact with external factors, even the number of bacteria present. Landsberg *et al.* (2006) also discussed four different scenarios for the sudden appearance of toxic *P. bahamense* in Florida, including change in environmental conditions and introduction of a toxic strain, previously unrecognized low-level toxin production, and increased susceptibility to toxins by biota. Different strains should be studied to gain a better understanding of the genetics and toxic profile of different populations in the region.

#### Acknowledgements

Support for this research was provided by a grant from CONACyT, project 37560-V C "Biology and molecular phylogeny of dinoflagellates from Mexican

**Table 1.** Environmental factors in 2001. The times of toxic blooms have been indicated in bold.

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
T °C	26	28	29	30	30	30	30	<b>32</b>	<b>31</b>	30.5	29	30
S ppm	30	30	31	33	35	34	34	<b>35</b>	<b>35</b>	33	33	34
NO <sub>2</sub> μM	0	1	1	2	2	1	2	<b>2</b>	<b>1.6</b>	1	1	1
NO <sub>3</sub> μM	8	10	10	11	11	12	13	<b>17</b>	<b>6.5</b>	5.5	7	9.8
PO <sub>4</sub> μM	7	9	10	12	13	15	15	<b>24</b>	<b>13</b>	8	7	3
NH <sub>4</sub> μM	0.14	0.19	0.21	0.25	0.30	0.34	0.39	<b>1.8</b>	<b>1.85</b>	1.8	0.75	0.75



**Figure 2.** *Pyrodinium bahamens* var. *compressum* from Mexican coasts. Material collected at Puerto Madero, Chiapas 24 Feb 2006; a, ventral view; b, lateral view; c, apical view; d, antapical view.

1'-4' - apical plates, 1''- 6''- precingular plates, 1'''-5''' - postcingular plates, 1''''- 2''''- antapical plates, vp- ventral pore, cp - closing platelet and pore of apical complex, pa - posterior attachment pore. Scales: 20 μm.

Pacific coasts". We are grateful to the Vicerrectoría de Investigación, Universidad de Costa Rica, for support through project N.810-A4043. We appreciate help with the English from Eréndira Cohen. Two anonymous reviewers are thanked for their comments on an earlier version of the manuscript.

## References

- Balech, E. (1985). *Rev. Paleobot. Palynol.* 45: 17-34.
- Cortés-Altamirano, R., Muñoz, L. & Sotomayor, O. (1993). *An. Inst. Cienc. Mar Limnol. Univ. Nal. Autón. México* 20: 43-54.
- Corrales, A.R. & Maclean, J.L. (1995). *J. Appl. Phycol.* 7: 151-162.
- Landsberg, J.H., Sherwood, H., Johannessen, J.N., White, K.D., Conrad, S.M., Abbott, J.P., Flewelling, L.J., Richardson, R.W., Dickey, R.W., Jester, E.L., Etheridge, S.M., Deeds, J.R., Van Dolah, F.M., Leighfield, T.A., Zou, Y., Beaudry, C.G., Benner, R.A., Rogers, P.L., Scott, P.S., Kawabata, K., Wolny, J.L. & Steidinger, K.A.. (2006). *Env. Health Perspect.* 114: 1502-1507.
- La Prensa Gráfica Newspaper, 31 January 2006 and 29 September 2006.  
<http://www.laprensagrafica.com/tortugas/>.
- Maclean, J.L. (1973). *Agric. J.* 24: 131-138.
- Mata, L., Abarca, G., Marranghello, L. & Viquez, R. (1990). *Rev. Biol. Trop.* 38: 129-136.
- Orellana, E., Martínez, E., Muñoz, L., López, P., Cabrera, E. & Ramírez, C. (1998). In: *Harmful Algae*. Reguera B., Blanco J., Fernández M. & Wyatt T. (eds), Xunta de Galicia, IOC of UNESCO, Vigo, Spain, p. 60.
- Ramírez-Camarena, C., Martínez-García A., Juárez-Ruíz, N., Rojas-Crisóstomo R. & Ramírez-García, H. (2004). Abstracts, XIII Reunión Nacional SOM-PAC, VI International Meeting of Planktology, 25-28 April, Nuevo Vallarta, Nayarit, México, p. 62.
- Rosales-Loessener, F. (1989). In: *Biology, Epidemiology and Management of Pyrodinium Red Tides*. Hallegraeff, G. & Maclean, J. (eds), Proceedings of the Management and Training Workshop Bandar Seri Begawan, Brunei Darussalam. 23-30 May, pp. 49-51.
- Steidinger, K.A., Tester, L.S. & Taylor, F.J.R. (1980). *Phycologia* 19: 329-337.
- Usup, G. & Azanza, R.V. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson D.M., Cembella A.D. & Hallegraeff G.M. (eds), vol. G41, Springer, Berlin, pp. 81-94.
- Vargas, M. & Freer, E. (2004). In: *Harmful Algae 2002*, Steidinger, K.A, Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography and IOC of UNESCO, St. Petersburg, Florida, USA, pp. 482-484.
- Vargas, M. & Freer, E. (2003).. In: *Molluscan Shellfish Safety*, Villalba, A., Reguera, B., Romalde L.J. & Beiras, R. (eds), Xunta de Galicia, IOC of UNESCO, Santiago de Compostela, Spain, pp. 211-217.

## On the genus *Alexandrium* (Dinoflagellata) in Vietnamese waters: - two new records of *A. satoanum* and *A. tamutum*

L. Nguyen-Ngoc<sup>1</sup> and J. Larsen<sup>2</sup>

<sup>1</sup>Institute of Oceanography, Cauda 01, Nhatrang city, Vietnam, habviet@dng.vnn.vn

<sup>2</sup>IOC Science & Communication Centre in Harmful Algae, Copenhagen University, Øster Farimagsgade 2D, DK-1353, Copenhagen K, Denmark, jacobl@bi.ku.dk

### Abstract

Based on observations of cell morphology and thecal plate pattern, two dinoflagellate species, *Alexandrium satoanum* Yuki & Fukuyo and *A. tamutum* Montresor, Beran & John are reported and illustrated for the first time from Vietnamese waters. *Alexandrium satoanum* belongs to subgenus *Gessnerium* and is characterized as follows: cells wider than long, ca. 40 µm wide and ca. 32 µm long, 1' plate not connected to the APC and without a ventral pore. *Alexandrium tamutum* belongs to the subgenus *Alexandrium* and is characterized as follows: isodiametric cells, 25-30 µm, 1' connected to the APC and with a ventral pore, 6'' as wide as long, and s.p. plate wider than long. With these records, 18 species of *Alexandrium* have now been reported from Vietnamese waters. Cysts of these two newly reported species were not documented.

### Introduction

Based on comparative morphology, Balech (1995) described 29 species of *Alexandrium* belonging to two subgenera *Alexandrium* and *Gessnerium*. Subsequently, from New Zealand and Italian waters, Mackenzie and Tod (2002) and Montresor *et al.* (2004) documented *A. camurasculatum* and *A. tamutum*, respectively, as new species to science. In Vietnamese waters, Nguyen & Larsen (2004) found 15 species of *Alexandrium*. Yoshida *et al.* (2000) reported the presence of PSP toxins for *A. minutum* isolated from Do Son (Tonkin Gulf). Recently, *A. foedum* was found in central Vietnam (Chu Van Thuoc & Ton That Phap, pers. commun.). In this paper, two new records of *Alexandrium* species are reported: *A. satoanum* and *A. tamutum*. Therefore, out of the 33 species presently included in the genus *Alexandrium*, 18 species have been found in Vietnamese waters.

### Materials and Methods

#### Materials

Samples were collected by 20-µm mesh-size plankton net from Phan Ri Bay in 2003-2004 and Nha Phu Lagoon (northern part of Nhatrang Bay) in 2004-2005.

#### Observations

Observations of plate pattern were made using Calcofluor White M2R (Fritz & Triemer 1985), sometimes in combination with hypochlorite. The samples were examined on an epifluorescence (violet excitation ca. 430 nm, blue emission ca 490 nm) Olympus

BH-2 microscope with phase contrast and differential interference contrast optics, or Leitz LDMB with similar accessories. A digital camera, C7070, was used for photography.

#### Identification

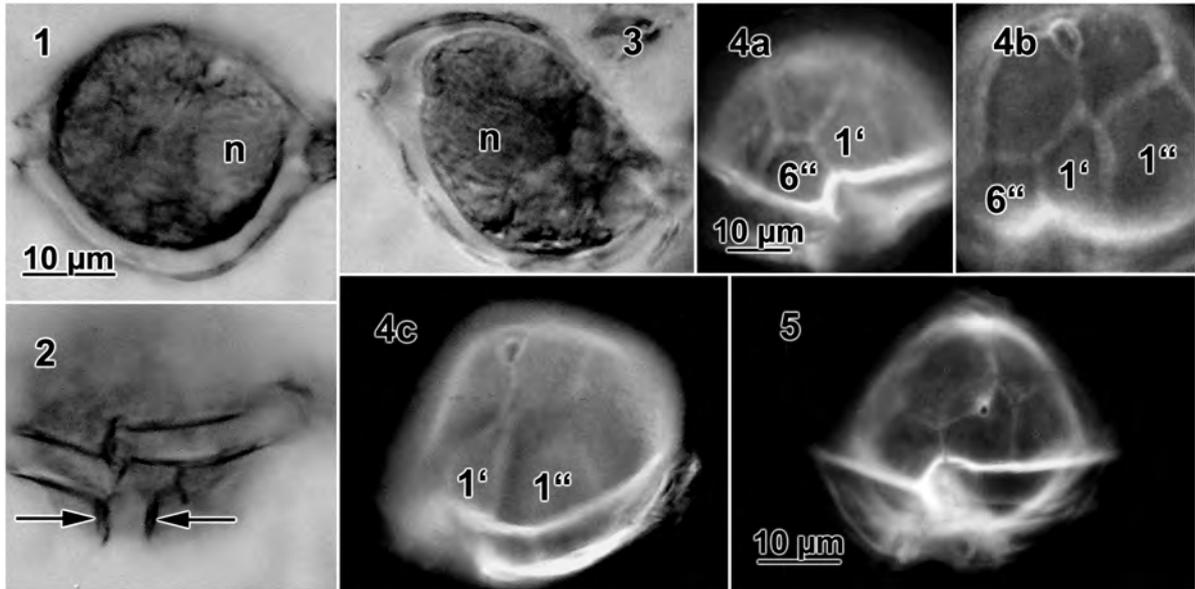
Identification of species was based on cell morphology and thecal plate patterns following Balech (1995), Steidinger and Tangen (1997), and Nguyen & Larsen (2004).

### Results and Discussion

#### New reports for Vietnamese waters

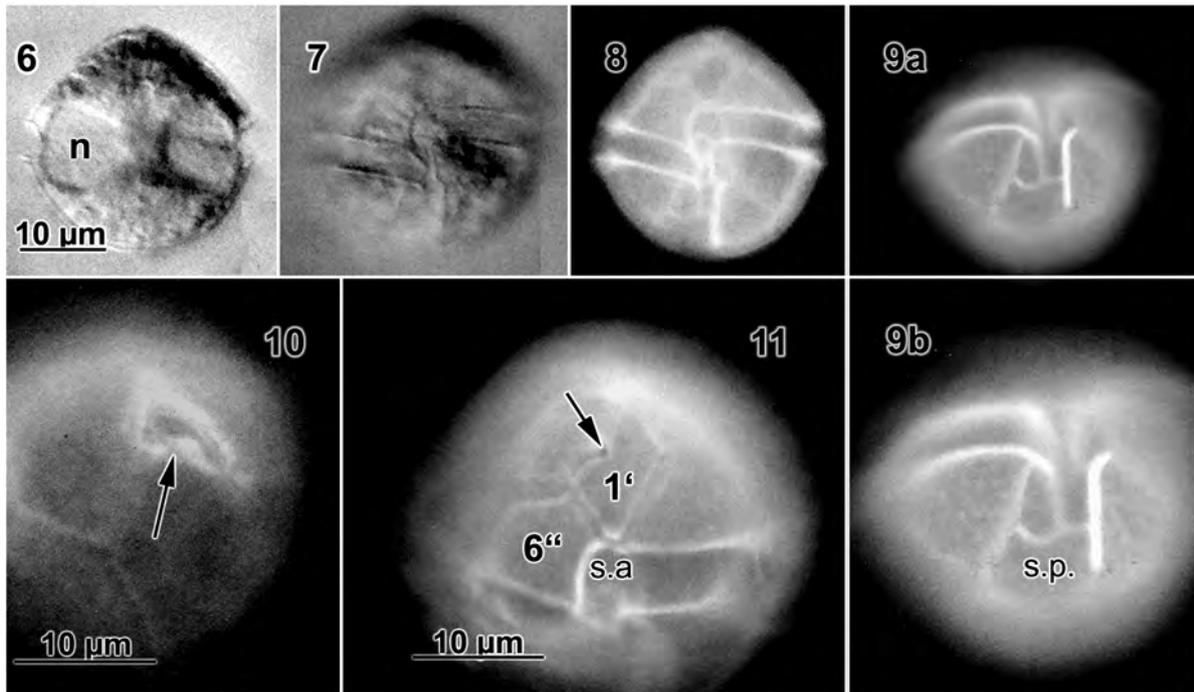
*Alexandrium satoanum* Yuki & Fukuyo 1992, Figs 1-5  
**Description.** The cells have a more or less flattened pentagonal appearance with obtuse and shortened conical epicone and hypocone. The ventral epitheca and dorsal hypotheca are flattened, whilst the dorsal epitheca and ventral hypotheca are convex causing cell obliqueness in lateral view (Fig. 3). The nucleus is U-shaped and located in the central part of the cell (Fig. 1). Plate 1' is not connected to the apical pore complex (APC), it is asymmetric pentagonal in shape. A ventral pore is not present (Figs 4a-c). Anterior and posterior attachment pores were not observed. Plate 6'' is rectangular in shape. The cingulum is descending, displaced one girdle width (Fig. 2). Sulcal lists are well developed (Fig. 2).

**Remarks.** The morphology and plate pattern of Vietnamese specimens are in good agreement with the description by Yuki and Fukuyo (1992) and illustrations provided by Balech (1995). The species belongs



**Figure 1-4.** *Alexandrium satoanum*: Fig. 1. Cell in ventral view showing position of nucleus (n); Fig. 2. Cell in ventral view, detail of the sulcus showing the marked development of the sulcal wings (arrows); Fig. 3. Cell in lateral view showing the oblique cell shape; Figs 4a-c. Cells showing plate 1' without the ventral pore.

**Figure 5.** *Alexandrium globosum* (after Nguyen-Ngoc and Larsen 2004); Figs 2, 3, 4b and 4c, scale as in Fig.1; Figs 1-3, LM, DIC; Figs 4-5, LM, epifluorescence.



**Figure 6-11.** *Alexandrium tamutum*: Fig. 6. Cell in ventral view showing the position of the nucleus (n); Figs 7, 8. Cells in ventral view ; Figs 9a-b. Sulcal area; Fig. 10. APC with a comma-shaped pore, note the small indentation (arrow); Fig. 11. Cell in ventral view showing the ventral pore on the right margin of plate 1' (arrow), the shape of plates 6'' and s.a.. Figs 7-9a, scale as in Fig. 6; Fig. 9b, scale as in Fig.11; Figs. 6-7, LM, DIC; Figs 8-10, LM, epifluorescence.

to subgenus *Gessnerium*. The asymmetric cell outline with protruded epitheca and hypotheca differed from that of *A. globosum*. The absence of ventral pore on plate 1' also characterizes *A. balechii*, but this latter species differs by its regular global shape and the sculptured plates.

*Distribution.* Japan. In Vietnam, the species was found in Phan Ri Bay in February.

*Alexandrium tamutum* Montresor, Beran & John 2004, Figs 6-11

*Description.* Cells are medium in size, isodiametric, 25-30 µm in diameter. This species does not form chains. The U-shaped nucleus is located at the centre of the cell, at the level of the cingulum (Fig. 6). Plate 1' is connected to the APC (Figs 10-11). A ventral pore is present at the middle right margin of 1' (Fig. 11). The posterior sulcal plate is rectangular in shape, wider than high (Figs 9a-b) as reported for *A. minutum*. The cingulum is descending, displaced one girdle width (Figs 7-8). The sulcus is wide and convex (Fig. 8), and the 6'' plate is as wide as long.

*Remarks.* In ventral view, cells are very similar to *A. tamarense*; they also have a similar morphology of plates 1', 6'' and the APC. The morphology of specimens from Vietnamese waters is in accordance with the original description by Montresor *et al.* (2004). Hansen *et al.* (2001) described *A. cf. tamarense*, which may be *A. tamutum*.

*Distribution.* Montresor *et al.* (2004) described *A. tamutum* from Italian waters. In Vietnam, this species has been commonly found in shrimp-ponds and shallow waters along south central coasts.

*Distribution of A. satoanum and A. tamutum in Vietnamese waters*

Most *Alexandrium* species are found in the coastal waters from north to south Vietnam (Tab. 1). *A. satoanum* was found to be common in samples from an upwelling area of the northern part of Binh Thuan waters (Phan Ri Bay), in the north east monsoon when temperature was low, 24-25 °C. This suggested that *A. satoanum* may be an oceanic species. Numerous cells of *A. tamutum* were found from shrimp ponds

and shallow waters of Nha Phu lagoon only, at low salinity (28-32 ‰) and high temperature (26-28 °C). *Alexandrium foedum* is known from Thua Thien-Hue only.

### Acknowledgements

Sampling programmes were funded by Danida / HABViet III Project and Vietnam National Project KC 09-19. We thank Dr. Chu Van Thuoc and Dr. Ton That Phap for sharing unpublished information about *A. foedum*, and Dr. Doan Nhu Hai, Mr. Ho Van The and Mrs. Nguyen Thi Mai Anh who helped during sampling. Thanks also go to two reviewers for their comments.

### References

- Balech, E. (1995). Sherkin Island Marine Station, 151 pp.
- Gaarder, K. (1954). Report on the Scientific Results of the 'Michael Sars' North Atlant. Deep-Sea Exped., University of Bergen, pp. 1-62.
- Hansen, G., Turquet, J., Quod, J.P., Ten-Hage, L., Lugomela, C., Ogongo, B., Tunje, S. & Rakotoarin-Jahary, H. (2001). IOC Manuals and Guides No. 41. Intergovernmental Oceanographic Commission of UNESCO, 105 pp.
- Montresor, M., John, U., Beran, A. & Medlin, L.K., (2004). *J. Phycol.* 40: 398-411.
- Nguyen-Ngoc, L. & Larsen, J. (2004). In: Potentially toxic microalgae of Vietnamese waters, Larsen, J. and Nguyen-Ngoc L. (eds), *Opera Botanica*, Copenhagen 140: 73-116.
- Steidinger, K. A. & Tangen, K. (1997). In: *Identifying Marine Phytoplankton*, Tomas, C.R. (ed.), Academic Press, San Diego, pp. 387-584.
- Yoshida, M., Ogata, T., Thuoc, C.V., Matsuoka, K., Fukuyo, Y., Hoi, N. C. & Kodama, M. (2000). *Fish. Sci.* 66: 177-179.
- Yuki, K. & Fukuyo, Y. (1992). *J. Phycol.* 28: 395-399.

## Mucilage phenomena in the North Aegean Sea, Greece: another harmful effect of dinoflagellates?

G. Nikolaidis<sup>1</sup>, K., Aligizaki<sup>2</sup>, K. Koukaras<sup>3</sup> and K. Moschandreu<sup>4</sup>

Department of Botany, School of Biology, Aristotle University of Thessaloniki,  
P.O. BOX 109, GR-54124, Thessaloniki, GREECE

<sup>1</sup>nikola@bio.auth.gr, <sup>2</sup>aligiza@bio.auth.gr, <sup>3</sup>koukaras@bio.auth.gr and <sup>4</sup>kkmosch@bio.auth.gr

### Abstract

Mucilage events in Greek marine waters constitute a common phenomenon of irregular frequency but always detected during the warm period. Mucilaginous aggregations were mainly detectable on fishing nets, impairing their function because of the weight of the mucilaginous material. Since 1982, at least 10 events have been recorded in different areas of the North Aegean Sea, most of them in Thermaikos Gulf. *Gonyaulax* species (*G. hyalina* and *G. fragilis*) were proposed as possible sources of the mucilage phenomena, thus attention is given to the occurrence of these species in the monitored areas. Data obtained from the monitored area indicate coincidence between mucilage aggregations and populations of *G. hyalina*. The highest abundances of *Gonyaulax* ( $7.9 \times 10^3$  cells L<sup>-1</sup>) were found on August 2005 in integrated water samples, while the abundance in 2004 and 2006 did not exceed  $1.40 \times 10^3$  cells L<sup>-1</sup>.

### Introduction

Massive mucilage aggregations like those of the Adriatic Sea (Molin *et al.* 1992) and New Zealand (MacKenzie *et al.* 2002) have created serious environmental and economic problems (Rinaldi *et al.* 1995). Because of their harmful effects, mostly in the fisheries and tourism industries, a considerable amount of scientific efforts has been devoted to understanding these events.

Mucilage formation is a very complex phenomenon involving several biological and hydrological interactions (Totti *et al.* 2005). Several studies have related the occurrence of mucilage to phytoplankton production, with diatoms (e.g. *Cylindrotheca closterium*, *Nitzschia* spp., *Skeletonema* spp.) and *Phaeocystis* species being implicated as the most important producers of polysaccharide exudates in these phenomena (Molin *et al.* 1992; Degobbi *et al.* 1995; Lancelot 1995); bacterial activity is suggested to contribute as well (Azam & Long 2001). Recently, however, such properties have been attributed to dinoflagellates (Alldredge *et al.* 1998; Passow 2002), such as *Gonyaulax hyalina* and *G. fragilis* (MacKenzie *et al.* 2002; Pompei *et al.* 2003; Pistocchi *et al.* 2005).

The appearance of mucilaginous aggregations in Greek marine waters started in the early 1980s. Most cases have been attributed to phytoplankton production without any direct relation to a specific organism (Gotsis-Skretas 1995; Nikolaidis, unpublished data).

This study presents information about mucilage formation in the North Aegean Sea during the last two

decades and investigates the potential relationship of these with the dinoflagellate *Gonyaulax hyalina* during 2004-2006.

### Materials and Methods

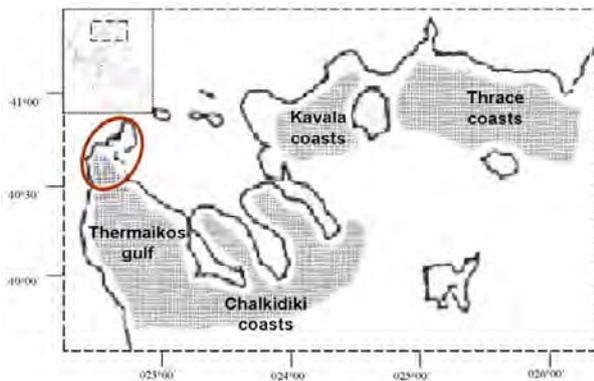
Records of mucilage phenomena in the North Aegean Sea are based on official documents from Greek authorities (Coast Guard, Fisheries Services) and on information collected by local fishermen associations. Lugol's iodine fixed surface water samples, collected by the Greek authorities, were analyzed for species identification and abundance measurements using the inverted microscope technique (Utermöhl 1958).

Fresh material of mucilage aggregations collected from the fishing nets was also examined. Observations on mucilage structure and species composition were made using light and electron microscopy. Chl-*a* and organic material concentration in mucilage aggregations were determined (Lorenzen 1967; Strickland & Parsons 1968).

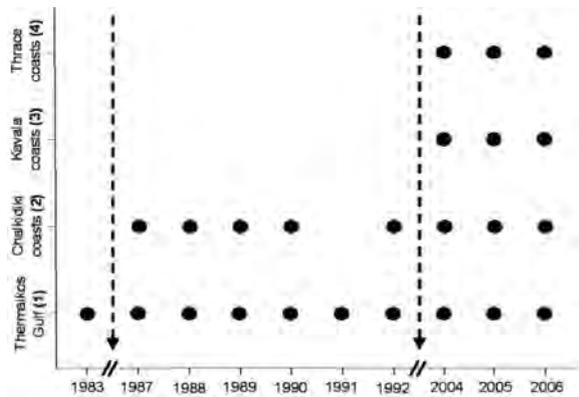
Monitoring data on phytoplankton were obtained from 19 fixed stations situated in the upper Thermaikos Gulf (NW Aegean Sea) during 2005 and 2006 (Fig. 1). Water samples, obtained by a PVC tube, from the whole water column, a Nansen-type sampler, from different depths, and a plankton net (20 µm), were used.

### Results

Since 1982 a total of at least 10 mucilage events (Fig. 2) have been recorded at different areas along North



**Figure 1.** Map of the North Aegean Sea showing the sampling area (the 19 stations located in the circle) and the areas affected by mucilage events (shaded areas).

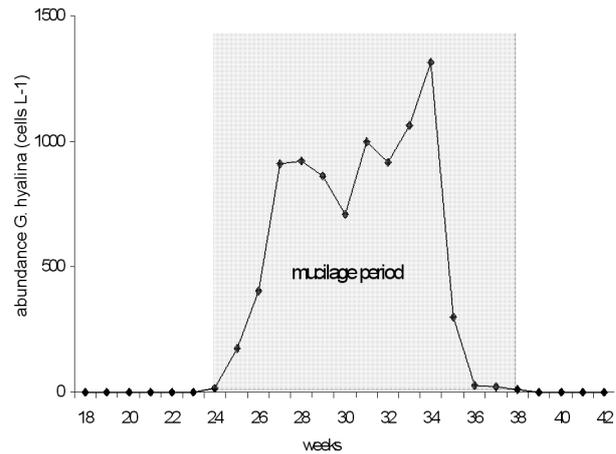


**Figure 2.** Mucilage phenomena in the North Aegean Sea during the last 23 years (arrows indicate the periods of no mucilage event detection).

Aegean coasts (Fig. 1) in the warm period (June-October). In most cases, mucilaginous aggregations were detected only on fishing nets (Fig. 4A), where the slimy masses made fishing difficult or impossible because of the added weight. According to fishermen, the interruption time of fishing activities was 2-6 weeks during mucilage events.

Examination of material in the microscope showed an amorphous construction, of yellow-brownish colour, composed of sand grains, mucus material, detrital components, together with empty diatom frustules and live cells (Fig. 4B), especially pennate diatoms (Fig. 4C). Mucilage aggregates were rich in Chl-*a*, the concentration being 22.8-33.2  $\mu\text{g g}^{-1}$  of fresh weight, and organic substances between 25 and 40 %.

Microscopic examination of water samples obtained at the peaks of these events showed a scarcity of mucilaginous aggregations but diatom dominance. The most common and abundant species were *Rhizosolenia setigera*, *Proboscia alata*, *Pseudosolenia calcar-avis*, *Skeletonema costatum*, *Hemiaulus hauckii*, *Nitzschia* spp., *Chaetoceros* spp. along with the dinoflagellates *Heterocapsa niei* and *Ceratium* spp.



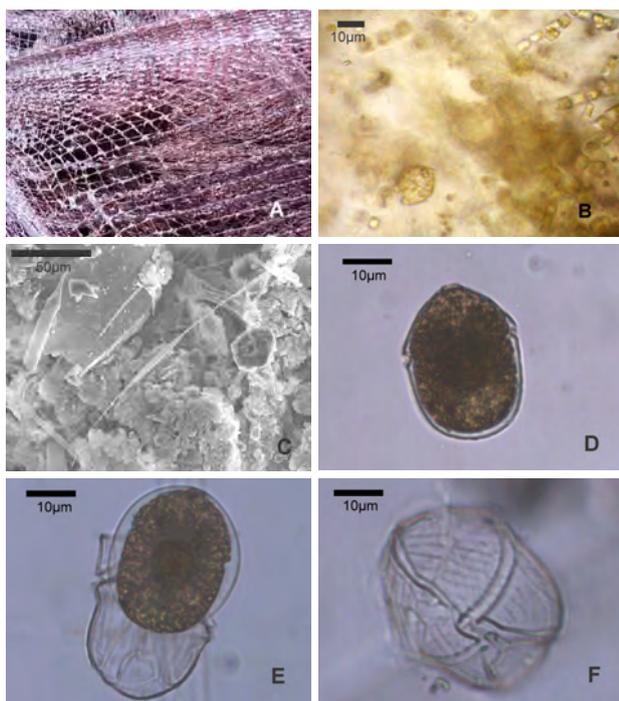
**Figure 3.** Temporal variations of *Gonyaulax hyalina* mean abundance (19 stations) during the period May-October 2005 in Thermaikos Gulf (shaded area corresponds to mucilage period).

Formation of mucilage aggregations occurred annually 1987-1992. After a 12-year period (1992-2004) without records, a strong outbreak was recorded in an extended area of the North Aegean Sea in the beginning of September 2004 (Fig. 1). In water samples collected during this event, the dinoflagellate *G. hyalina* was recorded for the first time along with the diatoms *Skeletonema costatum*, *Chaetoceros* spp., *Pleurosigma* sp. and *Cylindrotheca closterium*. The *G. hyalina* population was relatively small, not exceeding  $1.4 \times 10^3$  cells  $\text{L}^{-1}$ .

After this episode, attention was paid to the presence of *G. hyalina* in coastal waters in the upper Thermaikos Gulf, where a phytoplankton monitoring programme has been in progress. Cells of *G. hyalina* in the water samples were detected in early June 2005 at about the time mucilage aggregations were recorded (Fig. 3). Occurrence of these masses in coastal waters lasted until the end of September. During this time, *G. hyalina* populations reached abundances up to  $7.9 \times 10^3$  cells  $\text{L}^{-1}$ .

Formation of mucilage aggregates was also recorded in 2006, starting in early July. Monitoring data for the *G. hyalina* population during this last event, which ended in early October, showed relatively low abundances (40-400 cells  $\text{L}^{-1}$ ).

*Gonyaulax hyalina* cells were oval-shaped with a prominent apical crest (Fig. 4D). Cells had a very delicate theca, which was usually shed shortly after sample collection (Figs. 4E, F). The theca was characterized by strong parallel longitudinal ridges (Fig. 4F), which along with the fourth apical plate distinguishes this species from *G. fragilis*. Two size classes were observed: medium-sized cells ( $L=33.3-47.6$



**Figure 4.** Fishing nets bearing mucilaginous masses (A), LM (B) and SEM (C) micrographs of mucilage material, *G. hyalina* cell (D), discarding its theca (E) and abandoned theca (F) under LM.

µm, W=25.0-36.9 µm) and large-sized cells (L= 57.1-66.6 µm, W= 40.5-54.7 µm).

## Discussion

Mucilage aggregations in Greek marine waters seem to be a common phenomenon. Annual events occurred between 1987 and 1992, followed by a 12-year period (1992-2004) where no mucilage events were recorded, followed by the severe occurrences 2004-2006, a pattern previously suggested by Vollenweider *et al.* (1995). However, it should not be excluded that non detection of the phenomena during this period could be attributed to low intensity.

Mucilage phenomena usually intensify during the warm period as has also been observed in the Tyrrhenian and Adriatic Seas and New Zealand (Innamorati 1995; McKenzie *et al.* 2002; Pompei *et al.* 2003 ; Pistocchi *et al.* 2005). Furthermore, intense mucilage events in the Italian seas were recorded mainly in the upper water layers (Giani *et al.* 2005), whereas in the North Aegean Sea they were not visible in the surface waters but only detected on fishing nets.

The North Aegean Sea, and especially the Thermaikos Gulf, where water masses are classified as eutrophic (Nikolaidis *et al.* 2006), seem to be the most affected areas with regard to mucilage events (Gotsis-Skretas 1995; present study). However, a

connection between mucilage aggregates and anthropogenic pollution does not seem to exist, since mucilage aggregates in Greek waters occur also in regions where no point sources of pollution are documented and water masses are classified as oligotrophic (Ignatiades 2005). Furthermore, these phenomena have been known since the 18th century (Molin *et al.* 1992) when anthropogenic pressure on coastal waters was negligible.

For several years, diatoms were thought to be the most important causative organisms for mucilage aggregates. However, the role of *Gonyaulax* species has been pointed out recently (McKenzie *et al.* 2002; Pompei *et al.* 2003). Our data, obtained in 2004-2006, confirm the coincidence of mucilage events with the presence of *G. hyalina* populations. Despite the relatively low abundance of *G. hyalina*, its simultaneous presence with mucilage events could partially explain the formation of such events during the last three years.

A synergistic action between *Gonyaulax* cells and diatoms promoting mucilage formation should not be excluded. Further investigations on the allelopathic interactions among microalgae may clarify the factors determining mucilage phenomena.

## Acknowledgements

The authors would like to thank the Greek authorities (Coastal Guard and Fisheries Services of Thessaloniki, Kavala and Pieria) and fishermen for contributing to data collection.

## References

- Allredge, A.L., Passow, U. & Haddock, S.H.D. (1998). *J. Plankt. Res.* 20: 393-406.
- Azam, F. & Long, R.A. (2001). *Nature* 414: 495-498.
- Giani, M., Rinaldi, A. & Degobbi, D. (2005). *Sci. Total Environ.* 353: 3-9.
- Degobbi, D., Fonda-Umani, S., Franco, P., Malej, A., Precali, R. & Smodlaka, N. (1995). *Sci. Total Environ.* 165: 43-58.
- Gotsis-Skretas, O. (1995). *Sci. Total Environ.* 165: 229-230.
- Ignatiades, L. (2005). *J. Sea Res.* 54: 51-57.
- Innamorati, M. (1995). *Sci. Total Environ.* 165: 65-81.
- Lancelot, C. (1995). *Sci. Total Environ.* 165 : 83-102.
- Lorenzen, C.J. (1967). *Limnol. Oceanogr.* 12: 343-346.
- MacKenzie, L., Sims, I., Beuzenberg, V. & Gillespie, P. (2002). *Harmful Algae* 1: 69-83.
- Molin, D., Guidoboni, E. & Lodovisi, A., 1992. In:

- Marine Coastal Eutrophication, Vollenweider, R.A., Marchetti, R. & Viviani, R. (eds), Elsevier, Amsterdam, pp. 511–524.
- Nikolaidis, G., Moschandreu, K., Koukaras, K., Ali-gizaki, K., Kalopesa, E. & Heracleous, A. (2006). *Fresen. Environ. Bull.*, 15(9b), 1193-1198.
- Passow, U. (2002). *Mar. Ecol. Prog. Ser.* 236: 1–12.
- Pistocchi, R., Cangini, M., Totti, C., Urbani, R., Guerrini, F., Romagnoli, T., Sist, P., Palamidesi, S., Boni, L. & Pompei, M. (2005). *Sci. Total Environ.* 353: 307-316.
- Pompei, M., Mazziotti, C., Guerrini, F., Cangini, M., Pigozzi, S., Benzi, M., Palamidesi, S., Boni, L. & Pistocchi, R. (2003). *Harmful Algae* 2: 301-316.
- Rinaldi, A., Vollenweider, R.A., Montanari, G., Ferrari, C.R. & Ghetti, A., 1995. *Sci. Total Environ.* 165: 165-183.
- Strickland, J. & Parsons, T. (1968). *Bull. Fish. Res. Board Can.* 167: 1–311.
- Totti, C., Cangini, M., Ferrari, C., Kraus, R., Pompei, M., Pugnetti, A., Romagnoli, T., Vanucci, S. & Socal, G., 2005. *Sci. Total Environ.* 353: 204-217.
- Utermöhl, H. (1958). *Mitt. Int. Ver. Theor. Angew. Limnol.* 9: 1-38.
- Vollenweider, R.A., Montanari, G. & Rinaldi, A. (1995). *Sci. Total Environ.* 165: 213-224.

## Inter-annual variability of *Alexandrium* blooms in Cork Harbour, Ireland

A. Ní Rathaille, N. Touzet and R. Raine

Martin Ryan Institute, National University of Ireland, Galway, Ireland and aoife.nirathaille@nuigalway.ie, nicolas.touzet@nuigalway.ie, robin.raine@nuigalway.ie

### Abstract

Blooms of *Alexandrium* are a recurring problem in the retentive North Channel of Cork Harbour on the south coast of Ireland. Annual variations of the blooms include the timing of their initiation, their intensity and their duration. They often lead to toxic events and shellfish closures in the North Channel area. Field data from the 2004, 2005 and 2006 bloom seasons are presented. These data sets provide evidence that the inter-annual variability of the blooms is directly related to the physical regime, namely temperature and light levels within the water column and tidal dilution. Results of laboratory experiments investigating the effects of both temperature and light on the growth rates of *A. minutum* and *A. tamarensense*, species that co-exist within the North Channel, are presented. The control by tidal dilution in the North Channel varies substantially between spring and neap tides. It is hypothesised that the balance between the maximum growth rates, as determined by temperature and light, and the tidal dilution, as determined by the time of year, dictates the initiation, intensity and duration of the observed blooms.

### Introduction

Despite the widespread occurrence of *Alexandrium* sp. around the coastline of the Republic of Ireland, closures due to PSP have been confined to Cork Harbour on the south coast (FAO 2004). The North Channel of the harbour supports extensive shellfish culture and suffers from closures due to PSP contamination (Fig. 1). It is a naturally retentive area due to the tidal circulation, has the highest density of *Alexandrium* sp. resting cysts in the region and is the site of initiation of *Alexandrium* blooms in the harbour (Ní Rathaille and Raine 2006). Two species of *Alexandrium* co-occur there, a toxic form of *A. minutum* and a non-toxic form of *A. tamarensense* (Touzet *et al.* 2006).

Analysis of historical monitoring data for *Alexandrium* (1989-2003) in Cork Harbour reveals marked inter-annual variability. Bloom intensity varies from years with no blooms to years when blooms

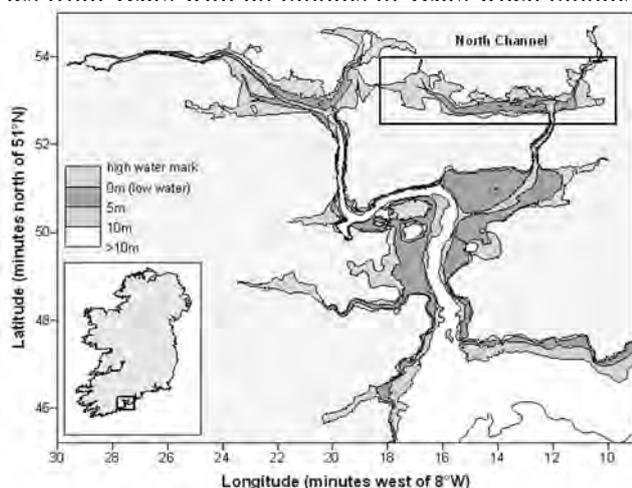
of up to 850,000 cells·L<sup>-1</sup> have been recorded (Irish Marine Institute, unpubl. records). The timing of the blooms also varies, with the highest density blooms most frequently occurring in June and occasionally in July. The blooms that infrequently occur in August or September are relatively low density blooms.

In this work we present an exploratory analysis of patterns in the recurring annual blooms in relation to environmental conditions.

### Methods

Field investigations were carried out over 3 bloom seasons (2004, 2005 and 2006). A wide range of environmental parameters were measured including *in situ* temperature and salinity, light attenuation, inorganic nutrients and chlorophyll-*a* concentrations. Meteorological data (incident irradiance, wind speed and direction) was provided by Met Éireann, the Irish national meteorological service, and was combined with vertical attenuation data to derive variations of *in situ* irradiance levels. Water samples were taken for the enumeration of *Alexandrium* sp. vegetative cells by light microscopy, so no distinction was made between *A. minutum* and *A. tamarensense*.

Batch culture experiments were undertaken in triplicate to investigate the effects of temperature and irradiance on the growth rate of each species. The temperature and irradiance levels examined were 5, 7, 11, 15 and 20 °C and 30, 50, 60, 100 and 125 μmol·m<sup>-2</sup>·s<sup>-1</sup>, respectively. Linear regressions were derived from the results, which were used to calculate the growth potential for each year based on the *in situ* temperature and irradiance data.



**Figure 1.** Map of Cork Harbour.

Tidal ranges were obtained from local tide tables. The data were correlated with tidal dilution rates using an existing 2D hydrodynamic model of the area (MarCon Computations Int. Ltd., Galway).

## Results and Discussion

### Laboratory studies

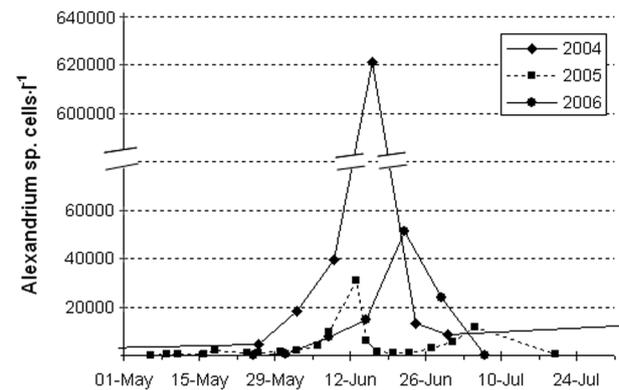
The batch culture experiments yielded a  $T_{\max}$  and an  $I_{\max}$  (the temperature and irradiance at which maximum growth rates were achieved) of 15 °C and 100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , respectively, with no difference observed between *A. tamarensis* and *A. minutum* (data not shown). A  $T_{\max}$  of 15 °C falls at the lower end of the scale of reported laboratory derived values (~15–20 °C) for *Alexandrium* sp. in temperate waters (Prakash 1967; Watras *et al.* 1982; Etheridge and Roesler 2005). Similarly, an  $I_{\max}$  of 100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  falls at the lower end of the scale of reported values (~100–250  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the genus in temperate waters (Anderson 1998; Parkhill and Cembella 1999; Etheridge and Roesler 2005). These results suggest that the species are well adapted to take advantage of the local *in situ* temperature (~5–22 °C) and irradiance (~10–200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) ranges. The low *in situ* irradiance range is due to high suspended matter levels related mainly to resuspension by winds and tides in a shallow water column.

Maximum growth rates of both species from strains generated from cysts from the North Channel were between 0.4 and 0.5  $\text{d}^{-1}$  (data not shown). This is typical for laboratory cultures of *Alexandrium* sp. (Anderson 1998) but higher rates have been reported under specific conditions, e.g. 0.68  $\text{d}^{-1}$  for *A. fundyense* from the Gulf of Maine incubated at high irradiances (Etheridge and Roesler 2005). Maximum *in situ* growth rates for *A. minutum* of up to 0.9  $\text{d}^{-1}$  have been reported from a Mediterranean harbour (Garcés *et al.* 1998).

### Field investigations

Figure 2 shows the numbers of *Alexandrium* sp. cells counted during each bloom season. Cell numbers in

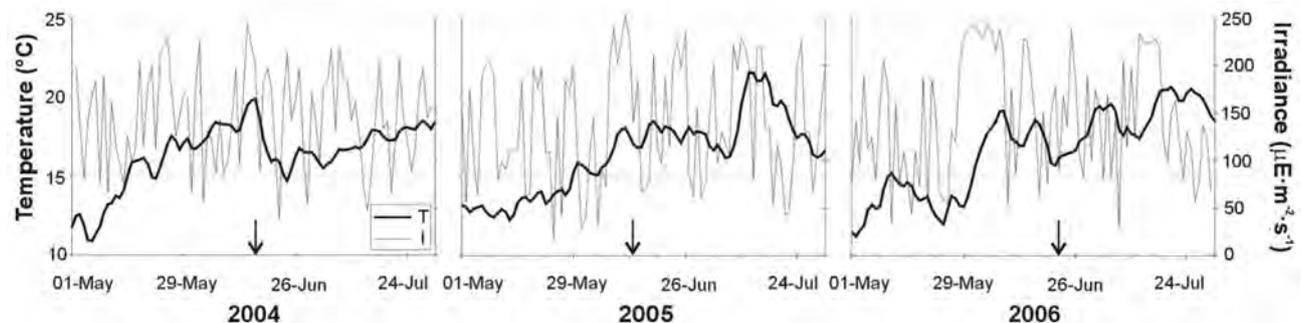
2004 peaked at 600,000 cells·L<sup>-1</sup> on 16 June, in 2005 at 35,000 cells·L<sup>-1</sup> on 13 June, and in 2006 they peaked at 50,000 cells·L<sup>-1</sup> on 22 June. PSP closures occurred on each occasion (Irish Marine Institute, unpubl. records). Figure 3 shows the *in situ* temperature and irradiance measurements recorded during each bloom. In each case, irradiance levels were far more variable than temperature. Figure 4 shows the calculated potential growth rates for each year. Figure 5 shows the pattern of tidal ranges for 2005, a pattern that repeats annually and is directly proportional to tidal dilution in the North Channel.



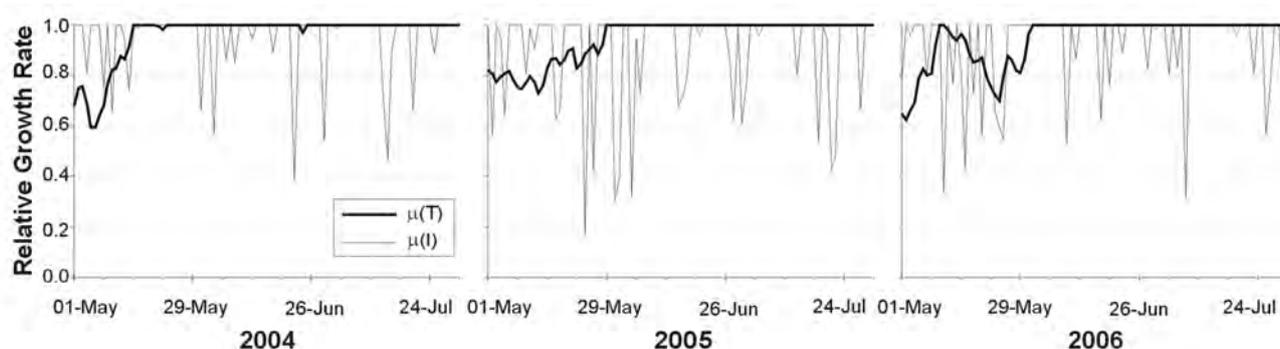
**Figure 2.** Numbers of *Alexandrium* sp. cells ( $\cdot\text{L}^{-1}$ ) in the North Channel during May, June and July of 2004, 2005 and 2006.

### Bloom intensity

The bloom that was observed in 2004 had a cell density that was an order of magnitude greater than those observed in 2005 and 2006. This seems to have been directly related to the patterns of *in situ* temperature and irradiance to which the cells were exposed. The 2004 bloom occurred 4 weeks after the onset of 15 °C water temperatures and co-occurred with the highest *in situ* irradiance levels. In 2005 the bloom occurred 2 weeks, and in 2006 3 weeks, after the onset of 15 °C water temperatures, and while the 2005 bloom co-occurred with the highest *in situ* irradiance, this was not the case in 2006. Globally, *Alexandrium* sp. bloom development has been reported to be correlated to a

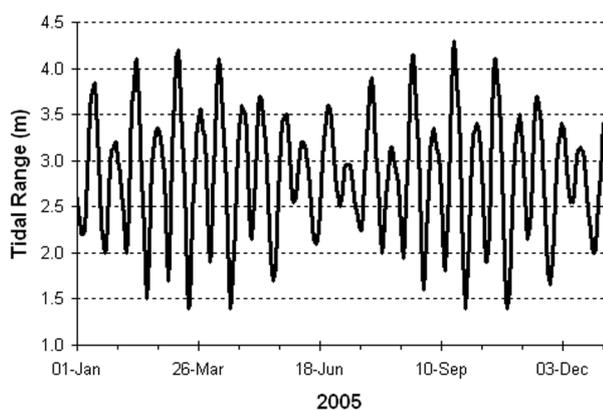


**Figure 3.** *In situ* temperature and irradiance levels during May, June and July of 2004 (left), 2005 (centre) and 2006 (right). (Arrows indicate time of peak cell numbers.)



**Figure 4.** Simulated growth rates for 2004 (left), 2005 (centre) and 2006 (right) based on *in situ* temperature and irradiance data and experimental growth rate results

wide range of both endogenous and exogenous factors that include temperature (Garcés *et al.* 1999; Vila *et al.* 2001) and the ambient light field (Townsend *et al.* 2001). While not exactly quantifiable, it was evident from the simulations (Fig. 4) that 2004 was the year with the greatest growth potential compared with 2005 and 2006.



**Figure 5.** Tidal ranges in Cork Harbour in 2005.

#### Bloom timing

Tidal patterns showed smaller ranges (2.0–3.5 m), and therefore smaller dilution, around the summer solstice. These dilution rates, 0.13–0.38 d<sup>-1</sup>, would do little to impede the development of a bloom of cells growing at 0.5 d<sup>-1</sup>, compared with equinoctial dilution rates (0.10–0.78 d<sup>-1</sup>). Tidal dilution increases with temporal distance from the summer solstice. In other words, the potential for bloom development, in terms of tidal dilution, is greatest in June and July.

#### Conclusions

The dynamics of *Alexandrium* blooms in the North Channel are controlled by interactions between the vegetative cells and the local environment, with *in situ* temperature and irradiance levels and tidal dilution exerting the greatest control. Inter-annual variations in bloom intensity and bloom timing depend on the co-occurrence of quality temperature and irradiance levels with weak tidal dilution.

#### Acknowledgements

The authors would like to acknowledge funding from the Marine Institute (Marine RTDI, NDP 2000–2006) and the European Commission (GOCE-CT-2005-003875 SEED). The authors also acknowledge Brian Byrne, Dónie Geary, Sandra Lyons, Hazel Farrell and David and Tristan Hugh-Jones for their help with field sampling.

#### References

- Anderson, D.M. (1998). In: *Physiological Ecology of Harmful Algal Blooms*, Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (eds), NATO ASI series G41, Springer-Verlag Berlin Heidelberg, pp. 29–48.
- Etheridge, S.M. & Roesler, C.S. (2005). *Deep-Sea Res. II* 52: 2491–2500.
- FAO (2004). *FAO Food and Nutrition Paper* 80.
- Garcés, E., Delgado, M., Vila, M. & Camp, J. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernandez, M.L. & Wyatt, T. (eds), Xunta de Galicia and IOC-UNESCO, Vigo, pp. 167–170.
- Garcés, E., Masó, M. & Camp, J. (1999). *J. Plankton Res.* 21: 2373–2391.
- Ní Rathaille, A. & Raine, R. (2006). In: *HABs in Coastal Embayments and Fjords*, Cembella, A. & Guzman, L. (eds), IOC-UNESCO, Paris, in press.
- Parkhill, J.-P. & Cembella, A.D. (1999). *J. Plankton Res.* 21: 939–955.
- Prakash, A. (1967). *J. Fish. Res. Bd. Can.* 24: 1589–1606.
- Touzet, N., Franco, J.M. & Raine, R. (2006). *Afr. J. Mar. Sci.* 28: 181–184.
- Townsend, D.W., Pettigrew, N.R. & Thomas, A.C. (2001). *Contin. Shelf Res.* 21: 347–369.
- Watras, C.J., Chisholm, S.W. & Anderson, D.M. (1982). *J. Exp. Mar. Biol. Ecol.* 62: 25–37.
- Vila, M., Garcés, E., Masó, M. & Camp, J. (2001). *Mar. Ecol. Prog. Ser.* 222: 73–83.

## Harmful algal blooms and eutrophication: nutrient sources, composition and consequences in the Arabian Gulf bordering Abu Dhabi Emirate

A. Rajan and T. Z. Al Abdessalaam

P.O.Box-45553, Marine Environment Research Center, Environment Agency Abu Dhabi, Abu Dhabi, UAE,  
arajan@ead.ae

### Abstract

The Arabian Gulf has witnessed an increase in algal blooms over the last decade, presumably due to pollution from land reclamation and urbanization. In response to growing concerns over the impact of harmful algal blooms on marine resources, ecosystems and human health, a survey was initiated in 2002 to study the harmful algae of the waters of Abu Dhabi Emirate bordering the Arabian Gulf. Subsequently in 2003 a harmful algal bloom was recorded in one of the sampling areas (Mussafah). The bloom with the maximum cell concentration of  $18 \times 10^7$  cells/L<sup>-1</sup> showed seasonal variation in species dominance; dinoflagellates during winter and cyanobacteria during summer. The winter bloom was represented by *Prorocentrum micans* and *P. minimum* and the water was brown in colour; whereas in summer the bloom was generated by *Oscillatoria* sp. and the water was green. Observations made on hydrographic parameters showed unusual conditions. The nutrient values were many-fold higher than the open sea. The blooms were associated with widespread harmful impacts including hypoxic events (0.20 mg/L), finfish kills (*Nematalosa nasus*) and subsequent bottom-living organism losses. The deterioration of water quality in Abu Dhabi waters was probably due to the human activities along the coastal areas, insufficient treatment of sewage water, and waste water discharge from industries. Extensive sediment load may also have exacerbated the situation and contributed to eutrophication and subsequent alteration of the ecosystem.

### Introduction

The occurrence of algal blooms in the Arabian Gulf has been increasing during the last two decades. Human activities and coastal development driven by economic growth and technological changes in bordering countries increased the eutrophication which enriched the marine environment with addition of nutrients (Anon. UAE Year Book 2006). Elevations in nutrient concentrations cause the algae to proliferate and disrupt the ecological integrity. This paper is one of the results of the HAB programme undertaken by Environment Agency Abu Dhabi since 2002. It illustrates the nutrient status, potential nutrient sources and their effects, particularly on harmful algal blooms in Abu Dhabi waters of United Arab Emirates.

### Materials and Methods

Abu Dhabi is located in the Southern Arabian Gulf (Fig. 1). It has a coastline of 420 km. Its marine environment is under pressure from human activities such as offshore oil exploration, dredging, land filling, and urbanization. The HAB survey sampling stations were selected randomly all over the Emirate. The Mussafah south channel, a dredged channel, is one of the sampling areas and is connected to the main Mussafah channel and the open sea. It is nearly 6 km long, 0.5 km wide and 7 m deep. The channel is characterized by the presence of number of industries, marinas, dry

docks, gas stations, oil storage facilities and municipal drainages on both sides.

Monthly samplings were carried out in the bloom area for a period of two years since 2002. Phytoplankton and nutrient samples were collected using a Niskin water bottle for quantitative analyses. The samples were fixed immediately using Lugol's solution and stored in dark bottles. Species identification and taxonomy was based on Tomas (1997) and Cupp (1943). Hydrographic parameters (temperature, salinity, pH, dissolved oxygen) were measured using a Hydrolab Surveyor IV at three depths (0, 3 and 7 m). Water samples for nutrient analysis ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ ,  $\text{SiO}_4$ ,  $\text{PO}_4^{3-}$ ) were collected, frozen immediately, and analyzed the same day using a Skalar autoanalyzer San<sup>++</sup> following standard methods (Anon., Skalar manual 2002).

### Results

#### Hydrography (Table 1)

The water temperature of Mussafah south channel varied between 20.43 and 33.57 °C. The salinity values showed fluctuation between surface and bottom and it varied between 45.00 and 49.52 in surface waters, 63.18, and 70.74 in bottom waters. The surface water had a pH range between 7.50 and 8.63 and the bottom waters varied between 5.51 and 8.46. The dissolved oxygen content of the surface water varied



Figure 1. Phytoplankton sampling site

Table 1. Hydrographic parameters and nutrient concentration in Mussafah, Abu Dhabi

Parameters	Non bloom Sea water			Bloom Area		
	Minimum	Maximum	Mean	Minimum	Maximum	Mean
Temperature (°C)	19.00	35.40	25.88	20.43	33.57	28.07
Salinity	37.00	50.10	42.16	45.00	70.74	54.46
pH	7.19	8.46	7.66	5.51	8.63	7.61
Dissolved oxygen (mg/L)	2.78	6.20	4.83	0.21	10.80	4.67
Nitrite (NO <sub>2</sub> <sup>-</sup> ) (µM/L)	0.00	0.25	0.05	0.57	14.25	4.99
Nitrate (NO <sub>3</sub> <sup>-</sup> ) (µM/L)	0.00	10.35	2.43	12.64	356.56	170.09
Silicate (SiO <sub>4</sub> ) (µM/L)	0.03	10.43	7.11	3.00	51.14	22.52
Ammonia (NH <sub>4</sub> <sup>+</sup> ) (µM/L)	1.05	27.41	9.43	13.17	30.50	17.68
Phosphate (PO <sub>4</sub> <sup>3-</sup> ) (µM/L)	0.16	4.83	0.52	0.08	20.53	7.91

Table 2. Dominance of different species during different seasons

Summer		Winter	
Species	No. of cells L <sup>-1</sup>	Species	No. of cells/L <sup>-1</sup>
<i>Oscillatoria</i> sp.	2.3 x 10 <sup>8</sup>	<i>Oscillatoria</i> sp.	4.8 x 10 <sup>6</sup>
<i>Prorocentrum micans</i>	7.2 x 10 <sup>6</sup>	<i>Prorocentrum micans</i>	3.9 x 10 <sup>7</sup>
<i>Prorocentrum minimum</i>	1.4 x 10 <sup>6</sup>	<i>Prorocentrum minimum</i>	1.8 x 10 <sup>8</sup>
<i>Prorocentrum sigmoides</i>	4.0 x 10 <sup>5</sup>	<i>Prorocentrum sigmoides</i>	2.0 x 10 <sup>6</sup>
<i>Gymnodinium</i> sp.	4.0 x 10 <sup>5</sup>	<i>Gymnodinium</i> sp.	0
<i>Gyrodinium</i> sp.	5.0 x 10 <sup>5</sup>	<i>Gyrodinium</i> sp.	0
<i>Scrippsiella</i> sp.	4.6 x 10 <sup>6</sup>	<i>Scrippsiella</i> sp.	0
<i>Nitzschia</i> sp.	2.4 x 10 <sup>7</sup>	<i>Nitzschia</i> sp.	7.2 x 10 <sup>5</sup>
<i>Cylindrotheca closterium</i>	1.0 x 10 <sup>5</sup>	<i>Cylindrotheca closterium</i>	0
<i>Thalassionema nitzschioides</i>	0	<i>Thalassionema nitzschioides</i>	1.7 x 10 <sup>6</sup>

from 2.73 to 10.84 mg/L and 0.21 to 2.76 mg/L in bottom waters.

#### Nutrients (Table 1)

The  $\text{NO}_2^-$  values varied between 0.57 and 14.25  $\mu\text{M/L}$ ,  $\text{NO}_3^-$  values fluctuated from 12.64 to 356.55  $\mu\text{M/L}$ ,  $\text{SiO}_4$  concentration varied between 3.00 and 51.14  $\mu\text{M/L}$ ,  $\text{NH}_4^+$  values between 13.17 and 30  $\mu\text{M/L}$ , and  $\text{PO}_4^{3-}$  concentration varied between 0.08 and 20.53  $\mu\text{M/L}$ .

#### Phytoplankton (Table 2)

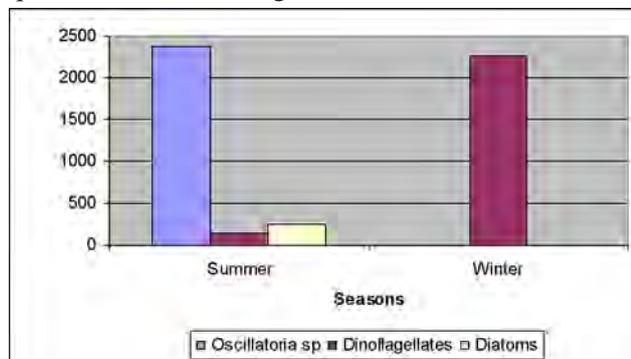
Phytoplankton numbers in Mussafah south channel varied seasonally; highest values ( $9.2 \times 10^7 \text{ cells/L}^{-1}$ ) during the summer and lowest ( $4.3 \times 10^7 \text{ cells/L}^{-1}$ ) during the months prior to summer. The samples collected showed that phytoplankton diversity was less compared to other areas of Abu Dhabi waters. The eutrophic channel was characterized by high concentrations of selected species including *Oscillatoria* sp., *Prorocentrum micans*, *P. sigmoides*, *P. minimum*, *Gymnodinium* sp., *Gyrodinium* sp., *Protoceratium* sp., *Protoperdinium* sp., *P. steinii*, *Scrippsiella* sp., *Nitzschia* sp., *Cylindrotheca closterium*, *Thalassiosira* sp., and *Thalassionema nitzschioides*.

#### Algal Blooms

Algal blooms occurred throughout the study period. Initially in October of 2003 the bloom was dominated by the *Oscillatoria* sp. which comprised 93.7 % of phytoplankton community abundance and had a maximum cell concentration  $1.5 \times 10^8 \text{ cells/L}^{-1}$ . This was followed by the *Nitzschia* sp. with  $2.0 \times 10^5$  to  $1.0 \times 10^7 \text{ cells/L}^{-1}$  (6.2 %). In winter the bloom was dominated by dinoflagellates ( $2.2 \times 10^8 \text{ cells/L}^{-1}$ ), followed by an increase in cyanobacteria (Fig. 2).

#### Fish kills

Fish kills of *Nematalosa nasus* (clupeid) were observed during blooms of cyanobacteria and subsequent blooms of the algae *Prorocentrum minimum*.



**Figure 2.** The dominance of different algal groups during different seasons.

## Discussion

The monitoring of water quality in Mussafah south channel indicates that the channel is eutrophic. The chemical analyses showed unusual values for water quality parameters, thus indicating poor water quality and possibly a concern for the health and/or sustainability of marine life. The area was highly stratified and the bottom salinity was higher than 70. The pH values were very low at the bottom, and the dissolved oxygen concentration fluctuated between 0.21 (bottom) and 10.84 mg/L (surface). The low dissolved oxygen and change in pH values could be due to the eutrophic condition of the area and photosynthetic activity and other related activities of the bloom. The oxidation of the organic matter in the study area also enhanced the pH variations.

The bloom was initially formed by *Oscillatoria* sp. and lasted for six months. During the following two months, the bloom was mixed with cyanobacteria and dinoflagellates, while the dinoflagellates dominated the area for the rest of the period. The nutrient enrichment through the outlets present on either side of the channel and Eolian dust with iron most probably produced blooms all through the year with seasonal succession. Increases in phytoplankton blooms due to increase in nutrient input have been reported from the South China Sea (Qi *et al.* 1993), Black Sea (Bodeanu and Ruta 1998), Hong Kong (Lam and Ho 1989), and Chesapeake Bay (Glibert *et al.* 1995; Malone *et al.* 1996). Besides, Shamal, the local wind that bring Eolian dust with iron content that also supports the cyanobacterial bloom in the Arabian Gulf (Subba Rao *et al.* (1999).

The eutrophication was probably caused by the continuous release of nutrient-rich discharge from outlets present on either side of the channel. Land reclamation and urbanization were important activities in Emirate of Abu Dhabi, which increased the nutrient level in the coastal waters. Additionally, topography of the area is such that the circulation and tidal influence was restricted, further enhancing the effect of nutrient enhancement. This continuous supply of nutrients and limited circulation led the channel to being highly stratified and the bottom became hypoxic. This condition can lead to a complex sequence of effects, as the change in water chemistry and biological community leads to the continuous formation of algal blooms, no life at the bottom, and fish kills. The nutrient enrichment and continuous algal blooms further increases the bottom deposits and hydrogen sulfide formation. This series of events is probably what led

to the massive deterioration that occurred in the Mus-safah south channel.

### Acknowledgements

Authors would like to express their sincere thanks to management of Environment Agency Abu Dhabi for their support. We thank the Secretary General of the EAD for his interest and support. We are especially grateful to our colleagues Dr. Himansu Das, Dr. Salem Javed and Dr. Shakeel Ahmed for their timely assistance at various stages of this research. Sincere thanks go to the Staff of the Environmental Laboratory Department for their help in analyzing the water samples.

### References

- Anon. (2006). United Arab Emirates Year Book 2005.
- Anon. (2002). Scalar manual for the Autoanalyzer San ++.
- Bodeanu, N. & Ruta, G. (1998). In: Harmful Algae, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T. (eds), Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Paris, France, pp. 188-191.
- Cupp, E.E. (1943). Bull. Scripps.Inst. Oceanogr. 5: 1-237.
- Glibert, P.M., Conley, D.J., Fisher, T.R., Harding, L.W. & Malone, T.C. (1995). Mar. Ecol. Progr. Ser. 122: 27-43.
- Lam, C.W.Y. & Ho, K.C. (1989). In: Okaichi, T. Anderson, D.M. & Nemo, T. (eds), Red Tides: Biology, Environmental Science and Toxicology, Elsevier, New York, pp. 49-52.
- Malone, T.C., Conley, D.J., Fisher, T.R., Gilbert, P.M., Harding, L.W. & Sellner, K.G. (1996). Estuaries 19: 371-385.
- Qi, Y.Z., Zhang, Z., Hong, Y., Lu, S., Zhu, C. & Li, Y. (1993). In: Smayda, T. & Shimizu, Y. (eds), Toxic Phytoplankton Blooms in the Sea, Elsevier, Amsterdam, The Netherlands, pp. 43-46.
- Subba Rao, D.V., Al-Yamani, F. & Nageswara Rao, C.V. (1999). Eolian Dust Affects Phytoplankton in the Waters off Kuwait, the Arabian Gulf.
- Tomas, C.R. (ed.) (1997). Identifying Marine Phytoplankton. Academic Press, 858 pp.

## Biology and seasonal distribution of *Hermesinum adriaticum* in the New River of North Carolina

Robert N. Reger<sup>1</sup> and Carmelo R. Tomas<sup>2</sup>

<sup>1,2</sup> University of North Carolina Wilmington, Center for Marine Science, 5600 Marvin K. Moss Lane, Wilmington NC 28409, 1rnr6195@UNCW.edu, <sup>2</sup>ctomas@UNCW.edu

### Abstract

The New River in southeastern North Carolina is a brackish water system having a variety of microalgae that form annual blooms. Some bloom species are considered toxic; however one rare microorganism that may play a significant role in the river's ecology has been overlooked. An ebridian, *Hermesinum adriaticum* Zacharias was found frequently in the New River during months when water temperatures are above ~22 °C. This unicellular organism contains a lanceolate internal skeleton making the cell about 50 µm by 20 µm. Cell populations in the New River commonly reached 40,000 cells/L, and once a population of 150,000 cells/L occurred at French Creek. Lugol-preserved monthly water samples from seven different stations in the New River collected 2001-2006 were observed to determine annual abundance and seasonal distribution of *H. adriaticum*. Live samples and cells maintained in the laboratory were observed to determine growth characteristics, including life cycle stages and feeding. Also, detailed studies were conducted using both scanning and transmission electron microscopy. SEM was used to examine external morphological changes including possible loss of skeletons, while TEM was used to study nutrition, possible endosymbiotic relationships, and internal structural changes.

### Introduction

*Hermesinum adriaticum*, first described in 1906 by Zacharias, is one of only two extant species in a group known as ebridians, which has been described under several different taxonomic categories. Most recently they have been described as the class Ebridea in the phylum Neomonada (Cavalier-Smith 1997). Both species, *H. adriaticum* and *Ebria tripartita*, are recognized by their internal siliceous skeleton (Hargraves 1974). Ebridians differ from silicoflagellates in many ways. First, ebridians have an internal rather than an external siliceous skeleton as do silicoflagellates. Silicoflagellates have chloroplasts, while these are absent in ebridians. Both groups have two flagella with bases near the nucleus; however ebridian flagellar bases diverge at a high angle while silicoflagellate flagellar bases diverge at an uncommonly low angle. Another difference is the presence of tentacles in silicoflagellates, which do not appear in ebridians (Moestrup and Thomsen 1990). *E. tripartita* has a triaxial skeleton with three branches while the skeleton of *H. adriaticum* is tetraaxial, with four branches (Hargraves 2002). *Hermesinum adriaticum* is 16-24 µm wide by 32-64 µm long, from tip to tip of the skeleton. It lives in an environment of temperatures of 20-25 °C or even higher, as well as a wide range of salinities from 12 to 38 PSU. The greatest abundance of *H. adriaticum* recorded occurred during 1955 in the Salton Sea of California. Environmental conditions that accompa-

nied cell concentrations of 450,000 cells/L were high water temperature and a salinity of 36 PSU (Tiffany 2002).

Since first discovered in the Adriatic Sea in Italy in 1906, *H. adriaticum* has been identified in many estuary systems throughout the world. Nutrition of *H. adriaticum* is not very well understood. *Hermesinum* is non-photosynthetic, however a possible endosymbiotic relationship between *H. adriaticum* and a cyanobacterium (possibly *Synechococcus*) commonly found inside suggests that cells could allow some photosynthetic capabilities (Hargraves 2002). While this species is considered predominantly herbivorous it was found at the chemocline indicating a complex nutrition strategy (Viličić *et al.* 1997). It is not known whether cells are dependent on whole cells of smaller species or whether they have the ability to ingest dissolved or suspended material.

### Materials and Methods

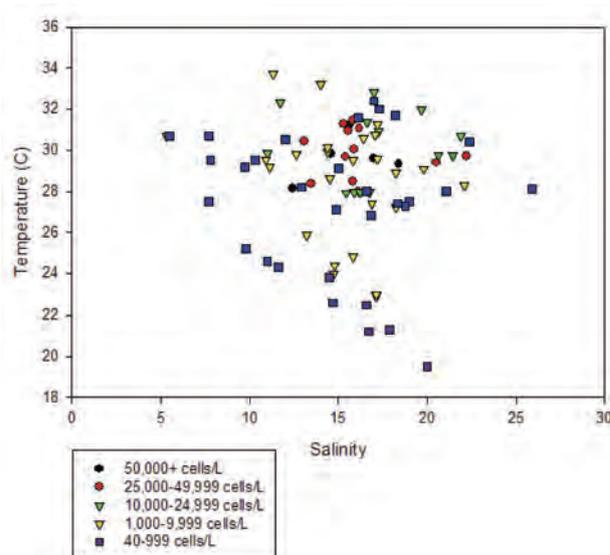
The New River is a brackish river located entirely in Onslow County, North Carolina. Part of the White Oak River Basin, the New River is 40 miles long and two to five miles in width. The river estuary system flows into the Atlantic Ocean just east of Jacksonville, NC. Water temperatures in the New River vary from 14 °C to 33 °C. There is a salinity gradient from 0 in the western river portions farthest from the ocean to 26 in French Creek (mid river) to higher salinities

closer to the ocean. The depth is only a few meters at all sampling stations, and the river is fairly shallow everywhere.

Monthly water samples were taken from seven locations in the New River by the North Carolina Department of Environment and Natural Resources. The locations include French Creek, Paradise Point, Northeast Creek, Southwest Creek, Wilson Bay, Brinson Creek and Highway 17. Sampling began in May 2000 and continued through the end of 2006. Unpreserved and preserved (in Lugols) water samples were observed and used for cell counts. The Utermöhl sedimentation method was used to determine cell number at each location each month. Live *H. adriaticum* cells were pipette isolated from New River samples and placed into 96-well plates, where they were exposed to a variety of media or live cultures of smaller organisms. Media included DY IV, Erdschreiber (Andersen 2005), and Gold and Baren heterotrophic media (Gold and Baren 1966). Also, live cultures of *Rhodomonas* and *Isochrysis* were added to isolated cells to determine if ingestion of whole cells occurred. Cells that successfully multiplied were moved to 24- and 6-well plates and eventually to 50- and 100-mL tissue culture flasks when necessary. Cells maintained in the laboratory were compared with those observed in natural assemblages to determine life cycle stages. Scanning electron microscopy (Phillips CM12) was used to study the siliceous skeletons of *H. adriaticum*. The skeletons were cleaned using von Stosch's (Hasle 1997) diatom cleaning method. Skeletons were compared to those described by Tiffany (2002) and similar stages of skeletal growth were observed in *Hermesinum* skeletons from the New River. Nutritional observations were made using both natural assemblages and isolated cells in an attempt to maintain a laboratory culture of *H. adriaticum*. Laboratory populations were monitored to observe effects of media on cell growth.

## Results

In the New River a direct relationship of increased cell numbers and increased water temperature was apparent (Fig. 1). Also, there was a decline of cells when water temperatures are lower (<25 °C). During the entire year of 2003 *H. adriaticum* was not present at any of the stations sampled. During that year, there was an extraordinary amount of rainfall causing salinities in the New River to become very low and in some areas zero. This suggests that although higher temperature was necessary for cell growth, salinity could also play an important role.

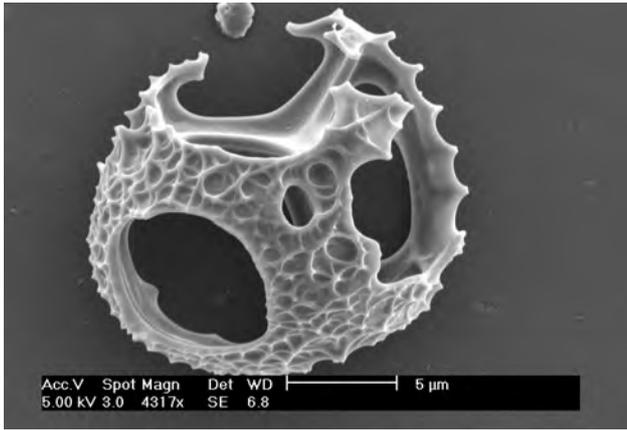


**Figure 1.** *Hermesinum adriaticum* cells from the New River, NC as a function of temperature and salinity during 2001-2006.

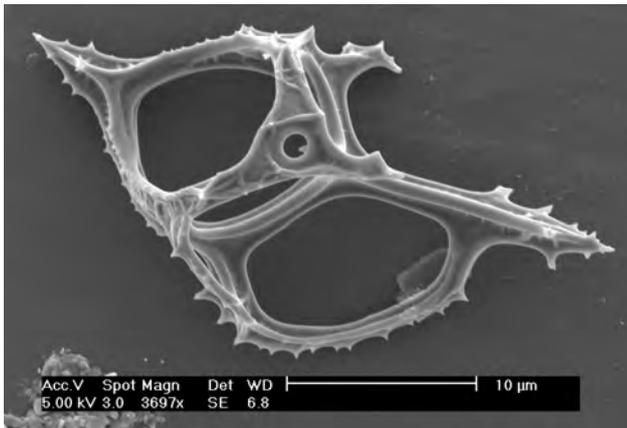


**Figure 2.** Live *H. adriaticum* cell from laboratory population seen under light microscope. Ingested *Isochrysis* cells can be seen inside the cells as small particles.

*Hermesinum* displays a very uncommon and somewhat unknown life cycle. They are found in the water column at times when the temperature is higher. Cells are easily recognizable due to their unique skeleton. A live *Hermesinum* cell is shown in Fig. 2. At times, possibly when the water begins to cool, cells become much harder to identify because the skeletons have a more rounded shape as seen in Fig. 3. These round skeletons were observed in New River samples taken at times after the summer when the water began to cool as well as in populations established from single cells and maintained in the lab. Some cells having a normal skeleton when originally isolated lost their skeletons and appeared as oval cells lacking skeletons. This suggests a possible change in morphology for the winter when the water gets cold.

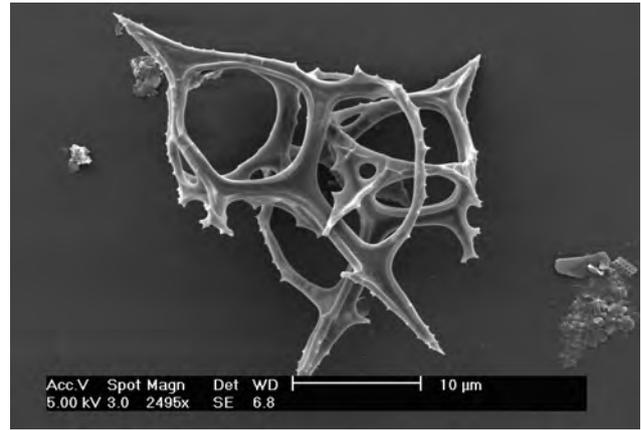


**Figure 3.** Rounded skeleton of *H. adriaticum* cell in a natural population from the New River, NC. SEM.



**Figure 4.** Normal skeleton of *H. adriaticum* from natural population in the New River, NC. SEM.

Nutritional studies of *Hermesinum* clearly show it to be capable of ingesting whole cells of smaller organisms. The media composition had no effect on cell growth, and laboratory populations did not survive in any media alone. However, cells placed in filtered seawater with *Isochrysis* added were maintained for several months. Cells were observed ingesting whole cells of *Isochrysis*. Reproduction is another life cycle feature of particular interest. *Hermesinum* was described as reproducing asexually by normal cell division (Hargraves 1974). In some samples, daughter cells were observed in an “x” shape, presumably from asexual reproduction. Figure 4 illustrates a normal skeleton while Fig. 5 shows the fused double skeleton of dividing daughter cells. While asexual reproduction is known to occur, observations suggest that it is very complex. Laboratory maintained cells as well as cells in natural samples divide into two, three or four daughter cells. In some cases large groups of up to fifteen or more cells are formed from one parent cell



**Figure 5.** Dividing *H. adriaticum* cells from natural population in the New River, NC. SEM.

in bursts of growth that lasted two to four days. They remained together continuing to reproduce in laboratory populations. Further studies are required to define the life cycle stages of this species.

#### Acknowledgements

We would like to thank Stephanie Garrett and the North Carolina Division of Environmental Natural Resources (NCDENR) as well as the Camp Lejeune Waste Water Treatment lab for help in acquiring New River water samples. This work was supported from CDC Grant #01-504-4 awarded to C. Tomas.

#### References

- Andersen, R.A. (2005). Algal Culturing Techniques. Elsevier Academic Press, New York.
- Cavalier-Smith, T. (1997). Arch. Protistenk. 147: 237-258.
- Gold, K. & Baren, C. F. (1966). J. Protozool. 13: 255-257.
- Hargraves, P.E. (1974). Arch. Protistenk. 116: 280-284.
- Hargraves, P.E. (2002). Plankton Biol. Ecol. 49: 9-16.
- Hasle, G.R. & Syvertsen E. (1997). In: Identifying Marine Phytoplankton, Tomas, C.R. (ed.), San Diego, p. 335.
- Moestrup, Ø. & Thomsen, H. (1990). Biol. Skr. 37: 1-57.
- Rhodes, R.G. (1981). Estuaries 4: 150-152.
- Tiffany, M.A. (2002). Hydrobiologia 473: 217-221.
- Viličić, D., Marasović, I. & Kušpilić, G. (1997). Arch. Protistenk. 147: 373-379.
- Zacharias, O. (1906). Arch. Hydrobiol. Planktonk. 1: 394-398.

## Dynamics of algal blooms in the Ukrainian coastal Black Sea

L. Terenko and G. Terenko

Odessa Branch of the Institute of Biology of the Southern Seas, National Academy of Sciences of Ukraine,  
37, Pushkinskaya St., Odessa, 65011, Ukraine, galla@paco.net

### Abstract

There were 66 cases of recorded microalgal blooms in the Ukrainian coastal Black Sea between 1995 and 2005. The primary findings of the studies performed during the time were 1) the number of harmful algal bloom species increased from 14 in the 1960s to 37 in the 1990s, 2) *Skeletonema costatum* (15 cases) was the single dominant species at all seasons, and a key indicator of eutrophication with peak densities up to  $5.0 \times 10^7$  cells $\cdot$ L $^{-1}$ , 3) blooms were frequently dominated by 2-4 species, which since 1999 included *S. costatum*, *Heterocapsa triquetra* and *Eutreptia lanowii*, 4) there were frequent blooms of coccolithophorids and Chryso-phyceae (*Emiliana huxleyi*, *Apedinella spinifera*) and Euglenophyceae (*Eutreptia lanowii*, *E. viridis*), 5) new bloom-forming species include *Gymnodinium simplex*, *Gyrodinium striatum*, and *Scrippsiella trochoidea*, 6) blooms are increasingly dominated by the invasive toxic species *Cochlodinium polykrikoides*, and 7) there was an increase in the occurrence of potentially toxic diatoms (*Pseudo-nitzschia seriata* aff., *P. delicatissima* aff.) and dinoflagellates (*Gymnodinium aureolum*, *C. polykrikoides*, *Alexandrium pseudogonyaulax*, *A. tamarense*). Analysis of long-term changes of blooms in the Ukrainian Black Sea showed an increase in number of blooms in the late 1990s.

### Introduction

The first recorded harmful algal bloom (HAB) event in the Black Sea was in 1909 off Sevastopol (Crimean coast), and caused by *Lingulodinium polyedrum* (Zernov 1913). More recently, a bloom of *Gonyaulax polygramma* was noted along the Ukrainian coast in 1958. During the 1960s, blooms of *Prorocentrum minimum* and *P. micans* were commonly observed during summer (Ivanov 1982), peaking with red tides of *P. minimum* (maximal densities  $2.24 \times 10^8$  cells $\cdot$ L $^{-1}$ ), with concomitant increase in the eutrophication of the northwestern part of the sea in the 1970s (Nesterova 1979). *Prorocentrum* blooms subsequently declined and red tides were formed by other dinoflagellate species, e.g. *H. triquetra* and *Akashiwo sanguinea*, in 1981-1990.

At least 30 species of harmful plankton algae were noted off the Ukrainian coasts of the Black Sea in the beginning of the 1990s (Nesterova 2001). Since 1995, this might be due to eutrophication of coastal waters and the global rise of temperature linked with climate change, as these have been correlated with not only frequency and magnitude of blooms, but also phytoplankton community composition (Sayce and Horner 1996; Hallegraeff 2003; Moestrup 2005).

In this paper we provide a review of historical HAB trends and results of our study on HABs in coastal waters of the Ukrainian part of the Black Sea between 1995 and 2005.

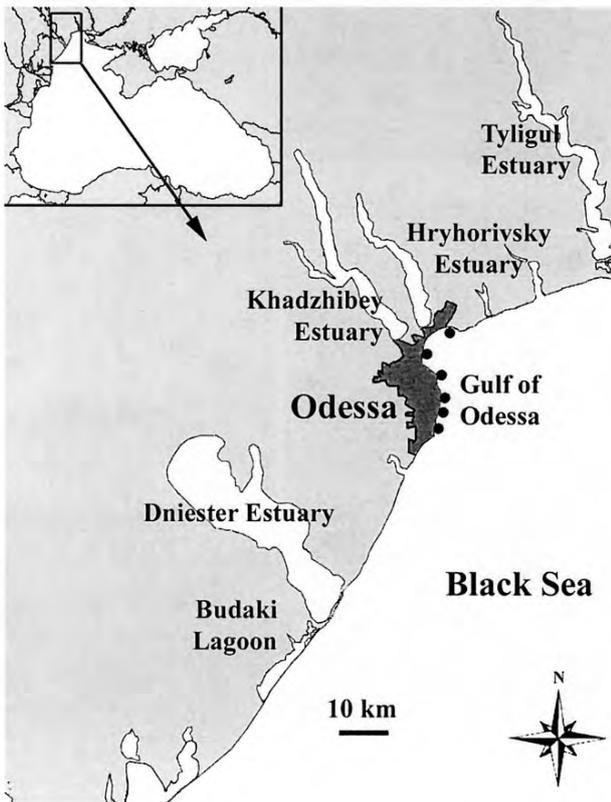
### Materials and Methods

Long-term studies of microalgal blooms were conducted annually with monthly collections at 5-7 stations in the coastal area of Odessa Bay (Fig. 1). Odessa Bay is under the influence of the Dnieper and Southern Bug Rivers, and industrial and domestic waste flows from the city of Odessa. Engineering complexes along all coastal zones extend for approximately 20 km, the three largest Ukrainian marine ports are located here, together with a recreation zone of sandy beaches.

Samples from the surface layer (1–2 litre) were concentrated by reverse filtration through Nuclepore 1- $\mu$ m filters and observed unfixed. Cell counts of the ultra- and nanoplankton were made with a Biolam light microscope at 600x; large forms were counted in a 5-mL chamber. Lugol's solution was subsequently added, followed by 40 % formaldehyde to a final 1:10 dilution.

### Results and Discussion

Many algal species involved in bay bloom events now reach densities  $>1 \times 10^6$  cells L $^{-1}$ . For the period 1995-2005, 66 blooms were identified in the Ukrainian coastal Black Sea. Eleven blooms occurred in 1998 and 1999, contrasting with a minimum of two blooms in 1997 and two in 2004. The blooms were formed by 37 species of microalgae including diatoms (19), dinoflagellates (13), euglenophytes (2), chrysophytes (2) and cyanophytes (1).



**Figure 1.** Map of study area with station locations in the Odessa Bay.

Winter blooms in the coastal zone of Odessa Bay were common, dominated by *Chaetoceros rigidus* ( $1.3 \times 10^7$  cells  $L^{-1}$ -1995), *Stephanodiscus socialis* ( $6.0 \times 10^6$  cells  $L^{-1}$ -2000) and *S. costatum* ( $1.8 \times 10^7$  cells  $L^{-1}$ -1998, 1999, 2000). Bloom events dominated by *S. costatum* have, however, become more frequent and are now possible at any season along the Black Sea coastal zone. The maximum number of bloom events (6) attributed to this species occurred in 1999.

The magnitude of *S. costatum* accumulations increased more than two-fold from the 1970s to the 1990s ( $1.04 \times 10^7$  in 1973-1980,  $1.37 \times 10^7$  in 1994-1996 and  $2.87 \times 10^7$  cells  $L^{-1}$  in 1998-1999), constituting 22.0 to 96.4 % (mean=67.3 %) of the total phytoplankton numbers.

In July 1996, the phytoplankton community was dominated by the coccolithophorid *E. huxleyi* ( $8.0 \times 10^6$  cells  $L^{-1}$ ). The chrysophyte *Apedinella spinifera* was first recorded in the spring of 1999 in Odessa Bay, increasing to a peak of  $1.8 \times 10^6$  cells  $L^{-1}$  in April, 2005.

Blooms in Odessa Bay are now caused by *A. sanguinea*, *G. simplex*, *H. triquetra*, *S. trochoidea*, *P. minimum* and *P. micans*. Two species, *H. triquetra* (March – April, 1999) and *A. sanguinea* (September

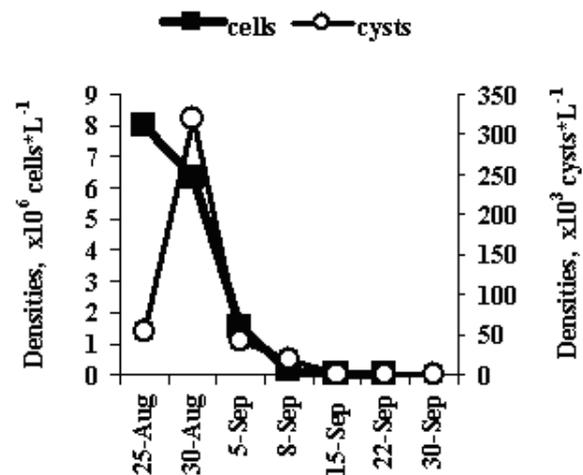
– October, 2000), commonly form red tides (Terenko and Kurilov 2001).

The number of dinoflagellate species causing blooms has increased from 1 (in 1960-70s) to 13 species at the present time. *P. minimum* blooms have declined in the Ukrainian Black Sea, with 10 cases in the 1970s, 3 in the 1980s – 90s and one during the last years (Terenko and Terenko 2005).

In eutrophic areas, mass development of mixotrophic (*A. sanguinea*, *G. simplex*) and heterotrophic (*Gyrodinium cornutum*, *Polykrikos schwartzii*) dinoflagellates is more often observed. This is likely connected to labile organic substances, particularly organic nitrogen, that has increased 2-3 times in the 1990s (Berlinsky *et al.* 2003) from river discharge, sediments and industrial discharge. Additionally, the increase in heterotrophic dinoflagellates could be a response to the frequent winter and early-spring diatom blooms.

New bloom formers are also apparent (*G. simplex*, *Gyrodinium instriatum*, *S. trochoidea*). In August-September 2005, after heavy rainfalls, red tides of *S. trochoidea* were recorded for the first time along the coast, reaching  $8.0 \times 10^6$  cells  $L^{-1}$  in the Odessa port area. Mass development of the species occurred at 23–25 °C and 11.8–13.6 ‰ salinity. Large numbers of *S. trochoidea* cysts ( $4.2 \times 10^4$  cells  $L^{-1}$ ) and motile intermediate stages were recorded (Fig. 2). On 15 September, a rise in salinity to 15.8 ‰ caused a sharp decline to  $1.6 \times 10^4$  cells  $L^{-1}$ . A decrease in *S. trochoidea* was observed during a subsequent accumulation of *A. sanguinea*, *P. schwartzii*, *Cochlodinium polykrikoides* and *Ceratium furca*.

Some potentially toxic species (diatoms aff. *P. seriata*, aff. *P. delicatissima* and dinoflagellates *G.*



**Figure 2.** Dynamics of vegetative cells and cysts of *S. trochoidea* 25 August-30 September 2005.

**Table 1.** Densities of potentially toxic algae in Ukrainian coastal waters of the Black Sea.

Species	Max. densities, cells L <sup>-1</sup>	Date
<i>Pseudo-nitzschia delicatissima</i> aff.	1.9×10 <sup>6</sup>	June 2001
<i>P. pungens</i>	4.50×10 <sup>5</sup>	November 2001
<i>P. seriata</i> aff.	3.50×10 <sup>5</sup>	June 2005
<i>Dinophysis acuminata</i>	1.60×10 <sup>5</sup>	August 2005
<i>D. caudata</i>	2.52×10 <sup>4</sup>	August 2005
<i>D. rotundata</i>	5.2×10 <sup>3</sup>	July 2005
<i>D. ovum</i>	4.6×10 <sup>3</sup>	July 1998
<i>D. recurva</i>	4.5×10 <sup>3</sup>	August 2002
<i>D. fortii</i>	2.6×10 <sup>3</sup>	October 2000
<i>D. baltica</i>	1.9×10 <sup>3</sup>	July 2005
<i>D. islandica</i>	882	December 2001
<i>Cochlodinium polykrikoides</i>	1.60×10 <sup>5</sup>	August 2005
<i>Gymnodinium aureolum</i>	7.00×10 <sup>4</sup>	August 2002
<i>Alexandrium tamarense</i>	8.5×10 <sup>3</sup>	March 2003
<i>A. pseudogonyaulax</i>	3.0×10 <sup>3</sup>	August 2002

*aureolum*, *C. polykrikoides*, *A. pseudogonyaulax*, *A. tamarense*) have often dominated Odessa Bay in recent years (Table 1).

The potentially toxic species *C. polykrikoides* was noted in August-September, 2001 (25.0 °C, 15.0 ‰), reaching 7×10<sup>2</sup> cells L<sup>-1</sup> (Terenko 2003, 2005). A maximum of 1.6×10<sup>5</sup> cells L<sup>-1</sup> were observed in 2005. The cysts of this species, subsequently germinated in the laboratory, were found in surface sediment of the Odessa port, together with *Alexandrium* spp. cysts (Terenko 2001).

Among the new taxa noted, marine and euryhaline dinoflagellates of boreal-tropical and tropical areas now comprise 70 % and cosmopolitans 30 % of the flora, likely preferring higher temperatures than previous dominants (Terenko 2006). This might reflect the penetration of Mediterranean species into the Black Sea (Zaitsev 1997). The potentially toxic dinoflagellates demand further attention for management

control to limit their development in the coastal zone of the Black Sea.

### Acknowledgments

The authors would like to thank the US National HAB Office for financial support to attend the 12th HAB Conference.

### References

- Berlinsky N.A., Garkavaya G.P. & Bogatova J.I. (2003). *Ekol. Sea* 63: 17-22 (in Russian).
- Hallegraeff G.M. (2003). In: *Manual on Harmful Marine Microalgae*, G.M. Hallegraeff, D.M. Anderson & A.D. Cembella (eds), UNESCO, Paris, pp. 25-50.
- Ivanov, A.I. (1982). Nauk Publ., Kiev, Ukraine (in Russian).
- Moestrup, Ø. (ed.) (2005). IOC Taxonomic Reference List of Toxic Algae, [ioc.unesco.org/hab/data.htm](http://ioc.unesco.org/hab/data.htm).
- Nesterova, D.A. (1979). *Biol. Sea* 5: 24-29 (in Russian).
- Nesterova, D.A. (2001). *Algology* 11: 502-513 (in Russian).
- Sayce, K. & Horner, R.A. (1996). In: *Harmful and Toxic Algal Blooms*, Yasumoto, T. & Oshima, Y. (eds), UNESCO, Paris, pp. 131-134.
- Terenko L. (2001). In: *The Port Biological Baseline Survey for the Port of Odessa GEF/UNDP/IMO Global Ballast Water Management Programme and the Government of Ukraine*, Zaitsev, Y.P. & Alexandrov, B.G. (eds), (Odessa, Ukraine), p. 168.
- Terenko, L.M. (2003). In: *Proc. 4th European Congr. Protistology and 10th European Conf. Ciliate Biology*, Cavallaro, G. & Luporini, P. (eds), San Benedetto del Tronto AP, Italy, pp. 126-127.
- Terenko, L. (2005). *Oceanol. Hydrobiol. Stud.* 34, Suppl. 3: 205-216.
- Terenko, L. (2006). In: *Proc. 25th Intl. Phycol. Conf.*, Burchardt, L. (ed.), Poznan, Poland, pp. 45-46.
- Terenko, L.M. & Kurilov, A.V. (2001). *Sci. Notes Ternopol State Univ., Hydroecol.*, Ternopil, Ukraine 3: 160-162 (in Russian).
- Terenko, L. & Terenko, G. (2005). In: *Proc. Intl. Conf., Centre of Excellence for Baltic Developmt., Educ. and Res., BALTDER*, (ed.), Gdansk, Poland, pp. 29-30.
- Zaitsev, Y.P. (1997). *GEF Black Sea Environmental Programme*, New York, p. 208.
- Zernov, S.A. (1913). *Acad. Sci. Phys. Mat. Br.* 32, 229 (in Russian).

## Distribution of dinoflagellate resting cysts in surface sediments from Changjiang River estuary before and during the spring bloom in 2004

Z. H. Wang, Y. Z. Qi and Y. F. Yang

Institute of Hydrobiology, Jinan University, Guangzhou, 510632, China, twzh@jnu.edu.cn

### Abstract

A dense *Prorocentrum donghaiense* bloom accompanied by *Alexandrium* sp., and covering more than 10,000 km<sup>2</sup>, occurred in southern Changjiang River estuary, East China Sea, in early May 2004. Surface sediments were collected to study cyst composition and distribution before and during the bloom. Only few differences in cyst composition were found in the two surveys. The average cyst concentrations in April and May were 374 and 482 cysts per gram dry weight sediment, respectively. Cysts of *Alexandrium* occurred commonly, but generally in low numbers, and the maximum concentration during the bloom was 219 cysts per gram dry weight sediment. High sand content in the sediments, high sedimentary rate and complex water currents, are thought to result in the low cyst concentrations. The fate of the *Alexandrium* cysts formed after the bloom is discussed.

### Introduction

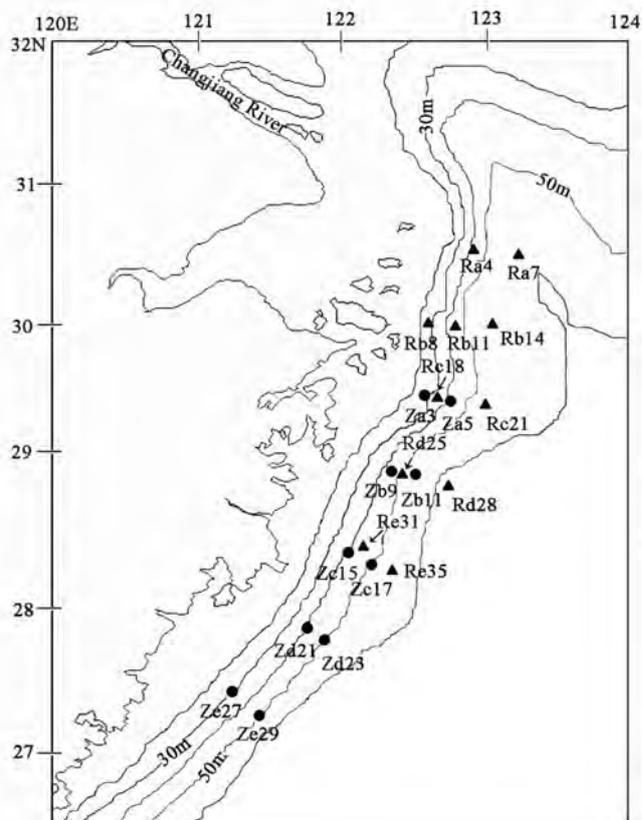
Changjiang River estuary is the largest estuary in China. The area is highly industrialized, and being the most densely populated region in China, there is heavy nutrient loading to the river and further into the estuary. Blooms caused by *Prorocentrum donghaiense* Lu (*P. dentatum* Stein), covering over 1,000 km<sup>2</sup> have occurred in this area almost in every spring since 1995 (Zhou *et al.* 2003).

Many dinoflagellates produce resting cysts as part of their life cycle and cyst formation is reported to play an important role in regulating the termination of blooms. A dense bloom of *P. donghaiense*, covering more than 10,000 km<sup>2</sup>, occurred in Changjiang River estuary in May 2004, concomitant with a bloom of *Alexandrium catenella* (Wang *et al.* 2004a). Two surveys were carried out in April and May of 2004, respectively, to study the formation of dinoflagellate cysts during the blooms.

### Materials and Methods

Ten and eleven stations, respectively, were sampled in the southern Changjiang River estuary (121.33°E-123.25°E, 27.27°N-30.50°N) along the coastline 2 - 6 April 2004 (April) and 28 April - 6 May 2004 (May). The stations and the water depths at the stations are shown in Fig. 1. Sediments were collected by a gravity corer, and surface sediments (0-2 cm) were sampled to analyze dinoflagellate cysts after sonication and sieving according to Matsuoka and Fukuyo (2000). Cells were studied under an inverted microscope (Leica DM IRB) at magnifications of 100-600 $\times$ . Only live

cysts with cellular contents were counted. For most samples, a minimum of 200 cysts were identified and counted. Cyst concentration is given as cysts per gram of dry weight sediment (cysts g<sup>-1</sup>).

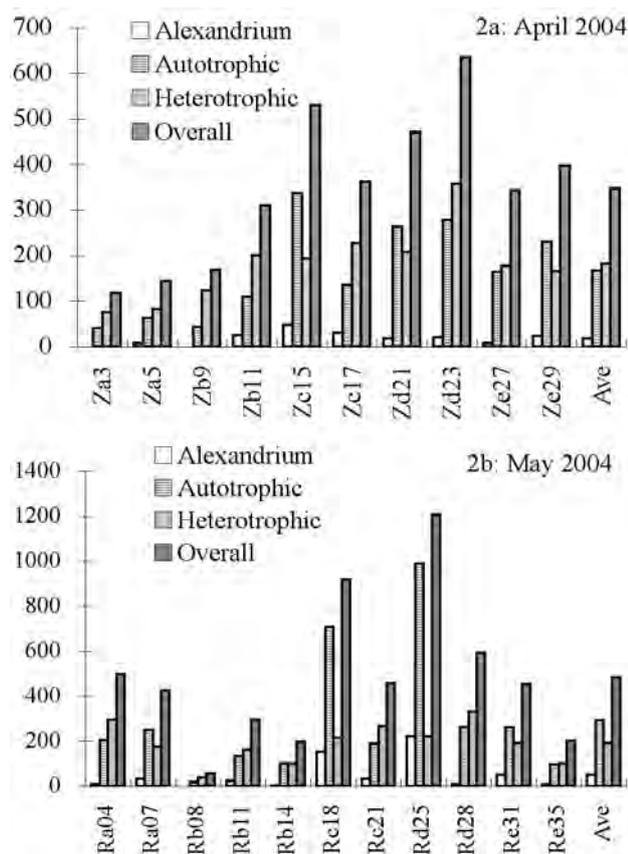


**Figure 1.** Sampling station in Changjiang River estuary, showing 20-60 m isobath. ●: Stations in April, 2004. ▲: Stations in May, 2004.

## Results

35 cyst types were identified in the surveys. No resting forms of *P. donghaiense* have been recognized so far (Wang *et al.* 2004a), suggesting that this species probably does not produce cysts. Cysts of heterotrophic dinoflagellates dominated the cyst assemblages in most samples. Cysts of *Protoperidinium* were the most abundant group with an average proportion of 48.1 % and 34.4 % in April and May, respectively. Cyst of *Scrippsiella trochoidea* was the most predominant autotrophic species, with a mean percentage of ca. 30. On average, cysts of *Gonyaulax* spp. and *Alexandrium* spp. constituted 5-10 % of total cyst assemblages.

Cyst concentrations ranged from 115 to 635 cysts g<sup>-1</sup> with an average of 374 cysts g<sup>-1</sup> in April. The concentrations were lower at stations near the river mouth such as Za3 and Za5, and increased at lower latitudes to reach a maximum at Zd23 near 28°N (Fig. 2a). Concentrations increased significantly in



**Figure 2.** Cyst concentrations of *Alexandrium* spp., autotrophic, heterotrophic and overall dinoflagellates in surface sediments from southern Changjiang River estuary in April and May 2004. 2a: April 2004, 2b: May 2004.

May, with maximum and average of 1206 and 482 cysts g<sup>-1</sup>, respectively (Fig. 2b). Cyst concentrations at Rc18 and Rd25 near 29°N were several times higher than in April.

Cysts of *Alexandrium catenella/tamarensis* occurred widely but in low numbers at most stations. The maximum concentration was 48 cysts g<sup>-1</sup> in April. Concentrations increased in May in the central area of the *Alexandrium* bloom near 29°N, where 153 and 219 cyst g<sup>-1</sup> in Rc18 and Rd25 were recorded, respectively.

## Discussion

Previous studies have reported low numbers of cysts in surface sediments from Changjiang River estuary (Cho and Matsuoka 2001; Wang *et al.* 2004a). The sampling stations in this study, which are located in the southern part of Changjiang River estuary, showed higher cyst abundance, but it was lower compared to other Chinese coastal areas (Wang *et al.* 2004b). High sand content in sediments, high sedimentary rate and complex water currents probably contributed to the low cyst abundance (Wang *et al.* 2004b).

Dinoflagellate cysts are formed following sexual reproduction, and high percentages of cyst formation have been observed during blooms of *A. tamarensis* elsewhere (20 % at Cape Cod, Anderson 1998; 30 % in northeastern Japan, Ichimi *et al.* 2001). During a bloom of *A. minutum* in northern Brittany, 40 % of the plankton population formed cysts (Probert *et al.* 2002). High cyst abundances have also been recorded in sediments from bloom areas such as Gulf of Maine, USA (Anderson *et al.* 2005) and Hiroshima Bay, Japan (Yamaguchi *et al.* 1995). Blooms of both *P. donghaiense* and *A. catenella* occurred during our second survey, with a maximum cell densities of 10<sup>7</sup> cells/L and 10<sup>5</sup> cells/L, respectively. However, the highest concentration of *Alexandrium* cysts was only 219 cyst g<sup>-1</sup> during the bloom, much lower than in other bloom areas. Low concentrations of *Alexandrium* cysts were also recorded after co-occurrence bloom of the two species in May 2002 in the same area (Wang *et al.* 2004a). This raises the question where the cysts formed during blooms accumulate?

The ellipsoidal cysts of *A. catenella/tamarensis* are distributed widely but at low concentrations along the Chinese coasts (Wang *et al.* 2004b). However, ellipsoidal *Alexandrium* cysts were observed in very high numbers (3,788 cyst g<sup>-1</sup>) in the central area of the

Yellow Sea, where no blooms of *Alexandrium* were recorded, and low PSP contents were detected nearby (Cho and Matsuoka 2001). The diluted water of the Changjiang River generally moves southeastward in the lower part of the river and then turns left to the northeast at the river mouth, in the direction of the central Yellow Sea (Le, 1986). The wind system in Changjiang River is predominated by the Southeast Monsoon during spring and summer. Thus, it is possible that cysts, formed during and after the *Alexandrium* blooms in the Changjiang River estuary, were transported northeastward by currents and settled down in the central Yellow Sea. Further studies are needed to verify this hypothesis and to understand the "seed bed" initiating the regularly recurring *Alexandrium* blooms.

#### Acknowledgments

The authors are thankful to Professor Mingjiang Zhou, Professor Mingyuan Zhu, Professor Songhui Lu, Professor Yahui Gao, Professor Douding Lu, and all the colleagues in 973 programme. This research project was supported by Chinese National Basic Research Priorities Programme (973): No. 2001CB409701, and by National Natural Science Foundation of China: No. 40306020.

#### References

- Anderson, D.M. (1998). *Limnol. Oceanogr.* 42: 1009-1002.
- Anderson, D.M., Stock, C.A., Keafer, B.A., Nelson, A.B., Thompson, B., McGillicuddy Jr. D.J., Keller, M., Matrai, P.A. & Martin, J. (2005). *Alexandrium fundyense* cyst dynamics in the Gulf of Maine. *Deep-Sea Research II* 52: 2522-2542.
- Cho, H.J. & Matsuoka, K. (2001). *Mar. Micropaleontol.* 42: 103-123.
- Ichimi, K., Yamasaki, M., Okumura, Y. & Suzuki, T. (2001). *J. Exp. Mar. Biol. Ecol.* 261: 17-29.
- Le, K.T. (1986). *Studia Marine Sinica* 27: 221-228.
- Matsuoka, K. and Fukuyo, Y. (2000). *Technical Guide for Modern Dinoflagellate Cyst Study*. WEST-PAC-HAB/WESTPAC/IOC, Japan Society of the Promotion Science, Tokyo.
- Probert, I., Lewis, J. & Erard-le Denn, E. (2002). *Cryptogamie* 23: 343-355.
- Wang, Z., Qi, Y., Lu, S., Wang, Y. & Matsuoka, K. (2004a). *Phycol. Res.* 52: 387-395.
- Wang, Z., Matsuoka, K., Qi, Y. & Chen, J. (2004b). *Mar. Ecol.* 25: 289-311.
- Yamaguchi, M., Itakura, S. & Imai, I. (1995). *Nippon Suisan Gakkaishi* 61: 700-706.
- Zhou, M., Yan, T. & Zou, J. (2003). *J. Appl. Ecol.* 14: 1031-1038.

## Role of short-term climate fluctuation the on outbreak of a large-scale dinoflagellate bloom along the east Chinese coast in 2005

M.J. Zhou<sup>1</sup>, M.Y. Zhu<sup>2</sup>, Y.F. Wang<sup>1</sup>, D.D. Zhu<sup>5</sup>, S.H. Lü<sup>4</sup>,  
D.D. Lu<sup>3</sup>, X.Y. Shi<sup>5</sup> and C.S. Zhang<sup>5</sup>

<sup>1</sup>Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China, mjzhou@ms.qdio.ac.cn, yfwang@ms.qdio.ac.cn, <sup>2</sup>First Institute of Oceanography, State Oceanic Administration, 6 Xixialing Road, Qingdao 266061, China, myzhu@public.qd.sd.cn, <sup>3</sup>Second Institute of Oceanography, State Oceanic Administration, 9 Xixihexia Road, Hangzhou 310012, China, ddzhu@sio.zj.edu.cn, dlu@mail.hz.zj.cn, <sup>4</sup>Jinan University, 601 West Huangpuadao Road, Guangzhou 510632, China, lvsh@jnu.edu.cn, <sup>5</sup>Ocean University of China, 5 Yushan Road, Qingdao 266003, CHINA, shixy@mail.ouc.edu.cn, zhangcs@mail.ouc.edu.cn

### Abstract

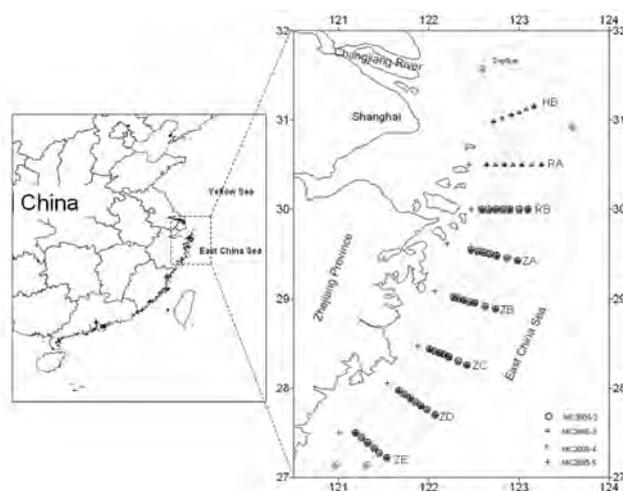
In 2005, as part of CEOHAB (Ecology and Oceanography of Harmful Algal Blooms in China) 4 cruises were carried out to further study the mechanisms of large-scale *Prorocentrum* blooms along the east Chinese coast, near the Changjiang River estuary and the Zhejiang coast. The blooms used to occur for several years in early May, but were delayed to the end of May in 2005. The data indicated that the delay was caused by the lower seawater temperature in early spring of 2005, which was unfavourable for *Prorocentrum* growth. It implied that short-term climate fluctuations can play an important role in the outbreak of large-scale dinoflagellate blooms along the east Chinese coast, and it should be taken into account in developing HAB prediction models for the area.

### Introduction

Large-scale dinoflagellate blooms along the east Chinese coast adjacent to the estuary of the Changjiang River were first reported in 2000. From then on, the bloom has appeared every year in early May, with the dominant species identified as *Prorocentrum donghaiense* (Lu *et al.* 2005). Supported by the Ministry of Science and Technology of China, a National Basic Research Priority Program (CEOHAB) was put into effect from 2002 to study the ecological and oceanographic mechanisms of the large-scale dinoflagellate blooms in this region and the potential approaches to bloom prediction and mitigation. Eight cruises were carried out 2002 - 2004 and a hypothesis on bloom formation was formulated that a suitable seawater temperature in the subsurface water column of about 0-20 m depth in early spring was a prerequisite for *P. donghaiense* to growth and proliferation into large-scale blooms in early May (Zhou and Zhu 2006). To validate the hypothesis, 4 cruises were organized to investigate the bloom area in the spring of 2005.

### Materials and Methods

The cruise stations in 2005 are shown in Fig. 1. Vertical profiles of water temperature, salinity and depth were recorded using a CTD (SBE 37) and the vertical profile of Chl.*a* was measured by a multi-parameter



**Figure 1.** Transects and stations of the cruises 2005.

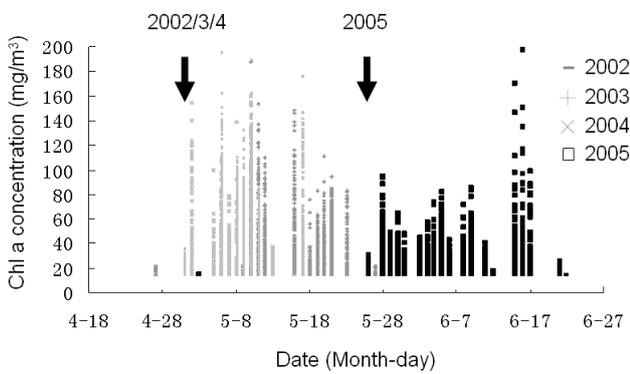
water quality sonde, YSI6600, at each station. Spatial distributions of water temperature, salinity and Chl.*a* were measured by another YSI6600 continuously during the cruises using a pumping system which could take seawater from about 1 m depth to a measuring chamber and remove most of air bubbles caused by the pumping. Both net and water phytoplankton samples were collected during the cruises and the algal species were identified and counted. Calibrations were made between the YSI readings of Chl.*a* and the phytoplankton counting. SST data were retrieved

from the dataset of [http://podaac.jpl.nasa.gov/pub/sea\\_surface\\_temperature/avhrr/pathfinder/data\\_v4.1](http://podaac.jpl.nasa.gov/pub/sea_surface_temperature/avhrr/pathfinder/data_v4.1)

**Results**

*Delayed outbreak of the dinoflagellate bloom*

The large-scale bloom of *P. donghaiense* occurred at the end of May in 2005, nearly 3 weeks later than in 2002-2004 (Zhou and Zhu 2006). The temporal distribution of Chl.a in the blooming area showed that proliferation of the algae began in early May in 2002-2004 but at the end of May in 2005 (Fig. 2). Since *Prorocentrum donghaiense* was the most dominant species (>90%, by cell counting), the level of Chl.a probably reflects the intensity of the *P. donghaiense* blooms.



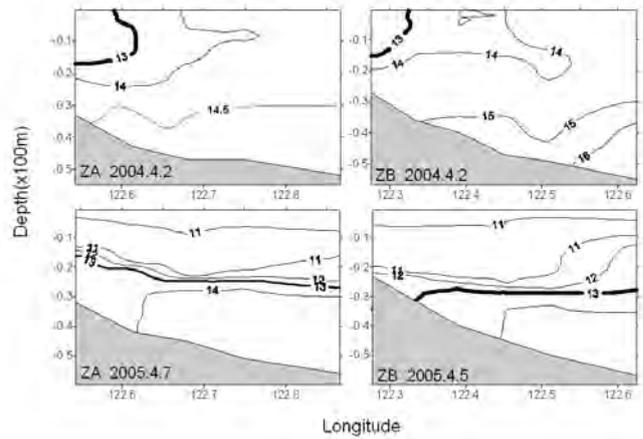
**Figure 2.** Chl.a levels recorded by YSI during the cruises indicated the delayed occurrence of *P. donghaiense* bloom in 2005 compared to 2002, 2003 and 2004 (*in situ* calibration indicated that a Chl.a level above 15mg/m<sup>3</sup> corresponds roughly to an algal cell density above 10<sup>6</sup>cells/L for *P. donghaiense* and could be used as the blooming index).

*Significant decrease of temperature*

Data recorded by CTD and YSI indicated that the seawater temperature in early spring 2005 was about 3 °C lower than in 2004, based on measurements of the vertical distribution of temperature (0-20 m depth) in two typical cruise transects (Fig. 3). The seawater temperature at 0-20m depth was only about 11 °C, compared to ~14 °C in 2004. The distribution pattern of satellite-measured sea surface temperature (SST, monthly average of March) agreed with the results from the field investigation (Fig. 4). The colder temperature is indicated by the different isolines of 13 °C.

**Discussion**

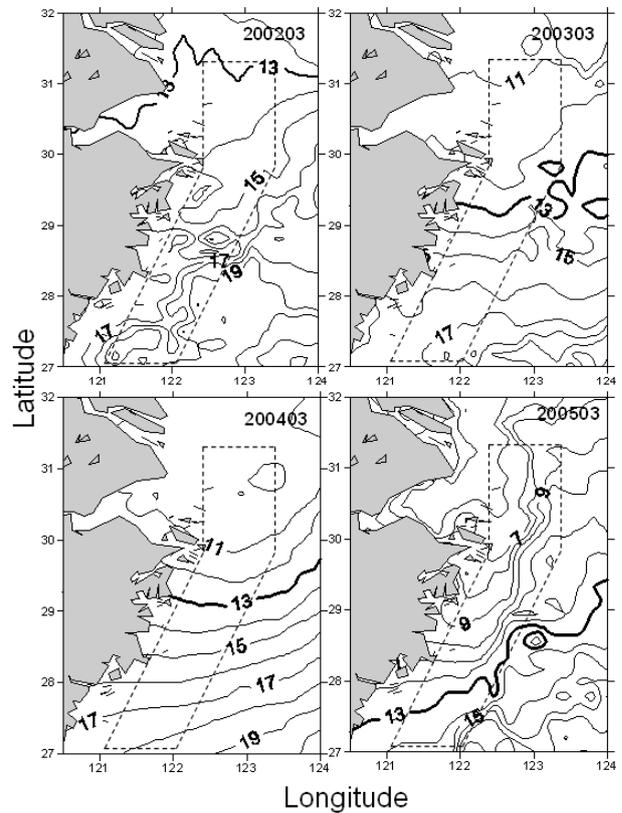
Previous investigations have indicated that an “incubation” stage of *P. donghaiense* in the subsurface layer (0-20m depth) in early spring is important for the



**Figure 3.** Comparison of seawater temperature in transects ZA and ZB 2004 and 2005.

outbreak of the large-scale bloom in early May. The relatively lower water temperature (<13 °C) made it impossible for *P. donghaiense* to grow. A laboratory study has shown that *P. donghaiense* can hardly grow below 13 °C (Chen *et al.* 2005).

The colder spring of 2005 can be considered a short-term fluctuation of climate change compared to the long-term data, in which an increasing trend of seawater temperatures was recorded (The International Ocean Database Management System for the



**Figure 4.** Comparison of SST in the investigation area in March, from 2002 to 2005.

China Seas and Adjacent Ocean, IOBMS, under development by Wang Fan). Peperzak (2003) indicated the importance of long-term climate change to HABs. The present study, however, emphasized that short-term climate fluctuation should also be taken into account, particularly in the effort of simulating and predicting annually recurring HABs.

#### **Acknowledgements**

The study was supported by the National Basic Research Priority Program 2001CB409700. The authors

would like to thank all participants in this project for their contribution.

#### **References**

- Lu, D.D., Goebel, J., Qi, Y.Z., Zou, J.Z., Han, X.T., Gao, Y.H. & Li, Y.G., (2005). Harmful Algae 4: 493–505.
- Chen, B.Z., Wang, Z.L., Zhu, M.Y. & Li, R.X. (2005). Adv. Mar. Sci. 23: 60-64 (in Chinese).
- Peperzak, L. (2003). Acta Oecol. 24(s1): 139-144.
- Zhou, M.J. & Zhu, M.Y. (2006). Adv. Earth Sci. 21: 673-679 (in Chinese).

## 8. TAXONOMY, BIOGEOGRAPHY



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## HAB-MAPS of toxic marine microalgae in coastal and shelf waters of South America

R. Akselman<sup>1</sup>, B. Reguera<sup>2</sup> and M. Lion<sup>2</sup>

<sup>1</sup>Instituto Nacional de Investigación y Desarrollo Pesquero, Mar del Plata, Argentina, rutaks@inidep.edu.ar;

<sup>2</sup>IOC-IEO Science and Communication Centre on Harmful Algae, Instituto Español de Oceanografía, Centro Oceanográfico de Vigo, Spain, beatriz.reguera@vi.ieo.es; monica.lion@vi.ieo.es

### Abstract

Geo-referenced distributions of potentially toxic microalgal species in coastal and shelf waters of South America have been created as part of the HAB-MAP project of the International Society for the Study of Harmful Algae (ISSHA). A total of 40 potentially toxic species - 9 diatoms, 23 dinoflagellates, 3 haptophytes and 5 raphidophytes - were recorded. The total number of toxic species could be greater than the apparent one because of dubious taxonomic identifications of some taxa, and low frequency of sampling in large areas of South America.

### Introduction

Information on the distribution of harmful microalgal species and their changes over time is essential to evaluate if harmful events are expanding/contracting in a given region, and to interpret dispersal routes. This information is also useful for risk assessment and planning of the exploitation of marine resources (Zingone and Wyatt 2004). Against this background, it is important to coordinate information at a regional scale to have reliable taxonomic knowledge and study the mechanisms underlying bloom dynamics.

The HAB-MAP project was developed under the guidance of ISSHA, and its main objective is to establish updated geo-referenced maps of the known distributions of harmful algae species. It is based on a literature overview, and will compile regional summaries of toxic marine microalgae from 11 regions of the world ocean. Future plans include publication of HAB-MAP on-line and on a CD-ROM. HAB-MAP registers the occurrence of toxigenic species, and complements the IOC-ICES Harmful Algae Events Database (HAEDAT). Species names and synonyms are according to those adopted by the IOC Taxonomic Reference List of Toxic Plankton Algae (Moestrup 2005). Maps for the Mediterranean Sea (Zingone *et al.* 2004) have been already produced. Here we present HAB-MAP activities for coastal and shelf waters of six countries of South America on the basis of a literature review of all available published papers and reports for the region.

### Materials and Methods

The geographic region considered in this work includes Brazil, Uruguay, Argentina, Chile, Peru and Ecuador.

Other South American countries, viz. Colombia, Venezuela, French and English Guyanas and Surinam, are included in the IOCARIBE region. Over 150 publications and reports from the region on toxigenic species included in the IOC Taxonomic Reference List were reviewed. Cyanobacteria were not considered. Reference lists were prepared for each species and country. The database was developed with Excel files for each species; different worksheets provided datasets extracted from each bibliographic reference. Factors considered included geo-referenced location (to allow for the generation of distribution maps), date, cell abundance, toxin content, harmful effects and relevant environmental information.

### Results

A total of 40 potentially toxigenic species - 9 diatoms, 23 dinoflagellates, 3 haptophytes, 5 raphidophytes - were recorded (Table 1). The real number could be greater than the apparent one because of dubious taxonomic identifications of some taxa, as is the case in the dinoflagellate genus *Karenia*, and low frequency of sampling in large areas of South America. Reported species included PSP, DSP and ASP-toxin producers. The regional distribution of *Alexandrium tamarense* and *A. catenella* are presented as examples of HAB-MAP applications that can be generated with the available information in the database. These species are without doubt the most threatening HAB species, both for human health and shellfish resources, in the six South American countries considered in this study. *A. tamarense* occurs on the Atlantic coasts, whereas *Alexandrium catenella* is recorded only on the Pacific coasts (Fig. 1).

**Table 1.** Toxic species registered at the South America database

## Diatoms

*Pseudo-nitzschia australis* Frenguelli*Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle*Pseudo-nitzschia delicatissima* (P.T. Cleve) Heiden*Pseudo-nitzschia fraudulenta* (P.T. Cleve) Hasle*Pseudo-nitzschia multiseriata* (Hasle) Hasle*Pseudo-nitzschia multistriata* (Takano) Takano*Pseudo-nitzschia pungens* (Grunow ex P.T. Cleve) Hasle*Pseudo-nitzschia seriata* (P.T. Cleve) H. Peragallo*Pseudo-nitzschia turgidula* (Hustedt) Hasle

## Dinoflagellates

*Alexandrium acatenella* (Whedon & Kofoid) Balech*Alexandrium catenella* (Whedon & Kofoid) Balech*Alexandrium monilatum* (Howell) Balech*Alexandrium ostenfeldii* (Paulsen) Balech & Tangen*Alexandrium tamarense* (Lebour) Balech*Dinophysis acuminata* Claparède & Lachmann*Dinophysis acuta* Ehrenberg*Dinophysis caudata* Saville-Kent*Dinophysis fortii* Pavillard*Dinophysis mitra* (Schütt) Abé vel Balech*Dinophysis rapa* (Stein) Balech*Dinophysis rotundata* Claparède & Lachmann*Dinophysis sacculus* Stein*Dinophysis tripos* Gourret*Gymnodinium catenatum* Graham*Karenia mikimotoi* (Miy. & Kom. ex Oda) G. Hansen & Moestrup*Ostreopsis ovata* Fukuyo*Pfiesteria piscicida* Steidinger & Burkholder*Pfiesteria shumwayae* Glasgow & Burkholder*Prorocentrum lima* (Ehrenberg) Stein*Prorocentrum minimum* (Pavillard) Schiller*Protoceratium reticulatum* (Clap. & Lachm.) Bütschli*Protoperidinium crassipes* (Kofoid) Balech

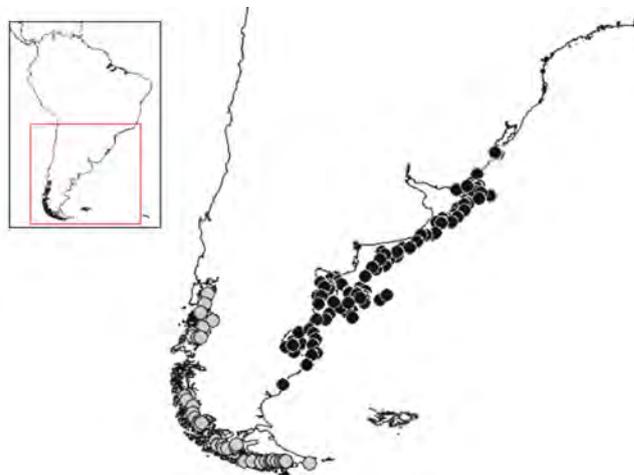
## Haptophytes

*Phaeocystis globosa* Scherffel*Phaeocystis pouchetii* (M.P. Hariot) G. Lagerheim*Prymnesium parvum* N. Carter

## Raphidophytes

*Chattonella globosa* Y. Hara & Chihara*Chattonella marina* (Subrahmanyam) Hara & Chihara*Chattonella subsalsa* Biecheler*Fibrocapsa japonica* Toriumi & Takano*Heterosigma akashiwo* (Hada) Hada ex Hara & Chihara

*References:* Akselman *et al.* 1998; Avaria 1979; Ferrario *et al.* 1999; Gayoso *et al.* 2002; Jiménez & Intriago 2001; Ochoa *et al.* 1999; Odebrecht & Abreu 1995; Proença *et al.* 2001; Santinelli *et al.* 1995; Torres-Zambrano 2001; Villac & Tenenbaum 2001.

**Figure 1.** Distribution of *Alexandrium tamarense* (black circles) and *A. catenella* (grey circles) in South America.

*A. tamarense* occurs over a large geographic range - from latitude 49°15' S in Argentina to 32°10' S in Brazil (Carreto *et al.* 1998; Méndez and Ferrari 2002; Persich *et al.* 1998). This species, first detected in 1980, may form very dense blooms (red tides) associated with frontal areas off Argentina and Uruguay, and has been the causative agent of dramatic PSP events that included fatal human cases and mass mortalities of marine fauna (Carreto *et al.* 1981; Méndez and Ferrari 2002; Montoya *et al.* 1996). The northernmost occurrence of *A. tamarense* in the region coincides with recent reports from Southern Brazilian shelf waters (Persich *et al.* 1998).

*A. catenella* was first reported in the 1970s from Magallanes, in the southernmost fjord region in Chile (Guzmán *et al.* 2002). Since then, a northwards expansion of this species has been detected. Distribution according to existing and confirmed records is from latitude 54°56' S, including the Beagle Channel, to 44°23' S (Benavides *et al.* 1995; Lembeye 1981; Muñoz *et al.* 1992). PSP toxicity associated with this species in southern Chile and Argentina caused human mortality and record levels (127 · 103 µg equiv. STX · 100 g<sup>-1</sup>) of PSP toxins in shellfish (Benavides *et al.* 1995).

## Acknowledgements

This work would not have been possible without the help of our colleagues from Argentina (M. Ferrario, R. Negri, N. Santinelli, V. Sastre), Brazil (M. Menezes, C. Odebrecht, L. Proença, M.C. Villac), Chile (P. Muñoz, G. Pizarro), Ecuador (G. Torres), Peru (S. Sánchez Ramírez) and Uruguay (S. Méndez), and fruitful discussions during regional IOC-FANSA WG meetings. We express our gratitude to A. Zingone and H. Enevoldsen, editors of the ISSHA HAB-MAP database, to the IOC of UNESCO for financial support, and to Cristina Sexto for continuous technical support at the IOC-IEO Science and Communication Centre on Harmful Algae in Vigo.

## References

- Akselman, R., Carreto, J.I. & Montoya, N.G. (1998). In: Harmful Microalgae, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T., (eds), Xunta de Galicia and IOC of UNESCO, Santiago de Compostela, pp. 122-123.
- Avaria, S. (1979). In: Toxic Dinoflagellate Blooms, Taylor, D.L. & Seliger, H.H., (eds), Elsevier, North Holland, pp. 161-164.
- Benavides, H.R., Prado, L., Díaz, S. & Carreto, J.I. (1995). In: Harmful Marine Algal Blooms, Lasso, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou, C., (eds), Lavoisier-Intercept Ltd, Paris, pp. 113-119.
- Carreto, J.I., Lasta, M., Negri, R.M. & Benavides, H.R. (1981). Contr. INIDEP 399, 55 pp., plus tables and plates.
- Carreto, J.I., Montoya, N.G., Cucchi Colleoni, A.D. & Akselman, R. (1998). In: Harmful Microalgae, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T., (eds), Xunta de Galicia and IOC of UNESCO, Santiago de Compostela, pp. 131-134.
- Ferrario, M., Sar, E., Castaños, C. & Hinz, F. (1999). Nova Hedw. 68: 131-147.
- Gayoso, A.M., Dover, S., Morton, S., Busman, M., Moeller, P., Fulco, V.K. & Maranda, L. (2002). J. Shellfish Res. 21: 461-463.
- Guzmán, L., Pacheco, H., Pizarro, G. & Alarcón, C. (2002). In: Floraciones Algales Nocivas en el Cono Sur Americano, Sar, E., Ferrario, M. & Reguera, B., (eds), Inst. Español Oceanogr., pp. 237-256.
- Jiménez, R. & Intriago, P. (2001). Harmful Algae News, IOC-UNESCO, 22, p. 7.
- Lembeye, G. (1981). Anales del Instituto de la Patagonia (Chile) 12: 273-276.
- Méndez, S. & Ferrari, G. (2002). In: Floraciones Algales Nocivas en el Cono Sur Americano, Sar, E., Ferrario, M. & Reguera, B., (eds) Inst. Esp. Oceanogr., Vigo, pp. 271-288.
- Moestrup, Ø., ed. (2005). At: IOC Taxonomic Reference List of Toxic Algae, [ioc.unesco.org/hab/data.htm](http://ioc.unesco.org/hab/data.htm)
- Montoya, N.G., Akselman, R., Franco, J. & Carreto, J.I. (1996). In: Harmful and Toxic Algal Blooms, Yasumoto, T., Oshima, Y. & Fukuyo, Y., (eds), IOC of UNESCO, Sendai, pp. 417-420.
- Muñoz, P., Avaria, S., Sievers, H. & Prado, R. (1992). Rev. Biol. Mar. Valparaíso 27: 187-212.
- Ochoa, N., Gómez, O., Sánchez, S. & Delgado, E. (1999). Bol. IMARPE 18, 1-14.
- Odebrecht, C. & Abreu, P.C. (1995). Harmful Algae News, IOC-UNESCO, 12/13, p. 4.
- Persich, G.R., Garcia, V.M. & Odebrecht, C. (1998). In: XI Semana Nacional de Oceanografía, Rio Grande (Editora e Gráfica Universitária UFPel, Brasil), pp. 261-263.
- Proença, L.A.O., Tamanaha, M.S. & Souza, N.P. (2001). Atlântica, Rio Grande 23: 59-65.
- Santinelli, N., Caille, G. & Lettieri, A. (1995). Harmful Algae News, IOC-UNESCO, 9: 6.
- Torres-Zambrano, G. (2001). Acta Oceanográfica del Pacífico (INOCAR, Ecuador) 10: 127-136.
- Villac, M.C. & Tenenbaum, D.R. (2001). In: Harmful Algal Blooms, Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J., (eds), IOC-UNESCO, Paris, pp. 34-37.
- Zingone, A., Montresor, M., Sacchi, U., Maio, G. & Ismael, A.A. (2004). In: Book of Abstracts of the XI International Conference on Harmful Algal Blooms, Cape Town, South Africa, p. 264.
- Zingone, A. & Wyatt, T. (2004). In: The Global Coastal Ocean: Multi-Scale Interdisciplinary Processes, Robinson, A.R. & Brink, K.H., (eds), Harvard Univ. Press, USA, pp. 867-926.

## Phytoplankton distribution, diversity and nutrient variations at the West Coast of Sweden, with special reference to harmful algae

A. Yousif Al-Handal\*, B. Karlson, L. Edler and A-T. Skjevik

Swedish Meteorological and Hydrological Institution, Nya Varvet 31, 426 71, V. Frölunda, Sweden,

\* adil.yousif@smhi.se

### Abstract

Species composition, abundance and distribution of phytoplankton as well as some major physico-chemical features and chlorophyll *a* concentration were investigated at three stations (Kosterfjord, Koljöfjord and Dana fjord) along the Swedish Skagerrak coast. Diatoms, dinoflagellates and other flagellates dominate phytoplankton populations. Highest abundance was in Koljöfjord, reaching  $3.32 \times 10^6$  cells  $L^{-1}$  in May 2005, with diatoms constituting 69.7 %. Lowest abundance was in Kosterfjord in March 2006, when cell densities dropped to  $11.7 \times 10^3$  cells  $L^{-1}$ . Diatoms were exceptionally scarce at this station in May 2005 ( $0.15 \times 10^3$  cells  $L^{-1}$ ) accompanied by a dominance of the harmful dinoflagellate *Dinophysis norvegica* and cryptophytes. Dinoflagellates reached the highest abundance of  $1.18 \times 10^5$  cells  $L^{-1}$  in Koljöfjord in August 2005. Surface chlorophyll *a* reached a minimum of 1.1  $\mu g/l$  in April 2005 (Danafjord) and a maximum of 8.1  $\mu g/l$  in September (Kosterfjord). Harmful algal taxa known from this region include species of *Alexandrium*, *Dinophysis*, *Pseudo-nitzschia* and *Chattonella*.

### Introduction

The West Coast of Sweden (Kattegat-Skagerrak) is an archipelago of rocky shores and fjords (Fig. 1). Salinity is rather variable owing to continuous mixing with Baltic Sea water that enters from the south, and with freshwater from Göta river, which averages  $500 \text{ m}^3 \text{ s}^{-1}$  (Lindström 2002). Freshwater discharge eutrophicates the coastal water, leading to high phytoplankton production. Nutrient concentrations, particularly P-phosphate and N-nitrate, increases from autumn onwards, following water disturbance and vertical mixing. The Water Quality Association of the Bohus coast with the collaboration of SMHI carries out regular, long-term phytoplankton monitoring programs from which the data of the present work were used. Three stations were selected; Kosterfjord in the north, Koljöfjord in the middle and Danafjord in the southern part of the coast.

### Material and Methods

Net and tube samples (0-10m depth) were collected monthly from each station. The net samples were examined before fixation within 24 h. Counting of cell numbers was by the Utermöhl technique (Utermöhl 1958), using 10- or 20-ml sedimentation chambers. Water samples for nutrients were collected using Niskin-type bottles from 0 m depth. Nutrients were analyzed according to Grasshoff *et al.* (1999) and chlorophyll *a* concentrations were determined according to Edler (1979).

### Results and Discussion

Water temperature fluctuated between a minimum of  $-2 \text{ }^\circ\text{C}$  in March and a maximum of  $19.5 \text{ }^\circ\text{C}$  in July (Fig. 2). Salinity ranged between 15.6 and 28.9 psu (Fig. 3). Lowest oxygen values were in summer (Fig. 4) coinciding with high temperature readings. Nitrate and phosphate values ranged between 0.1 and  $12.9 \mu\text{mol/l}$ , and between 0.02 and  $0.87 \mu\text{mol/l}$ , respectively (Figs 5, 6). Chlorophyll *a* reached a minimum of  $1.1 \mu\text{g/l}$  in April 2005 in Danafjord and a maximum of  $8.1 \mu\text{g/l}$  in September in Kosterfjord (Fig. 7).

A total of 148 species were encountered, of which 70 belong to Bacillariophyceae and 59 to Dinophyceae. Species occurrence was rather similar at the three stations. The most common taxa, which appeared most of the year, were the diatoms *Chaetoceros* spp., *Rhizosolenia* spp., *Proboscia alata*, *Thalassiosira* spp. and *Pseudo-nitzschia* spp. Dinoflagellates were most commonly represented by *Ceratium* spp., *Protoperidinium* spp., *Gyrodinium* spp. and *Dinophysis* spp. Cell densities reached a peak during April-June at all stations, with a maximum of  $3.32 \times 10^6$  cells  $L^{-1}$  at Kosterfjord. Lowest values were recorded during winter (Fig. 8). Although diatoms and dinoflagellates were most species rich, their contribution to the total cell counts were low (Fig. 9). The small flagellates, particularly cryptophytes and prasinophytes, as well as many other unidentified nano- and picoplankton, were the major contributors. The Utermöhl counting method used (Utermöhl 1958) did not allow for accu-

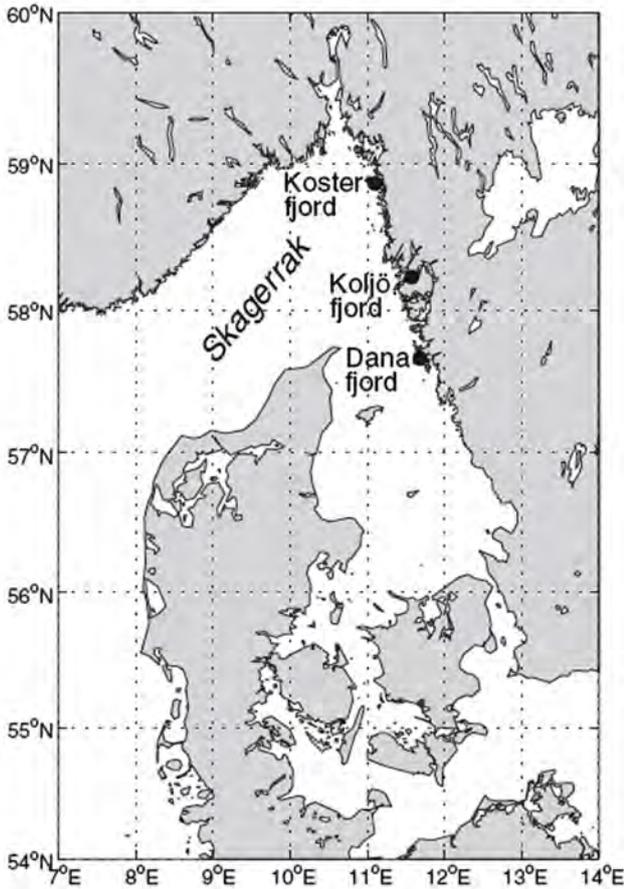


Figure 1. West Coast of Sweden with sampling locations indicated.

rate estimate of picoplankton density, but it has been shown previously (Kuylenstierna and Karlson 1994) that they may reach a peak of  $250 \times 10^6$  cells  $L^{-1}$  on the West Coast of Sweden.

The West Coast is subjected to occasional blooms of toxic algae, causing fish mortality and shellfish contamination. The first harmful bloom of *Chattonella* aff. *verruculosa* occurred in 1998 and caused mortality of both wild and cultivated fish (Karlson and Anderson 2003). Similar incidents were reported in 2001. *Dinophysis* spp. however, have for many years caused DSP in blue mussels. The fjord nature of the coast enhances cyst formation of several toxic dinoflagellates, that may lead to harmful blooms (Godhe and McQuoid 2003). The toxic species reported in this study include *Alexandrium tamarense*, *Dinophysis acuminata*, *D. acuta*, *D. dens*, *D. norvegica*, *Lingulodinium polyedrum*, *Phalacroma rotundatum*, *Fragilidium* spp., *Protoperidinium crassipes*, *Chattonella* aff. *verruculosa* and *Pseudo-nitzschia* spp. During April 2005-March 2006 (this study), no harmful blooms were observed.

As shown in Fig. 10, *Dinophysis norvegica* occurred all the year round at all stations, but it never

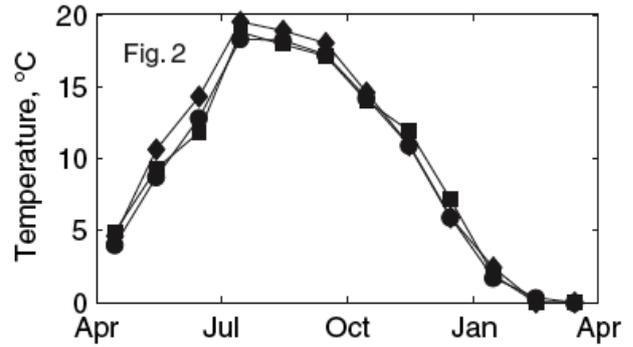


Figure 2. Monthly variations of temperature 2005-06.

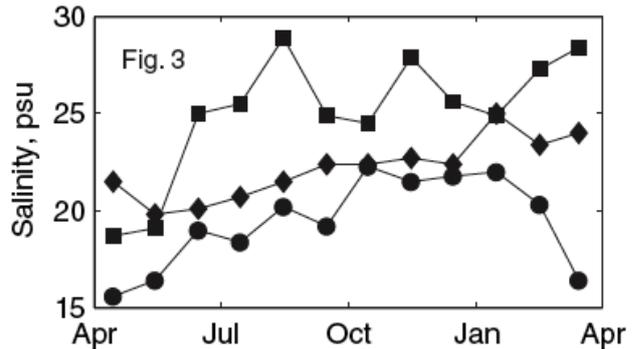


Figure 3. Monthly variations of salinity 2005-06.

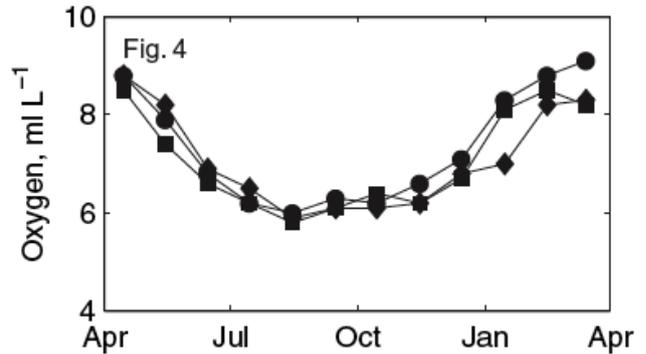


Figure 4. Monthly variations of oxygen 2005-06.

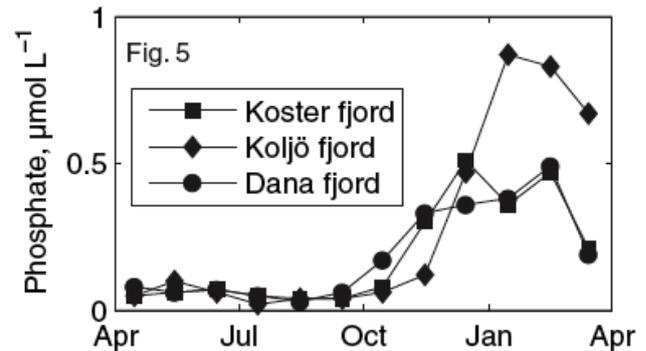


Figure 5. Monthly variations of phosphate 2005-06.

formed a harmful bloom. The highest cell density of  $1325$  cells  $L^{-1}$  was in August at Koljöfjord, and this cell density is below its critical limit of  $2000$  cells  $L^{-1}$ , as recommended by the Swedish National Food Administration, but considered rather high. The relatively high numbers of *Dinophysis* spp. coincided with sharp declines in both occurrence and cell densities of

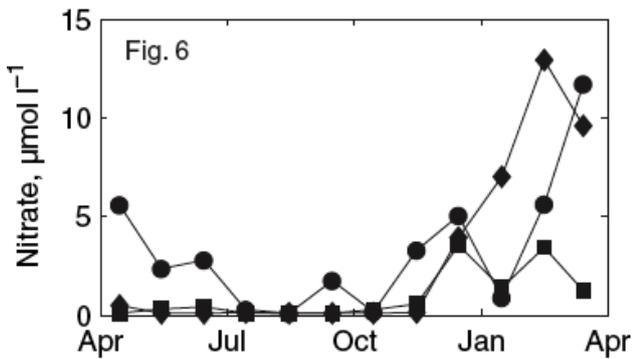


Figure 6. Monthly variations of nitrate 2005-06.

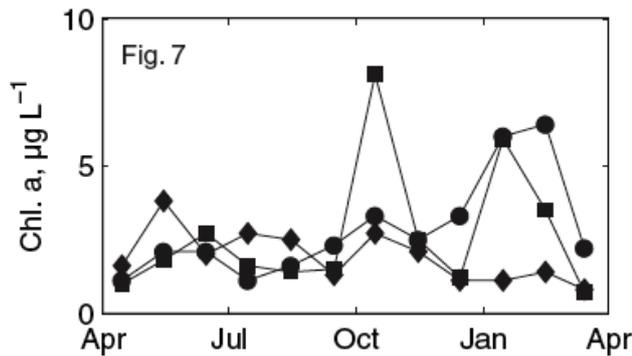


Figure 7. Monthly variations of chlorophyll a 2005-06.

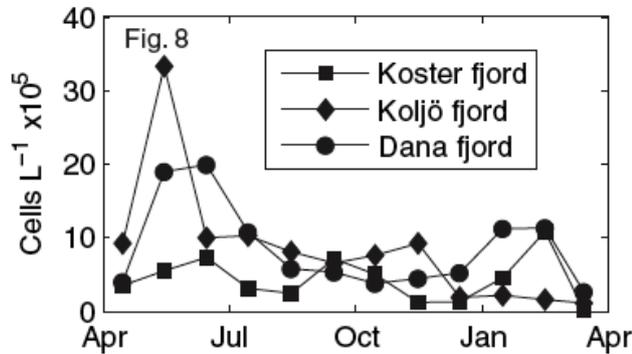


Figure 8. Monthly variations of total phytoplankton abundance 2005-06.

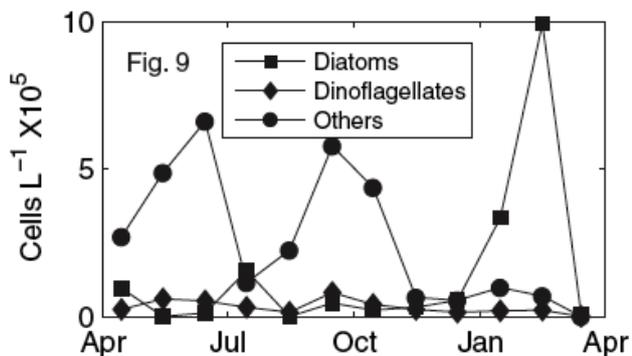


Figure 9. Monthly variations of the average abundance of diatoms, dinoflagellates and other phytoplankton 2005-06.

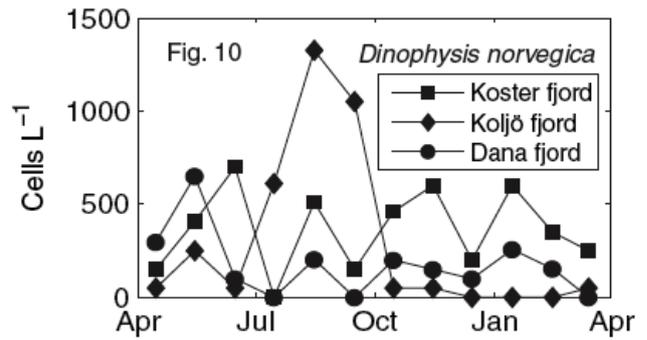


Figure 10. Monthly variations of the abundance of *Dinophysis norvegica* 2005-06.

diatoms. This was particularly evident in May, June and August (Figs 9, 10) and requires further investigation. *Dinophysis* spp. may have an allelopathic effect on other phytoplankton, and diatoms may be more sensitive to this effect. Laboratory experiments on the allelopathic effect of *Dinophysis* spp. are not yet feasible as keeping this species in cultures is difficult.

*Chattonella* aff. *verruculosa* reached high cell densities along the Danish coasts in spring 2006 ( $6 \times 10^6$  cells  $L^{-1}$ , Per Andersen, pers. comm.) and resulted in harmful effects on farmed fish, yet the cell densities were rather low along the Swedish coasts. The highest cell numbers of  $145 \times 10^3$  cells  $L^{-1}$  were encountered in March at Dana fjord but it appeared in small numbers at all stations during spring.

**Acknowledgments**

Sampling and analysis were made by SMHI on commission from the Water Quality Association of the Bohus coast.

**References**

Edler, L. (1979). Recommendations for marine Biological Studies in the Baltic Sea. Phytoplankton and chlorophyll. Baltic Marine Biologists (publication No. 5).

Godhe, A. & McQuoid, R. (2003). Aquat. Microb. Ecol. 32: 185-201.

Grasshoff, K., K. Kremling & M. Erhardt (eds) (1999). Methods of Seawater Analysis. 3rd ed. Wiley-VCH.

Karlson, B. & Andersson, L. (2003). The *Chattonella* bloom in year 2001 and effects of high freshwater input from river Göta to the Kattegat-Skagerrak area. Rep. No.32, SMHI.

Kuylenstierna, M. & Karlson, B. (1994). Bot. Mar. 37: 17-33.

Lindström, G. (2002). Vattentillgång och höga flöden i Sverige under 1900-talet. Reports Hydrology No 18, SMHI.

Utermöhl, H. (1958). Mitt int. Ver. ther. angew. Limnol. 9: 1-38.

## Morphological and phylogenetic description of an unusual *Amphidinium* (Dinophyceae) species

T. Cyronak<sup>1</sup> and C. Tomas<sup>2</sup>

<sup>1,2</sup>Center for Marine Science, 5600 Marvin K. Moss Ln., Wilmington, NC 28409,

<sup>1</sup> tjc4596@uncw.edu, <sup>2</sup>tomasc@uncw.edu

### Abstract

*Amphidinium carterae*, an important harmful algal species that produces powerful antifungal and hemolytic compounds (amphidinols) and cytotoxic macrolides (amphidinolides) is ubiquitous in coastal waters. Samples from coral rubble contained an unusual and previously unreported *Amphidinium* (D2) with a circular outline. Genetic analysis of clone D2 of this species, involving the sequencing of large subunit (LSU) rDNA, revealed a relationship between *Amphidinium* sp. D2 and both *A. carterae* and *A. massartii*. However, morphological and genetic differences suggest that *Amphidinium* sp. D2 is not conspecific with *A. carterae* or *A. massartii*. Further studies to describe this species are presently underway.

### Introduction

*Amphidinium* Claparède & J. Lachmann is a genus of epibenthic dinoflagellates with approximately 120 described species (Murray and Patterson 2001). The concept of the genus has recently been narrowed by Flø Jørgensen *et al.* (2004), to the exclusion of many species, including all known freshwater species (Calado and Moestrup 2005). Hypotheses used to explain past systematic confusion about the species within *Amphidinium sensu stricto* included the concept of an *Amphidinium operculatum* Claparède & J. Lachmann species complex as well as the notion that there were many forms of *A. operculatum* (Barlow and Triemer 1988; Al-Qassab *et al.* 2002; Murray and Patterson 2002; Murray *et al.* 2004). Murray *et al.* (2004) used phylogenetic analysis combined with morphological descriptions to show at least 9 distinct species within *Amphidinium sensu stricto*.

Within the genus *Amphidinium* the ubiquitous *Amphidinium carterae* Hulbert is probably the most studied species. Along with *A. klebsii* Kofoid & Swezy (= *A. gibbosum sensu* Murray *et al.* 2004), *A. carterae* is known to produce amphidinols, compounds having both antifungal and hemolytic properties and cytotoxic macrolides called amphidinolides (Satake *et al.* 1991; Ishibashi and Kobayashi 1997; Echigoya *et al.* 2005). Ecologically, the role of *Amphidinium* species as primary producers is likely to be significant in coastal, benthic communities (Flø Jørgensen *et al.* 2004).

The defining morphological characteristic of the genus *Amphidinium* is an epicone smaller than the hypocone; within *Amphidinium sensu stricto* the epi-

cone is minute, triangular or crescent-shaped, and deflected to the left. Morphological characters used to differentiate species include epicone size, presence or absence of plastids, plastid appearance, positioning of the sulcus and cingulum, and general cellular proportions (Flø Jørgensen *et al.* 2004). *Amphidinium* species within the smallest size range (10–20 x 7–17 μm) include *A. carterae* and *A. massartii* Biecheler. The two species are similar in size and appearance and are often difficult to differentiate using light microscopy (Murray *et al.* 2004).

The objective of this study is the identification to the species level of the two clonal isolates of *Amphidinium* (D1 and D2) which have a circular morphology. Morphological and phylogenetic results were compared to known cultures of *A. carterae* and *A. klebsii*, as well as data from Murray *et al.* (2004).

### Materials and Methods

All cultures of *Amphidinium* species were established using single cell pipette isolation and maintained non-axenically. *Amphidinium* clones D1 and D2 were isolated from a sample of live rock obtained at a local aquarium store and *A. carterae* UNCW and *A. klebsii* UNCW were both isolated from water samples taken in the Bahamas. All cultures were maintained in K media (Keller and Guillard 1987) at 39‰ and 28 °C on a 14:10 h L:D cycle.

Micrographs were obtained using a Zeiss Axio Imager Z1. Images for measurements were taken on a Nikon Diaphot inverted microscope and measured in Axio Vision v4.5.0.0. Chloroplast epifluorescence was observed using a Rhodamine filter.

**Table 1.** Morphometric variation among species of *Amphidinium*

Species	n	Length ( $\mu\text{m}$ )				W/L					
		Maximum	Minimum	Mean	SD	Maximum	Minimum	Mean	SD		
<i>A. massartii</i> *	-	19	13	16	1.8	15	8	12	1.8	0.77	-
<i>A. carterae</i> *	-	16	11	14	1.2	11	7	9	1.0	0.67	-
<i>A. carterae</i> UNCW	50	18	12	15	1.5	13	8	10	1.2	0.67	0.05
<i>Amphidinium</i> sp. D2	102	15	10	12	1.1	14	10	12	1.0	0.97	0.05

\*From Murray *et al.* 2004.

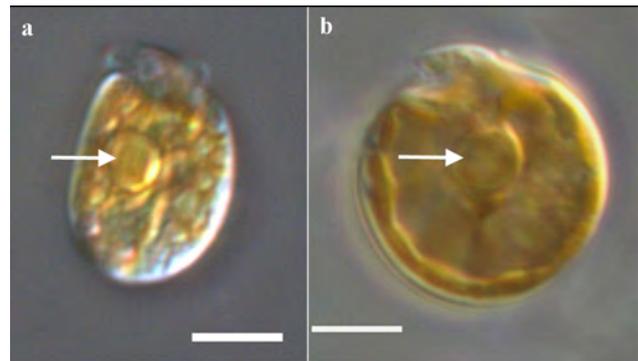
A volume of 8-10 ml of culture was centrifuged at 2,000 rpm for 15 min. The resulting pellet was frozen in liquid nitrogen and kept frozen in a  $-80\text{ }^{\circ}\text{C}$  freezer until DNA extraction. Extraction of genomic DNA was done using a Power Soil DNA kit (MoBio Labs, Inc.). PCR of the LSU rDNA gene was performed with previously published primers (Flø Jørgensen *et al.* 2004). LSU rDNA, sites D1-D6, was sequenced using internal primers (D1R, D3B, D3AC, D2C, D2Ra, and 1483R) and read on an Applied Biosystem's Hitachi 3100 Genetic Analyzer (Tokyo, Japan).

Sequences were assembled using the program Sequencher v4.6 and aligned using Clustal W (European Bioinformatics Institute). Aligned sequences were proofread using MacClade v4.06. The hypervariable D2 region was removed according to the alignment of Murray *et al.* (2004) resulting in sequences of 1189 base pairs.

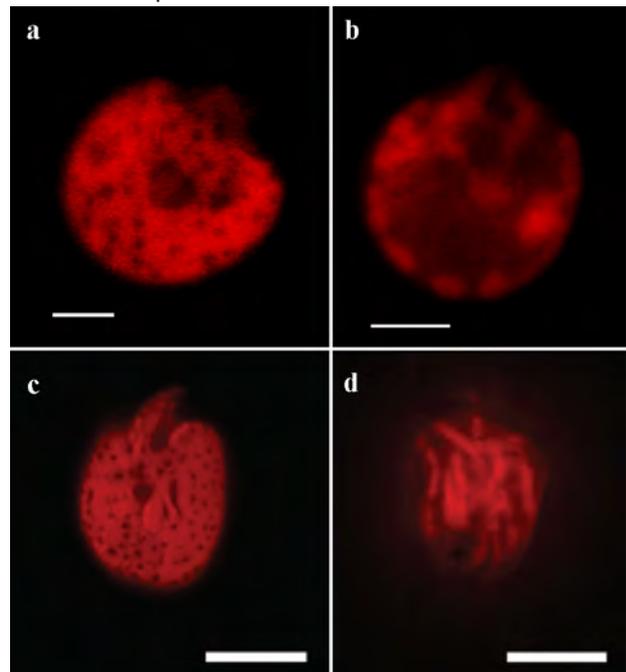
Maximum likelihood analysis was done with PAUP v4.0b10 (Swofford 2000) using heuristic searches with 10 random addition replicates and a TBR branch swapping algorithm. The optimized model chosen by Modeltest version 3.06 (Posada and Crandall 1998) was a general time reversal (GTR+I+G). The specific parameters were a rate matrix of (1, 2.8547, 1, 1, 6.2407, 1), proportion of invariable sites = 0.1811, gamma distribution shape  $a=0.6863$ , and nucleotide frequencies of  $a=0.2585$ ,  $c=0.196$ ,  $g=0.288$ ,  $t=0.2567$ . Bootstrap values were obtained using heuristic searches and 100 replicates.

## Results

Width and length measurements of *Amphidinium* sp. D2 overlap in size range with both *A. carterae* and *A. massartii*. The length to width ratio for *A. carterae* and *A. massartii* ranges from 0.67 to 0.77, whereas in the almost circular *Amphidinium* sp. D2 it is 0.97 (Table 1). *Amphidinium carterae* has an ovoid cell shape with a starch-sheathed pyrenoid located in the

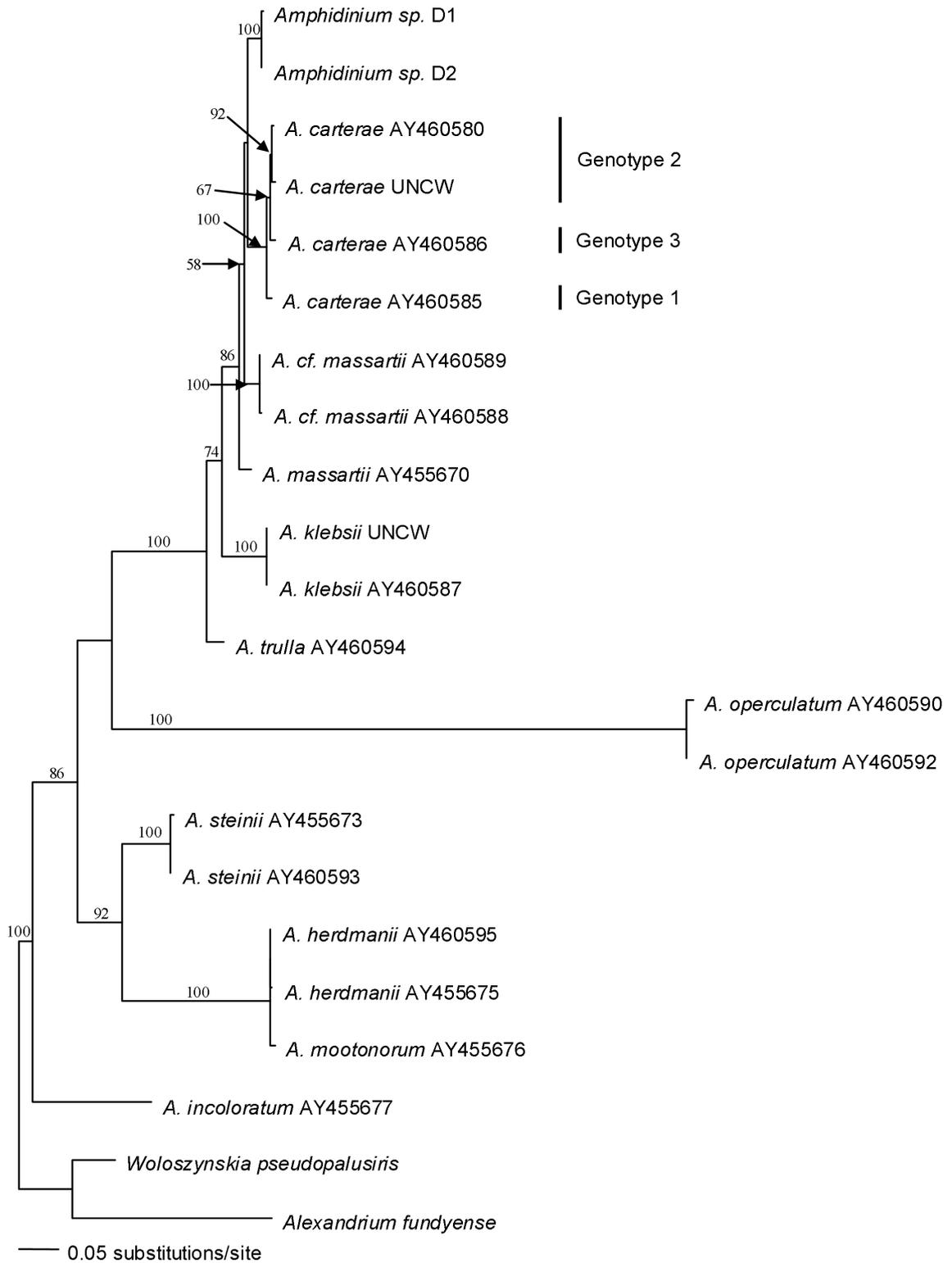


**Figure 1.** Light micrographs of (a) *A. carterae* and (b) *Amphidinium* sp. D2 in dorsal view. Arrows point to pyrenoid. Scale bars =  $5\text{ }\mu\text{m}$ .



**Figure 2.** Epifluorescence micrographs of *Amphidinium* species. (a, b) Ventral views of *Amphidinium* sp. D2, (c) ventral view of *A. carterae*, (d) dorsal view of *A. massartii*, \*(c,d) from Murray *et al.* 2004. (a, b) Scale bars =  $5\text{ }\mu\text{m}$ . (c, d) Scale bars =  $10\text{ }\mu\text{m}$ .

left anterior region of the hypocone, while *Amphidinium* sp. D2 has a more centrally located pyrenoid (Fig. 1 a, b).



**Figure 3.** Phylogram obtained by maximum likelihood analysis of LSU rDNA sites D1-D6 excluding the hypervariable site D2. Bootstrap values above 50% are shown.

*Amphidinium* sp. D2 has a dorsally located, perforated, peripheral plastid which is connected to a central radiating chloroplast (Fig. 2 a, b). This morphology is distinct to *A. carterae* and found within all genotypes of the species (Murray *et al.* 2004). *Amphidinium massartii* (Fig. 2 d) was described as having a more internal plastid with many lobes radiating from the pyrenoid (Murray *et al.* 2004).

Maximum likelihood analysis of LSU rDNA places *Amphidinium* clones D1 and D2 as a sister group to *A. carterae* (Fig. 3). The intraspecific variation of LSU rDNA, domains D1-D6 excluding domain D2, for all *A. carterae* genotypes is 0.4-1.7%. The variation between *Amphidinium* clones D1 and D2 and *A. carterae* (4.1-4.4 %) is higher than *A. carterae*'s intraspecific variation (unpublished). *Amphidinium* D1 and D2 formed their own clade distinct from both *A. carterae* and *A. massartii*.

### Discussion

Morphologically *Amphidinium* sp. D2 is distinct from both *A. carterae* and *A. massartii*. Phylogenetic analysis shows that *Amphidinium* sp. D2 is related to *A. carterae* and *A. massartii* but not close enough to call it either species. By combining the morphological and phylogenetic data it is shown that *Amphidinium* sp. D2 is not conspecific to either *A. carterae* or *A. massartii*. More environmental and physiological studies are needed to determine the plasticity of this unusual morphology. The morphological and phylogenetic proximity of *Amphidinium* sp. D2 to *A. carterae* is cause for interest in the production of bioactive compounds. More toxin assays need to be run to determine *Amphidinium* sp. D2's potential to produce toxins.

### Acknowledgements

This work was supported by CDC grant # 01-584-4 awarded to C. Tomas. We would like to thank Dr. BK Song for his help with this project.

### References

- Al-Qassab, S., Lee, W. J., Murray, S., Simpson, A. & Patterson, D. J. (2002). *Acta Protozol.* 41: 91-144.
- Barlow, S. & Triemer, R. E. (1988). *Phycologia* 27: 413-420.
- Calado, A. J. & Moestrup, Ø. (2005). *Phycologia* 44: 112-119.
- Echigoya, R., Rhodes, L., Oshima, Y. & Satake, M. (2005). *Harmful Algae* 4: 383-389.
- Hansen, G., Daugbjerg, N. & Henriksen, P. (2000). *J. Phycol.* 36: 394-410.
- Ishibashi, M. & Kobayashi, J. (1997). *Heterocycles* 44: 543-572.
- Jørgensen, M.F., Murray, S. & Daugbjerg, N. (2004). *J. Phycol.* 40: 351-365.
- Keller, M. D., Selvin, R. G., Claus, W. & Guillard, R. (1987). *J. Phycol.* 23: 633-638.
- Murray, S., Jørgensen, M.F. & Daugbjerg, N. (2004). *J. Phycol.* 40: 366-382.
- Murray, S. & Patterson, D. J. (2002). *Eur. J. Phycol.* 37: 279-298.
- Posada, D. & Crandall, K.A. (1998). *Bioinformatics* 14: 817-818.
- Satake, M., Murata, M., Fujita, T., Naoki, H. & Yasumoto, T. (1991). *J. Am. Chem. Soc.* 113: 9859-9861.
- Swofford, D.L. (2000). Sinauer Associates. Sunderland, MA.

## Harmful algae can be transported via relocation of bivalve shellfish

Hélène Hégaret<sup>1</sup>, Sandra E. Shumway<sup>1</sup> and Gary H. Wikfors<sup>2</sup>

<sup>1</sup> Department of Marine Sciences, University of Connecticut, Groton, CT 06340, Helene.Hegaret@uconn.edu and Sandra.Shumway@uconn.edu; <sup>2</sup> NOAA-NMFS, Milford, CT 06460, Gary.Wikfors@noaa.gov

### Abstract

Our study tested the hypothesis that harmful algae can be introduced into new environments by shellfish relocations, a common practice for commercially-exploited bivalve molluscs. We identified which managed shellfish species and HABs co-occur geographically and established a protocol to assess the potential of the bivalve species as vectors for transport of harmful algae. Cultured strains of harmful algae, *Alexandrium fundyense*, *Heterosigma akashiwo*, *Prorocentrum minimum*, and *Karenia mikimotoi* were fed to bivalve molluscs for two days at natural bloom concentrations to assess the ability of the algal cells to pass intact through the digestive tract and subsequently grow. After feeding, the bivalves were kept for two days in ultrafiltered seawater. Biodeposits were collected and observed under the microscope after 24 and 48 h to evaluate the presence or absence of intact, possibly-viable cells or temporary cysts. Subsamples of biodeposits were transferred into both algal culture medium and filtered seawater and monitored microscopically for algal growth. Intact algal cells of the various harmful algae were seen in biodeposits (feces) and generally these re-established growing populations.

### Introduction

During the last decade, many introductions of non-native species, including toxic and ecosystem-disruption microalgae, have occurred. The dominant vectors are thought to be shipping, and fisheries activities for marine, invasive introductions (Ruiz *et al.* 2000a, b).

Occurrences of harmful algal blooms are increasing worldwide in intensity, frequency, and geographic distribution (Hallegraeff 1993). Ballast water has been suggested as one of the important vectors transporting harmful algae (Hallegraeff 1998).

Displacement of shellfish molluscs has been described or suggested as another possible vector for introduction into new environments of non-native microorganisms, including parasites (Carriker 1992) and harmful algae (Bricelj *et al.* 1993; Scarratt *et al.* 1993 Vila *et al.* 2001; Lilly *et al.* 2002; Penna *et al.* 2005).

This study assessed the potential introduction of new harmful algal species associated with the transport of bivalve molluscs. Bivalves are moved from one body of water to another very frequently, and better risk-management strategies can be developed with knowledge of which HAB species may be transferred inadvertently to new geographic locations by shellfish transplantation, potentially with negative consequences to the transplanted bivalves themselves (Shumway 1990).

### Materials and Methods

Six species of bivalve molluscs (softshell clams *Mya arenaria*, northern quahogs *Mercenaria mercenaria*, bay scallops *Argopecten irradians*, eastern oysters, *Crassostrea virginica*, blue mussels *Mytilus edulis* and Manila clams *Venerupis philippinarum*) were fed cultured HAB species obtained from the NOAA, Milford Laboratory (CT USA) collection: *Alexandrium fundyense* strain BF2, *Prorocentrum minimum* (strain JA-98-01), *Heterosigma akashiwo* (strain OL). A fourth species of harmful algae was also tested, *Karenia* (= *Gymnodinium*) *mikimotoi* (Stock GM95TIN), isolated at Tinduff (Rade de Brest, France); this was obtained from the culture collection of IFREMER, Brest, France.

Each of six species of bivalve molluscs was exposed to the four different species of harmful algae (Table 1) experimentally for 48 h in an 80-l basin, at a concentration equivalent to a natural bloom:  $10^3$  cells ml<sup>-1</sup> for *Alexandrium fundyense* (Shumway *et al.* 1988; Townsend *et al.* 2005),  $10^4$  cells ml<sup>-1</sup> for *Karenia mikimotoi* (Nakamura *et al.* 1995; Matsuyama *et al.* 1999),  $10^4$  cells ml<sup>-1</sup> for *Heterosigma akashiwo* (Rensel & Whyte 2003),  $10^4$  cells ml<sup>-1</sup> for *Prorocentrum minimum* (Hégaret & Wikfors 2005).

After 48 h of exposure to the simulated bloom, each of 10 individual bivalves was transferred into a

1.2-l basin containing only filtered seawater (FSW) to allow the animals to clear their guts and produce feces. After 24 h, each individual bivalve was transferred into a new, 1.2-l basin with FSW for an additional 24 h. Feces were collected and observed under the microscope for presence of intact, possibly-viable cells. Then, 1 ml of concentrated fecal suspension was collected from each shellfish and transferred into a culture tube containing either 5 ml of FSW or 5 ml of the culture medium in which the algal cells had been grown. Growth of harmful algal cells was assessed weekly in each tube under an inverted microscope for up to two months.

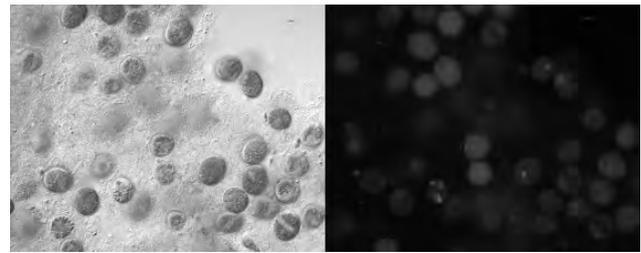
**Results**

Microscopic observations of feces after the bivalves had been in FSW for 24 h showed the presence of intact, possibly-viable cells in almost every interaction, with the exception of softshell clams (Fig. 1, Table 1). Usually the feces produced 24-48 h of depuration in FSW contained fewer intact algal cells (Table 1).

In tubes inoculated with biodeposits, growing populations of harmful algae were usually observed, with first detection after various periods of time. The tubes inoculated with biodeposits from bivalves exposed to *H. akashiwo* and *K. mikimotoi* usually

**Table 1.** Bivalve-HAB interactions tested: Grey represents interactions that have not been tested; white shows interactions where algae were not observed in the biodeposits and did not recover; 1. Presence of intact cells in the biodeposits of bivalves after 24 h of incubation in FSW, 2. Presence of intact cells in the biodeposits of bivalves after 48 h of incubation in FSW, 3. Recovery of HAB cells from the biodeposits cultured in the tubes containing FSW after 24 h of depuration in FSW, 4. Recovery of HAB cells from the biodeposits cultured in the tubes containing FSW after 48 h of depuration in FSW, \* no biodeposits produced after 24 h of incubation.

<i>Argopecten irradians</i>	1,2,3		1,2,3,4	1,3
<i>Crassostrea virginica</i>	1,2,3		* 4	1,2,3
<i>Mercenaria mercenaria</i>	1,2,3		3	1,3
<i>Mya arenaria</i>				
<i>Mytilus edulis</i>	1,3	3		1,3
<i>Venerupis philippinarum</i>		3		



**Figure 1.** Photomicrographs, paired light (A) and epifluorescence (B) to show chlorophyll a, of fecal pellets produced by hard clams (=quahogs, *Mercenaria mercenaria*) after being exposed to simulated blooms of cultured *Alexandrium* spp. and then moved to ultrafiltered seawater for 24 h (scale bar = 20 μm): Temporary cysts and cells of *A. fundyense* were seen in fecal material from *Mercenaria mercenaria*.

showed a recovery of the cells after 1-2 wk. The tubes inoculated with biodeposits from bivalves exposed to *P. minimum* revealed recovery after 3-4 wk; whereas it took 6-8 wk for the tubes inoculated with biodeposits from bivalves exposed to *A. fundyense* to form detectable, growing populations (Table 1).

Biodeposits from the softshell clam, *Mya arenaria*, were thoroughly processed, and not a single intact cell of any harmful alga was observed in the fecal pellets after 24 or 48 h of depuration in FSW. Moreover, no culture tubes inoculated with biodeposits from softshell clams showed any growth of HAB populations (Table 1).

*Alexandrium fundyense* was observed in the biodeposits in two different morphologies (Fig. 1): as vegetative cells and also as temporary cysts (Persson *et al.* in press), which could have formed during gut passage. The three other harmful algal species did not show any cyst formation; only vegetative cells were observed in the biodeposits or in the tubes where the cultures re-established.

**Discussion**

Results of these experiments clearly demonstrate that harmful algal cells can be found intact in the biodeposits of bivalves which have been feeding upon these, and at least some of these cells have the ability to recover from the feces and re-establish new populations. These findings demonstrate a clear risk of introducing new species of harmful algae through movement of shellfish.

These results also demonstrate that a period of 24 h or more in seawater allows partial clearing of gut contents. Indeed, usually when the bivalves were held in seawater for 24 h or more, the harmful algae did not

recover from the feces. Thus, keeping the animals for 24 h in seawater before reintroducing them into a new environment may be one way to mitigate the risk of introducing harmful algae into new environments.

These findings present but a small number of bivalve-harmful algal bloom interactions; many others are known to occur, thus a systematic approach is needed to test for specific bivalve-algal interactions. Indeed, the responses observed in our experiments are species-specific: softshell clams for example do not seem to present a risk of introducing HABs; whereas, oysters and scallops seem to present more risk of transfer.

Information generated in the present study may be used in shellfish hatcheries, but it will also be applicable to public education as recreational fishing or harvesting may precipitate introductions.

These results demonstrate a clear potential for transplanted bivalves to be vectors transporting HAB species into new areas, but they also show that a 24-h depuration period in seawater may mitigate this risk. Current studies are investigating alternative, less expensive or more convenient methods, to mitigate the risk of harmful algal introductions through movement of bivalve shellfish.

#### Acknowledgements

We thank all the people who provided shellfish to conduct experiments and have worked on the project: N. Bloom, S. Mattison, D. Motherway, W. Blogoslawski, J. Widman, J. Fajans, N. Saliou, J. Alix, M. Dixon and B. Smith, G. Arzul and M.P. Crassous. We are indebted to Rick Karney, Chris Danes and Leslie Sturmer, shellfish growers, who drew our attention to this issue and have contributed their expertise.

This work was supported by EPA/ECOHAB - GRANT 523792 to S.E. Shumway, G.H. Wikfors, and J.M. Burkholder. We also acknowledge the Lerner Grey Fund from the American Museum of Natural History, the Feng Student Activities Fund from University of Connecticut, and National Oceanic and Atmospheric Administration Center for Sponsored Coastal Ocean Research for financial support.

#### References

- Bricelj, V.M., Greene, M. & Cembella, A.D. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J. & Shimizu, Y. (eds), pp. 371-376.
- Carriker, M.R. (1992). *J. Shellfish Res.* 11: 507-510.
- Hallegraeff, G.M. (1993). *Phycologia* 32: 79-99.
- Hallegraeff, G.M. (1998). *Mar. Ecol. Progr. Ser.* 168: 297-309.
- Hégaret, H. & Wikfors, G.H. (2005). *Harmful Algae* 4: 201-209.
- Lilly, E.L., Kulis, D.M., Gentien, P. & Anderson, D.M. (2002). *J. Plankton Res.* 24: 443-452.
- Matsuyama, Y., Uchida, T. & Honjo, T. (1999). *Fish. Sci.* 65: 248-253.
- Nakamura, Y., Suzuki, S.Y. & Hiromi, J. (1995). *Mar. Ecol. Progr. Ser.* 125: 269-277.
- Penna, A., Garcés, E., Vila, M., Giacobbe, M.G., Fraga, S., Luglie, A., Bravo, I., Bertozzini E. & Vernesi, C. (2005). *Mar. Biol.* 148:13-23.
- Persson, A., Smith, B.C., Wikfors, G.H., Quilliam, M. (in press). *Harmful Algae*.
- Rensel, J.E. & Whyte, J.N.C. (2003) In: *Manual on Harmful Marine Microalgae*, Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D. (eds), UNESCO, Paris, pp. 693-722.
- Ruiz, G.M., Fofonoff, P.W., Carlton, J.T., Wonham, M.J. & Hines, A.H. (2000a). *Ann. Rev. Ecol. Syst.* 31: 481-531.
- Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A. & Colwell, R.R. (2000b). *Nature* 408: 49-50.
- Scarratt, A.M., Scarratt, D.J. & Scarratt, M.G. (1993). *J. Shellfish Res.* 12: 383-388.
- Shumway, S.E. (1990). *J. World Aquacult. Soc.* 21: 65-104.
- Shumway, S.E., Sherman-Caswell, S. & Hurst, J.W. (1988). *J. Shellfish Res.* 7: 643-652.
- Townsend, D.W., Pettigrew, N.R. & Thomas, A.C. (2005). *Deep-Sea Research Part I -Topical Studies in Oceanography* 52: 2603-2630.
- Vila, M., Garcés, E., Maso, M. & Camp, J. (2001). *Mar. Ecol. Progr. Ser.* 222: 73-83.

## The toxic benthic dinoflagellate *Prorocentrum arabianum* Morton et Faust isolated from Phan Ri Bay, South Central Vietnam

T. Ho-Van<sup>1</sup>, L. Nguyen-Ngoc<sup>1</sup> and S.L. Morton<sup>2</sup>

<sup>1</sup>Institute of Oceanography, Cauda 01, Nhatrang city, Vietnam, habviet@dng.vnn.vn

<sup>2</sup>NOAA National Ocean Service, Charleston, United States of America, steve.morton@noaa.gov

### Abstract

Dinoflagellates isolated from *Liagora* sp. (Rhodophyta) in a tidal area of Phan Ri Bay, Binh Thuan Province included a species identified as *Prorocentrum arabianum*. This is a new record for Vietnam. The cells were broadly oval, 30-40 µm wide and 40-45 µm long. Thecal surface was rugose with scattered large and small pores except at the centre. A large nucleus and pyrenoid were positioned at the lower part and the centre of the cell, respectively. Intercalary bands were horizontally striated. This strain of *P. arabianum* produced two cytotoxic fractions, a non-polar and a polar fraction. The polar compound was found to be ichthyotoxic and the non-polar compound cytotoxic.

### Introduction

The genus *Prorocentrum* was named by Ehrenberg (1834) with *P. micans* as the only species. Up to now, about 70 species have been described, divided into two genera, *Prorocentrum* and *Exuviaella* which are usually regarded as synonymous (Dodge 1975; Fukuyo 1981; Steidinger 1997; Faust *et al.* 1999; Nguyen-Ngoc *et al.* 2004).

Most harmful species of *Prorocentrum* are benthic or tychoplanktonic, and in the tropics are particularly common on dead coral in lagoons (Grzebyk *et al.* 1994). They may produce toxins: thus *P. concavum* is ciguatoxic (Tindall & Morton 1998). Several toxins have been isolated from *P. concavum* including water-soluble fast-acting toxins, OA, and DTX-1 (Faust *et al.* 1999); several different types of toxins are produced by *P. lima*: OA and isomers of OA, DTX-1, DTX-4, and prorocentrolide (Faust *et al.* 1999; James *et al.* 2000); this species may be implicated in ciguatera (Tindall & Morton 1998); *P. rathymum* may produce toxins with hemolytic activity (Nakajima *et al.* 1981), a water-soluble fast-acting toxin (Tindall *et al.* 1989); *P. faustiae* is a diarrhetic shellfish poison (DSP) producing species that forms okadaic acid (OA) and dinophysistoxin-1 (DTX1) (Morton 1998); *P. arabianum* produces two cytotoxic compounds, one of which is ichthyotoxic; OA is apparently not produced (Morton *et al.* 2002).

*Prorocentrum arabianum* is a new record of the benthic microalgal flora of Vietnam. This work documents its morphology and toxicity.

### Materials and Methods

#### *Sampling site and isolation*

Sampling was conducted at 11°14' N and 108°47' E in Phan Ri Bay, Binh Thuan Province. The temperature was 24-28 °C. The lowest salinity, about 32 ‰, was recorded in February-March (Nguyen-Ngoc *et al.* 2004). Dinoflagellate cells were isolated from the red alga *Liagora* sp. and maintained in TL-medium in the laboratory at 25 °C, 30 psu, 50 µmol photons m<sup>-2</sup> s<sup>-1</sup>, and a L:D regime of 12:12.

#### *Observation and identification of species*

Cultured cells were stained by Calcofluor White M2R (Fritz & Triemer 1985) and observed on an epifluorescence microscope Leica LDMB. Micrographs were taken with an OLYMPUS DP-12 digital camera. For SEM, a sample was dehydrated in an alcohol series of 10, 30, 50, 70, 90, 96 and 99.99 %, and subsequently dried in an oven at 40-50 °C for 24 h. A Jeol 5410-LV SEM was used to identify cells. Cell morphology was compared with closely resembling species such as *Prorocentrum concavum*, *P. faustiae*, and *P. arabianum* using the descriptions of Fukuyo (1981), Morton (1998), and Morton *et al.* (2002).

#### *Toxicity*

**GH4C1 cytotoxicity assay.** Cytotoxicity assays were used as a first screen for toxic activity. Cytotoxicity was measured in a microtiter plate assay using the mitochondrial indicator MTT for an endpoint measurement. 30,000 cells per well were plated in 0.1 ml medium in 96-well tissue culture plates. Cell lines to be used for toxicity measurements were GH4C1 rat

pituitary cells. Test samples were added to each well and incubated for approximately 24 h. After incubation, 15  $\mu$ l of 3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium (MTT, 5mg/ml in PBS) was added to each well and incubated for 4 h at 37 °C. Mitochondrial dehydrogenases in live cells converted the MTT to an insoluble formazan crystal which has a purple colour. The crystals were solubilized by addition of 1 % SDS in 0.1N HCl, and the absorbance was read at 570 nm with a Titre Tek 96 well plate reader (Flow Laboratories, McLean, VA). The plate reader subtracts non-specific absorbance by the medium and the non-converted MTT.

**Colourimetric phosphatase inhibition assay.** The protein phosphatase inhibition assay was carried out in 96-well tissue plates, following Tubaro *et al.* (1996). For the assay, 50  $\mu$ l samples and standards (0.1-1000 nM OA; LC Laboratories, Cambridge, MA), 100  $\mu$ l reaction buffer and 50  $\mu$ l purified PP2A enzyme (Upstate Biotechnology, Lake Placid, NY) were added to

duplicate 96-well plates (Costar, Corning, NY). To start the reaction, 50  $\mu$ l pNPP (50 mM; Sigma, St. Louis, MO) was added to each well and the reaction was allowed to proceed for 1 h at room temperature. Protein phosphatase activity was determined (405 nm) using a plate reader (Titertek Multiscan Plus, Hutitsville, AL).

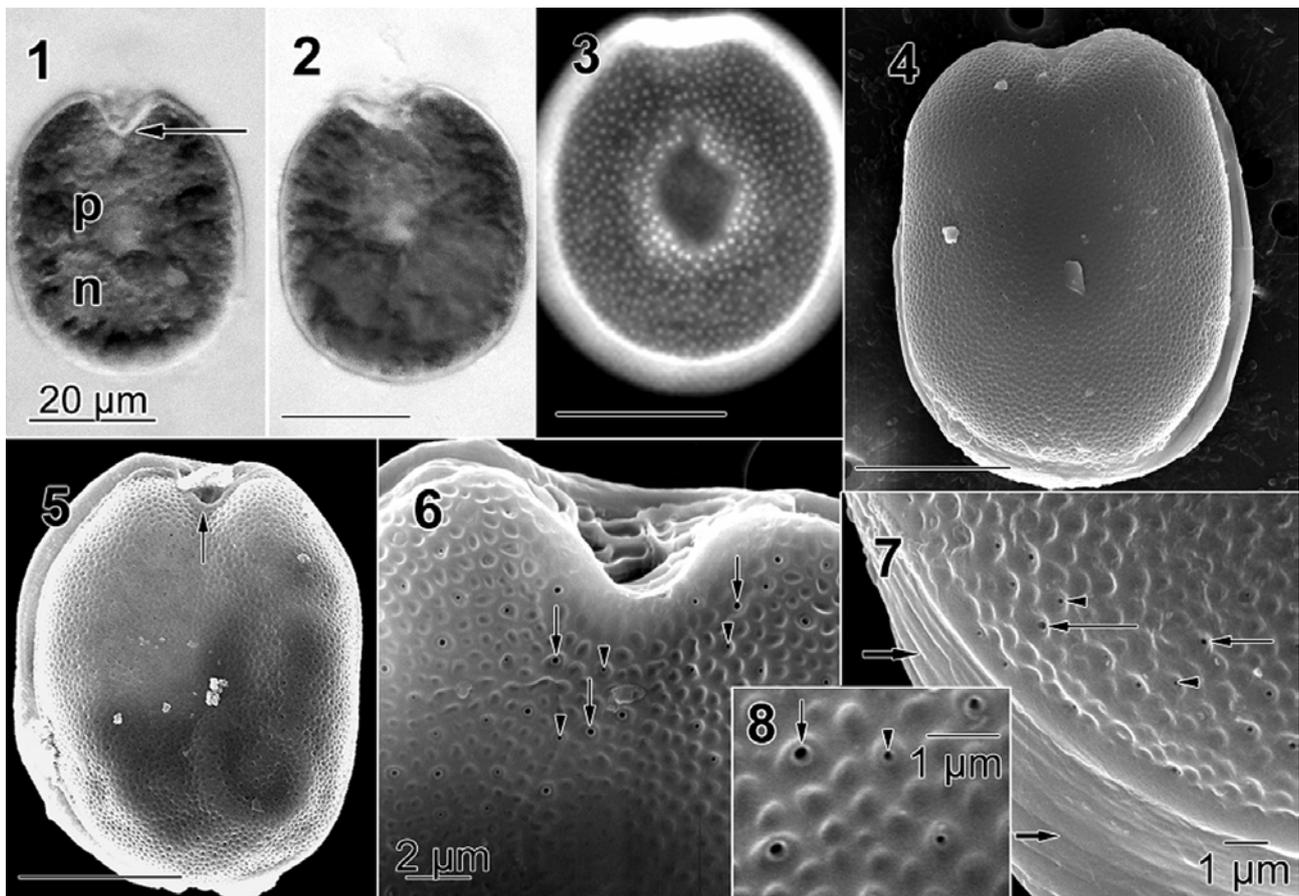
## Results and Discussion

### Light microscopy

Cells of *P. arabianum* were broadly oval, 30-40  $\mu$ m wide and 40-45  $\mu$ m long, with a centrally located pyrenoid (Fig. 1). A large nucleus was situated posteriorly (Fig. 1). No valve pores could be seen at the centre of the cell. The right valve had a v-shaped depression in the peri-flagellar area (Figs 1, 5, 6).

### SEM

The surface was laced with valve pores. Large and small pores (Figs 6-8) were measured as  $162 \pm 27.5$



**Figure 1-8.** *Prorocentrum arabianum*. – Fig. 1 (DIC). The right valve showing the shallow v-shaped indentation (arrow), central position of pyrenoid (p) and posterior position of nucleus (n). Fig. 2 (DIC). The left valve. Fig. 3 (Epifluorescence). Theca showing the surface laced with valve pores. Figs 4-5 (SEM). Left and right valve of different cells showing the surface in detail. Fig. 6 (SEM). The peri-flagellar area is shallow v-shaped (arrow) with two kinds of pores, large (arrow) and small (arrowhead). Figs 7, 8 (SEM). Details of thecal surface: large pores (arrows), small pores (arrowheads), and the intercalary band with fine horizontal lines (left arrows). Scale bars in Figs 2-5 = 20  $\mu$ m.

nm (n=18) and  $92 \pm 15$  nm (n=22); cells lacked marginal pores (Fig. 3). The intercalary band was horizontally striated (Fig. 7).

#### Distribution

This species is known from Oman Gulf (Arabian Sea) (Morton 1998), Nha Trang and Phan Ri Bays (south central Vietnam).

#### Remarks

The species is very similar to *P. faustiae* in shape and scattered valve pores, but it differs in the possession of marginal pores. The present species matches the original description of *P. arabianum* (Morton *et al.* 2002) but it was found on macroalgae (*Liagora* sp.) and cells grew well on the bottom of culture flasks, while Morton *et al.* (2002) isolated the original *P. arabianum* from a surface bloom in the Arabian Sea.

Benthic dinoflagellates, such as *P. rathymum*, *P. lima*, *Ostreopsis ovata* and *Coolia monotis* can be found in the plankton in Nha Trang Bay during the north east monsoon season. The waves and strong winds at this time dislodge benthic dinoflagellates from the substratum. We suggest that the *P. arabianum* of Morton *et al.* (2002) is benthic. Normawaty Mohammad Noor (2006, pers. comm.) has found this benthic *Prorocentrum* in Malaysian waters.

Several benthic dinoflagellates of the genus *Prorocentrum* have been observed as epiphytes on macroalgae, mainly on *Padina*, with occasional records on *Sargassum* (Nguyen Ngoc Lam, pers. observ.). Here for the first time *P. arabianum* was observed on *Liagora*. Hei *et al.* (1998) investigated the distribution of five benthic dinoflagellates as epiphytes on different substrata on Heron Island (Australia). They claimed that *P. lima* attaches by a mucus strand to macroalgal surfaces such as *Chnoospora implexa*, *Giffordia mitchelliae*, and the flat substrates of *Colpomenia sinuosa*. Macroalgae provide exudates and hence may release nutrients for *P. arabianum* (Hei *et al.* 1998).

#### Toxicity

*Prorocentrum arabianum* in culture produced two cytotoxic compounds, which appear unique among planktonic prorocentroids. The first compound was non-polar, and was extracted with ethyl acetate. It was active in the cytotoxicity assay and displayed a dose response curve. EC50 was 6 pg of extract. The second compound was polar, and was extracted with methanol. It was active in the cytotoxicity assay and found to be ichthyotoxic. It displayed a dose response curve with an LD50 of 15 pg of extract. The polar

compound produced an unusual green end point to the cytotoxicity assay. The end point of this assay is usually yellow. The fractions did not inhibit protein phosphatase activity, indicating that this species does not produce OA or related compounds. The culture tested showed the same activity as the type culture examined by Morton *et al.* (2002).

#### Acknowledgements

L. Nguyen Ngoc thanks the Council of Life Science, Fundamental Research Programme (MOST, Vietnam) for financial support of project 61-04-02. The two first authors thank Danida /HABViet Project III for providing funds to attend the HAB 12 Conference in Copenhagen. Thanks also to Peter Moeller for toxicity testing. Two reviewers are thanked for editing and improving the English.

#### References

- Dodge, J. D. (1975). Bot. J. Linn. Soc. 71: 103-125.
- Faust, M.A., Larsen, J. & Moestrup, Ø. (1999). Potentially toxic phytoplankton. 3. Genus *Prorocentrum* (Dinophyceae). – ICES Identification Leaflets for Plankton, leaflet no. 184: 1-24.
- Fukuyo, Y. (1981). Bull. Jap. Soc. Sci. Fish. 47: 967-978.
- Grzebyk, D., Berland, B., Thomassin, B.A., Bosi, C. & Arnoux, A. (1994). J. Exp. Mar. Biol. Ecol. 178: 51-66.
- Morton, S.L., (1998). Bot. Mar. 41: 565-569.
- Morton, S.L., Faust, M.A., Fairey, E.A. & Moeller, D.R.P. (2002). Harmful Algae 1: 393-400.
- Nakajima, I., Oshima, Y. & Yasumoto, T. (1981). Bull. Jap. Soc. Sci. Fish. 47:1029-1033.
- Nguyen-Ngoc, L., Larsen, J. & Chu, T.V. (2004). In: Larsen, J. & Nguyen-Ngoc, L. (eds), Potentially Toxic Microalgae of Vietnamese Waters, Opera Botanica 140, Copenhagen, pp. 1-216.
- Steidinger, K. A., (1997). In: Tomas, C. R. (ed.), Identifying Marine Phytoplankton, Academic Press, San Diego, pp. 387-584.
- Tindall, D. R. & Morton, S. L., (1998). In: Anderson, D. M., Cembella, A. D. & Hallegraeff, G. M. (eds), Physiological Ecology of Harmful Algal Blooms, NATO ASI series, Series G, Ecological Sciences, 41, Springer-Verlag, Berlin, pp. 291-313.
- Tindall, D. R., Miller, D. M. & Bomber, J. W. (1989). Toxicon 27: 83. (Abstract).
- Tubaro, A., Florio, C., Luxich, E., Soso, S., Della Loggia, R. & Yasumoto, T. (1996). Toxicon 34: 743-752.

## A new Mediterranean genotype of *Fibrocapsa* sp.

S. Kloeppe, U. John and A.D. Cembella

Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12,  
27570 Bremerhaven, Germany, skloeppe@awi-bremerhaven.de

### Abstract

Phylogenetic studies were conducted on monoclonal cultures of *Fibrocapsa* sp. isolated from a mixed, but raphidophyte dominated, bloom in the northern Adriatic Sea. The investigation focused on classification within the marine raphidophytes, based upon phylogenetic analysis of the ribosomal DNA marker ITS and the *psaA* subunit of Photosystem 1 of *Fibrocapsa* sp. from the Adriatic Sea and of strains of *Fibrocapsa japonica* from other populations. Among the raphidophytes, the ITS and *psaA* genotypes of the Adriatic *Fibrocapsa* strains were recovered in a sister clade to the clade containing all other *Fibrocapsa* strains examined. Constitutive detailed studies of the morphology may indicate the existence of a new species within the currently monospecific genus. Alternatively, the phylogenetic patterns expressed evidence of cryptic diversity among isolates of *F. japonica*, hitherto believed to form a single global population.

### Introduction

The marine flagellate *Fibrocapsa japonica* Toriumi et Takano, Raphidophyceae; Hara and Chihara (1985), has been identified as the causative organism for several fish killing events, particularly in Asian waters (Toriumi and Takano 1973; Yoshimatsu 1987). The increasing frequency of occurrence reports (Hallegraff 2003) may reflect progressive global spreading, but it is also possible that these delicate flagellates, which are badly preserved in most permanent samples, have been frequently overlooked.

The Italian coast of the northern Adriatic Sea (Mediterranean) is well known for the regular occurrence of raphidophyte blooms during summer (Milandri, pers. comm.). It also is a region in which aquaculture becomes more and more important (Anonymous 2005). Therefore, harmful algal blooms, including those caused by raphidophytes such as *Chattonella* and *Fibrocapsa*, are subject to monitoring. *Fibrocapsa* has been described as a monospecific genus, and based upon analysis of the ITS (internal transcribed spacer) region of rDNA it is currently believed to form a single global population, documented by strains from coastal habitats in the Pacific and Northern Atlantic Ocean (Kooistra *et al.* 2001).

Classification of raphidophytes is an ongoing process, combining molecular data with morphological and life history studies. Raphidophytes are difficult to identify reliably by means of light microscopy, because of their variable cell shape, depending on physiological condition and state of preservation. Therefore, information obtained from fast-evolving

genetic markers and from EM observations is essential for correct identification.

During a raphidophyte bloom in the northern Adriatic near Rimini, Italy, *Fibrocapsa cf. japonica* was the dominant species, accompanied by substantial amounts of *Chattonella* sp. We established clonal strains of *Fibrocapsa* for phylogenetic comparison with established strains from culture collections and sequence data available from public data bases. As genotypic markers for species identification and characterization, we analysed the ITS 1 and ITS 2 region of the nuclear encoded rDNA and chloroplast encoded subunit *psaA* of Photosystem I.

### Methods

#### Sampling

Surface water samples were collected by bucket at the shallow sandy coast in front of Rimini, Italy in August 2004. Monoclonal cultures were established in 100-ml culture glass flasks in K growth medium (Keller *et al.* 1987) and maintained at 21 °C on a 14:10 L:D photocycle at a photon flux intensity of 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . All cultures were harvested during exponential growth phase by centrifugation.

#### DNA extraction, amplification and sequencing

DNA isolation was performed with DNeasy Mini Kit according the protocol of the manufacturer (Qiagen, Hilden, Germany). The PCR reaction mix for all primer pairs consisted of 5  $\mu\text{l}$  of 10x HotMaster Taq Buffer, 1  $\mu\text{l}$  dNTP Mix 10 mM and 0.5  $\mu\text{l}$  Taq DNA polymerase (all Eppendorf, Hamburg, Germany) in

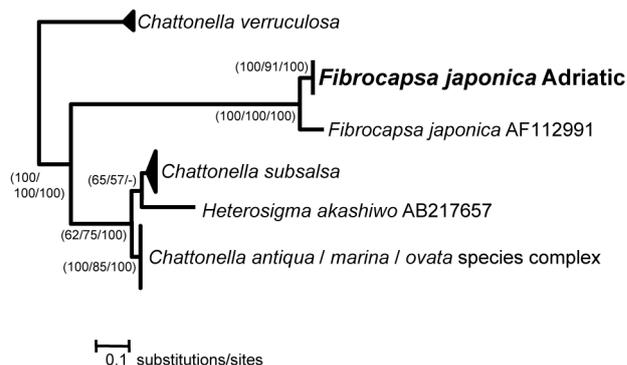
40.5 µl water. Different primers (0.1 µM final concentration) were added pairwise.

For amplifying the ITS, 0.5 µl of “ITS A” and “ITS B” (Adachi *et al.* 1996a), and for psaA “130F” and “1600R”, were added, respectively (Yoon *et al.* 2002). The temperature profile for the PCR of the ITS region was performed with Mastercycler Gradient (Eppendorf, Hamburg, Germany), started with 94 °C for 5 min (initial denaturation), followed by 35 cycles at 94 °C for 20 s (denaturation), 57 °C for 10 s (annealing) and 70 °C for 5 min (ex-tension). The PCR temperature profile for psaA was 94 °C for 3 min, followed by 39 cycles of 94 °C for 30 s, 44.5 °C for 30 s and 70 °C for 5 min. PCR products were purified with a Qiagen PCR Purification Kit and sequenced with an ABI 3130XL sequencer, using Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Primers were the same for sequencing as for PCR with the additional primers “870F” and “970R” (Yoon *et al.* 2002) for the psaA region.

Publicly available and generated sequences were aligned with ClustalW and finally manually edited in Bioedit (Version 7). Alignments and sequences are available upon request. The appropriate model of evolution for the phylogenetic analysis of the 43 ITS and 11 psaA sequences, consisting of 778 and 1388 base pairs, were determined by Modeltest (Posada and Crandall 1998, 2001). Maximum likelihood (ML) trees were inferred with PAUP\*4.0b8 (Swofford 1998). Bootstrap support for clades was obtained using ML, neighbour joining (NJ) and maximum parsimony (MP) methods with 1000 replicates. The likelihood settings for ITS from best-fit model (SYM + G) selected by AIC in Modeltest Version 3.06 for base substitution frequencies were A-C = 0.7453, A-G = 2.5607, A-T = 1.1881, C-G = 0.4517, C-T = 4.2231 and G-T 1.000; gamma distribution shape parameter = 1.1507. The best fitting model (GTR+I+G) for psaA likelihood settings, selected by AIC in Modeltest Version 3.06., were A-C = 0.9684, A-G = 3.6966, A-T = 3.1366, C-G = 1.4146, C-T = 14.5688 and G-T 1.000. The proportion of invariable sites = 0.5292 and gamma distribution shape parameter = 3.4541.

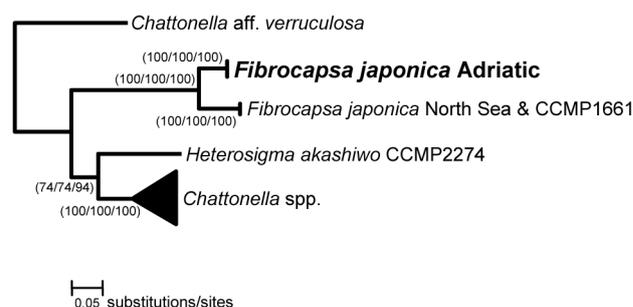
## Results

The generated sequences did not reveal any intra-strain genetic variation. Therefore no cloning of PCR amplicons was necessary. For phylogenetic analysis of the ITS rDNA region, strains of *Chattonella verruculosa* Hara et Chihara were chosen as outgroup (Fig. 1). Within the ingroup, remainder of the *Chat-*



**Figure 1.** ML phylogenetic tree of 43 ITS rDNA sequences. *Chattonella verruculosa* strains from public databases were used as outgroups. ML, NJ and MP bootstrap values (>50%) are shown respectively in brackets at each node.

*tonella* strains were recovered in two clades as sister to the strains of *Heterosigma akashiwo* (Hada) Hada ex Hada et Chihara. The strains of *Fibrocapsa*, grouped as sister to this clade. The strains of *Fibrocapsa* themselves were recovered in two clades of which one included all of the Adriatic stains and the other contained all of the remainder. Most of the clades obtained sufficient ( $\geq 75\%$ ) bootstrap support. Similar to the results obtained with the ITS marker, the psaA sequences revealed an early separation of *Fibrocapsa* from the other raphidophytes (Fig. 2). Contrary to the results of the ITS analysis, the psaA analysis showed a monophyletic clade of *Chattonella* spp., which is collapsed for simplification. A detailed study on *Chattonella* spp. is in preparation for publication.



**Figure 2.** ML phylogenetic tree of 11 sequences of the psaA region of PS I. *Chattonella aff. verruculosa* (revision in progress, pers. comm. B. Edvardsen) were used as outgroup. ML, NJ and MP bootstrap values (>50%) are shown respectively in brackets at each node.

## Discussion

The phylogenetic findings based on ITS and psaA regions suggest the existence of two genetically distinct species within the genus *Fibrocapsa*. These findings were supported by results of phylogenetic analysis of

the LSU (large subunit) and even the more conservative SSU (small subunit) rDNA region (Kloepper *et al.*, unpub. data), which also showed differences, even if only at the level of a few bases. Kooistra *et al.* (2001) investigated the ITS region of 16 widely distributed strains and reasoned that *Fibrocapsa japonica* forms a single global population, as Connell (2000) described for the raphidophyte *Heterosigma akashiwo*. The potentially endemic strains of the Adriatic might represent a different species, compared to the widespread strains of *Fibrocapsa japonica*. To clarify the distribution patterns, extensive sampling in the Adriatic and the bordering marine regions is necessary. Moreover there is exigency for morphological structure analysis of whole cells and ultrastructural details from thin sections of the type material, to clarify if the two clades of *Fibrocapsa* are morphologically distinct or cryptic species from a globally distributed morphotype. The inconsistency among the phylogenetic trees regarding *Chattonella* spp. seems to be due to a low resolution of the concerning branch within the ITS tree.

Regarding the intra-population structure, Kooistra *et al.* (2001) found clear polymorphisms within the ITS regions. The number of ambiguities increased with more recent collection date, while a phylogeographic structure is lacking. The authors assumed a mixing of formerly isolated populations, e.g. by ballast water transport and ongoing sexual reproduction. Our investigated strains did not display any of those ambiguities within both described markers.

### Acknowledgements

The authors thank Silvia Pigozzi and Anna Milandri (Centro Ricerche Marine in Cesenatico, Italy), Marina Montesor (Stazione Zoologica Anton Dohrn, Naples,

Italy), Bank Beszteri (Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany) and the RMP MarPLAN funding within the NoE MarBEF for support.

### References

- Adachi, M., Sako, Y. & Ishida, Y. (1996a). *J. Phycol.* 32: 424-432.
- Anonymous (2005). FAO Fisheries Report R779.
- Connell, L.B. (2000). *Mar. Biol.* 136: 953-960.
- Dolenec, T., Lojen, S., Kniewald, T., Dolenec, M. & Rogan, N. (2007). *Aquaculture* 262: 237-249.
- Hallegraeff, G.M. (2003). In: *Manual on Harmful Marine Microalgae*, Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D. (eds), UNESCO, Paris, pp. 25-49.
- Hara, Y. & Chihara, M. (1985). *Arch. Protistenk.* 130: 133-141.
- Keller, M.D., Selvin, R.C., Claus, W. & Guillard, R.R.L. (1987). *J. Phycol.* 23: 633-638.
- Kooistra, W.H.C.F., de Boer, M.K., Vrieling, E.G., Connell, L.B. & Gieskes, W.W.C. (2001). *J. Sea Res.* 46: 213-222.
- Posada, D. & Crandall, K.A. (1998). *Bioinformatics Applications Note* 14: 818.
- Posada, D. & Crandall K.A. (2001). *Systems Biol.* 50: 580-601.
- Swofford, D.L. (1998). *PAUP\**. Phylogenetic Analysis Using Parsimony (\* and Other Methods). Version 4. Sinauer Association, Sunderland, MA.
- Toriumi, S. & Takano, H. (1973). *Jap. Bull. Tokai Reg. Fish. Res. Lab.* 76: 25-35.
- Yoon, H.S., Hackett, J.D. & Bhattacharya, D. (2002). *Proc. Nat. Acad. Sci. USA* 99: 11724-11729.
- Yoshimatsu, S. (1987). *Bull. Plankton Soc. Japan* 35: 25-31.

## The species concept and cryptic diversity

D.G. Mann and K.M. Evans

Royal Botanic Garden, Edinburgh EH3 5LR, Scotland, United Kingdom, d.mann@rbge.org.uk

### Abstract

Meiosis and sexual reproduction are probably symplesiomorphic for all protists and in many they produce a real but fuzzy boundary between reticulate relationships (the outcome of sexual recombination) and hierarchical relationships (reflecting only ancestor–descendant relationships). This boundary creates a special status for the species category. Speciation in protists is often decoupled from morphological divergence, creating complexes of ‘cryptic’, ‘semicryptic’ and ‘pseudocryptic’ species (we clarify the definitions of these terms). Gene sequence–based molecular methods provide valuable insights concerning species boundaries, but divergence in neutral markers may not be synchronous with speciation. Cryptic, semicryptic and pseudocryptic species can differ functionally and to ignore those that have been detected by molecular or mating methods is irrational unless their properties and origins are well understood. Historical examples indicate that purely informal naming of cryptic, etc, species is unsatisfactory; once groups have been shown to have the properties of biological species, they need to be given a formal epithet and data about them circulated widely and effectively.

### Introduction and Definitions

Many species concepts and definitions have been proposed and we cannot analyse their merits here. Overall, however, there are two views: (1) species are in some sense ‘real’, having properties that distinguish them from all other systematic groupings and taxonomic ranks, or (2) species are artificial and ultimately arbitrary constructs, which do not differ conceptually from groups that are assigned higher or lower taxonomic ranks (families, genera, subspecies, varieties). We take the former view, on the basis that there is a real though wide boundary between populations that are related to each other only by descent from a common ancestor, and those that exhibit reticulate relationships created by sexual reproduction. This oversimplifies our position, which corresponds more or less to the ‘biological species concept’ (BSC), and we refer to the excellent account by Coyne and Orr (2004) for detailed discussion. To those who consider that some other species concept is in some sense better than the BSC, we would reply that, whatever the intrinsic merits of any alternative, the BSC has been and remains remarkably stimulating in evolutionary biology and is central to most discussions of ‘speciation’. The BSC cannot be applied to organisms that lack sexual reproduction and so, among HAB taxa, cyanobacteria have to be treated separately and differently. Culture studies and genetic analyses suggest increasingly, however, that meiosis and sexual reproduction are symplesiomorphic for all eukaryotes (Ramesh *et al.* 2005) and are present in most, though

they may be difficult to detect (e.g. Figueroa and Rengefors 2006).

Among organisms that use visual cues for social interactions, mate selection or pollination, or that are eaten or parasitized by sighted animals, the correlation between speciation and morphological differentiation is likely to be strong (e.g. Coyne and Orr 2004, chap. 6; Harder and Barrett 2006). In other kinds of organisms, morphological differences will often have functional and evolutionary significance, reflecting present or past selection, but there will be a less consistent relationship between morphology and species boundaries. Some species show considerable morphological variation or plasticity. Others are so similar to each other in shape, size and structure that telling them apart is difficult or impossible: variation among close relatives is often considerable (e.g. in physiology) but ‘cryptic’. Even among metazoa and higher plants, cryptic species are not infrequent (e.g. Barbosa *et al.* 2006).

There is a large and rapidly growing literature on cryptic species, but even a cursory examination reveals that different authors have very different ideas as to what ‘cryptic’ means. For example, Saunders and Lehmkuhl (2005) referred to several new *Plocamium* species as ‘cryptic’ and yet were able to provide a key for identifying them based on morphological and anatomical features. Others use the terms ‘semicryptic’ or ‘pseudocryptic’ for such cases, apparently interchangeably. We suggest the following clarifications:

- species are *cryptic* if it is impossible to identify individuals consistently and accurately from mor-

phology alone (e.g. *Peridinium limbatum*: Kim *et al.* 2004).

- species are *semicryptic* if individuals can be identified consistently and accurately from morphology if and only if the provenance is known, either in terms of geographical origin (e.g. of the five known molecular clades of the left-coiling foraminiferan morphospecies *Neogloboquadrina pachyderma*, only one is found in the N Atlantic: Darling *et al.* 2004), or population characteristics.
- species are *pseudocryptic* if individuals can be identified from morphology providing sufficient care is taken, but are so similar that there is a high probability of misidentification, even by a competent scientist (e.g. *Sellaphora*: Kelly *et al.* 2002; Mann *et al.* 2004; and see below).

Each of these definitions specifies ‘individuals’ as the basis for discrimination. Populations of different species may have different mean values for one or more morphological characteristics but if the ranges of values overlap, *individuals* cannot be identified and the species are cryptic or semicryptic. On the other hand, species do not qualify to be termed ‘cryptic’ merely because the diagnostic characters are seldom used, e.g. heterochromatin distributions discriminate between putative species in *Navicula cryptocephala* agg. (Pouličková and Mann 2004) but they have not yet appeared in any species diagnosis. There can be no nonarbitrary definition of how difficult identification must be before groups of species qualify to be ‘pseudocryptic’: the term basically means that identification is tricky for some particular (usually unspecified) group of ‘experts’.

Classification of groups of microalgal species as semicryptic or pseudocryptic must usually be provisional, because sampling is rarely adequate. Some species complexes contain mixtures of cryptic, semicryptic and pseudocryptic species, e.g. in the freshwater diatom *Sellaphora* (Behnke *et al.* 2004; Mann *et al.* 2004).

### Detection

Pseudocryptic protist species should reveal themselves to anyone who looks for them carefully with appropriate instruments. Hence, the introduction of TEM and SEM (from the 1960s onwards) has led to the detection of many species within taxa of microalgae previously thought unitary (e.g. in *Pseudo-nitzschia*: Hasle 1965). Semicryptic and cryptic species are by definition undetectable or doubtfully detectable by this means. Originally, ‘cryptic’ protist species were

discovered by mating experiments (e.g. in the ciliates *Paramecium* and *Tetrahymena*, or the chlorophyte *Pandorina*: Sonneborn 1975, Nanney 2004; Coleman 1959) or through observations of sexualized natural populations (e.g. in *Sellaphora*: Mann 1984). Just as in higher plants (e.g. the Californian transect studies carried out by Clausen, Keck and Hiesey in the mid-20th century: Baldwin 2006), breeding relationships among microalgae, and also their correlation with morphological variation, can be complex (e.g. *Micrasterias*: Blackburn and Tyler 1987).

During the last 15 years, molecular genetic methods have increasingly been used to investigate the nature of morphospecies in protists. Some morphospecies, especially those that have been well sampled from a wide geographical range or ecological spectrum (e.g. *Skeletonema costatum*, *Cyclidium glaucoma*: Sarno *et al.* 2005; Finlay *et al.* 2006), have been shown to be genetically diverse with respect to commonly-used markers, such as rDNA (SSU, partial LSU or ITS), *rbcL* or *rbcL-rbcS* spacer sequences. However, single gene trees never give unambiguous evidence about species boundaries. For example, a study of the avian parasite *Tetratrichomonas gallinarum* using ITS sequence data and RAPD markers (Cepicka *et al.* 2005) showed several levels of variation within the nominal species, but deciding which of the groups represent biological species is currently impossible. However, by using multiple nuclear markers or a combination of markers from different genomes, it is possible to detect recombination, making it easier to make decisions about species limits. Microsatellite markers have recently been used to assess the extent of gene flow between populations. Some sexual microalgal morphospecies are not only highly variable genetically but divided into several, largely isolated breeding populations (*Ditylum brightwellii*: Rynearson and Armbrust 2004); others behave as large panmictic units (*Pseudo-nitzschia pungens*: Evans *et al.* 2005).

The complex relationship between sequence variation – even in fast-evolving parts of the genome – and speciation is well illustrated by Coleman (2005), who examined ITS variation in *Paramecium aurelia sensu lato*. In this paradigmatic species complex, Sonneborn discovered several mating groups (originally referred to as ‘syngens’, later recognized as *P. primaurelia*, *P. biaurelia*, etc), each reproductively isolated from all the others although an F1 is produced in some intersyngen crosses in the laboratory (Sonneborn 1975). Three pairs of species (Sonneborn’s syngens 3+5,

4+9, 8+12) have identical ITS1–5.8S–ITS2 sequences (Coleman 2005), showing that ribotyping alone would underestimate biological species diversity. However, the syngen pairs with identical ribotypes are not the pairs that one might predict to be most similar from Sonneborn's assessment of the relative intensity of residual mating reactions. Overall, Coleman found that if two strains can produce an F1, their ITS2 sequences are identical, but the converse is not true: ITS identity does not necessarily imply compatibility or conspecificity. Hori *et al.* (2006) studied the *P. aurelia* complex using the hsp70 gene family; here too some species-pairs are indistinguishable, but not the same pairs as those that are identical with respect to ITS.

In *Paramecium* and other examples, Coleman (2005) has found that secondary structure analyses of ITS provide extra insights into species limits. Compensatory base changes (CBCs) in conserved regions of ITS-2 seem to correlate well with reproductive isolation (and even the ability to pair) and gamete interactions are rare where there is even one hemiCBC. If this correlation continues to hold, then ITS sequences alone can be used to make a minimum estimate for the number of cryptic biological species in a particular morphospecies, but the *Paramecium* example demonstrates that this may be a low estimate. *Stephanodiscus yellowstonensis*, a morphospecies that evolved within c. 4000 y of deglaciation in Yellowstone Lake, Wyoming (Theriot *et al.* 2006), has not diverged in ITS sequence from its likely ancestor in the *S. niagarae* clade (Zechman *et al.* 1994).

By contrast, demes (groups of individuals of a specified taxon: Gilmour and Heslop-Harrison 1954) that can mate in the laboratory may be significantly different genetically. *Sellaphora blackfordensis* (= *S. pupula* 'rectangular') and the related *S. pupula* agg. deme 'pseudocapitate' are clearly differentiated with respect to all five genetic markers that have been examined, viz. ITS, SSU, *rbcL*, *psaA* and *cox1* (Behnke *et al.* 2004, Evans *et al.*, submitted and in prep.); *S. blackfordensis* and "pseudocapitate" differ in one-sided CBCs in two helices of ITS2, as well as in loop sequences; and *S. blackfordensis* has a highly unusual amino-acid substitution (A→T) at position 42 in *rbcL*. Nevertheless, cells of these two demes can pair and produce a viable F1 in the laboratory (Behnke *et al.* 2004). Presumably they do not do so in nature, either because they do not meet (so far, we have not found them growing together) or because hybrids have low fitness or are infertile. Lack of pre- or postzygotic re-

productive isolation in the laboratory must be interpreted with caution.

Thus, there is a somewhat fuzzy relationship between the clades evident in gene trees and biological species. This is scarcely surprising, given (1) genetic recombination below the species level, achieved through sexual reproduction, (2) the complications caused by lineage sorting, and (3) the fact that most genes analysed have no direct involvement in mate recognition and sexual reproduction.

### **Is it always necessary to differentiate between cryptic, semicryptic and pseudocryptic species?**

We asserted earlier that 'biologically' defined species are real in a way that higher taxa are not. However, although their ranking is arbitrary, higher taxa may nevertheless possess characteristics that make them suitable as recording units in biogeographical, ecological or other studies. For example, oak (*Quercus*) species are generally forest trees with large seeds dispersed by animals and *Nothofagus* is restricted to the southern continents. In the context of temperate forest ecology, or biogeography, therefore, meaningful statements can be made at the genus level about '*Quercus*' and '*Nothofagus*'. So, it could be argued by analogy that, although a protist morphospecies may comprise several or many cryptic, semicryptic or pseudocryptic species, it may be legitimate to continue to treat this species complex as a single, named entity in some kinds of non-systematic research and inventory.

There are several problems with adopting this approach in protists, including the possible non-monophyly of morphospecies and their heterogeneity with respect to important functional characteristics and biogeography. Essentially, the decision to continue using morphospecies as recording units and to ignore cryptic variation is rational only if the nature of the variation can be shown to be irrelevant to the particular research or other activity being undertaken. Thus, for example, if Hubbell's assumption of neutrality ('the assumption of per capita ecological equivalence of all individuals of all species in a trophically defined community': Hubbell 2001) applies to epipelton or phytoplankton, then many ecologists might consider it a waste of effort to differentiate between virtually indistinguishable, functionally equivalent species within a community. However, it must first be demonstrated that the species are indeed equivalent. 'An alternative is that the sibling [i.e. cryptic *sensu lato*] species of protists exploit a much more fine-grained ecological

landscape than is imagined by those lumping morphologically similar organisms' (Nanney 2004).

Our own 'model system', in the diatom genus *Sellaphora*, illustrates the complex relationship between morphological variation and sequence data from genes commonly used for phylogeny estimation. A phylogeny based on *rbcL* and SSU sequences reveals that very closely related demes usually possess similar morphology, but the converse is not true and overall there is only a weak relationship between morphological and molecular evolution (Fig. 1, Evans *et al.*, submitted). Furthermore, although the *S. pupula* complex is easily identified as a whole (Hustedt 1930), it is in fact paraphyletic with reference to an-

other long-recognized and easily identified species, *S. bacillum*. Since *S. bacillum* is known from 12 Mya deposits (e.g. Saint Martin and Saint Martin 2005), the *S. pupula* group must have existed for much longer.

In relation to biological attributes (e.g. mating system, physiology) and ecology, several studies have already shown that there are significant differences among cryptic, semicryptic and pseudocryptic protist species. For example, Kucera and Darling (2002) have demonstrated the value of differentiating between similar foraminifera for palaeo-temperature reconstruction (also see Darling *et al.* 2004). In the *S. pupula* complex, closely related cryptic, semicryptic and pseudocryptic species can vary with respect



**Figure 1.** Molecular phylogeny of *Sellaphora*, with representative valves of demes in the *S. pupula* – *S. bacillum* species complex (formal names for described taxa, informal names for undescribed species); all demes belonging to *S. pupula sensu* Hustedt (1930) are marked by an asterisk. Maximum likelihood tree (lnL = -8620.273) based on a 1365-bp *rbcL* alignment (codon-partitioned: codon 1 GTR+I+G, codon 2 GTR+I+G, codon 3 GTR+G; parameters and details available from the authors). Thick stems indicate nodes with > 50 % bootstrap support (500 replicates). Valves are aligned horizontally with the corresponding branch.

to seasonality, parasite sensitivity and mating system (Mann 1999; Mann *et al.* 2004). There is no consistent relationship in *S. pupula sensu lato* between phylogenetic proximity and overall ecological preference. Species that co-occur in the same lake are often drawn from different major clades, and nearest relatives often occur in lakes of different nutrient status or type (dys/mesotrophic and eutrophic).

The short answer to the question we posed in this section ('Is it *always* necessary to differentiate between cryptic, semicryptic and pseudocryptic species?') is 'no': it will sometimes be possible to obtain and communicate useful information by using a coarse-grained classification that ignores cryptic variation. However, where a crude taxonomy (one that ignores the likely mismatches between morphological divergence and speciation in protists) is adopted as the basis for dependent science, the resulting data will also be crude, sometimes misleading, and often untransferable.

#### **How common is cryptic–semicryptic–pseudocryptic species-level variation?**

Until more cases have been examined, there can be no answer. All that can be said is that where long-established species have been sampled fairly intensively (several 10s to 100+ isolates) over a wide geographical area, sensitive molecular and morphological analyses have generally detected what appears to be significant hidden variation. Some examples among HAB species (detected using molecular methods) are in *Pseudonitzschia* (e.g. Lundholm *et al.* 2006; Amato *et al.* 2007, in press) and *Scrippsiella* (Montresor *et al.* 2003; Gottschling *et al.* 2005). Other phytoplankton examples include *Skeletonema costatum sensu lato* (Sarno *et al.* 2005) and *Micromonas pusilla*, where divergence of extant cryptic species has been provisionally dated at 66 Mya (Šlapeta *et al.* 2006).

In diatoms, where homeostatic mechanisms (well-regulated size control during auxosporulation, limited flexibility of the frustule, internalized morphogenesis of new wall elements, centrifugal rib formation during valve deposition) tend to produce a high constancy of size, shape and pattern, preliminary surveys of pseudocryptic variation can be executed fairly easily through population studies and morphometrics. Our experience suggests that many of the diatom species named in the 19th and early 20th century are heterogeneous (Mann 1999; Droop *et al.* 2000; Mann and Chepurnov 2005).

#### **Is it necessary to recognize cryptic, semicryptic and pseudocryptic species formally?**

Conversations with other biologists suggest to us that most people support the formal recognition of pseudocryptic biological species, considering that, if there are diagnostic morphological characters, a species should be given a name even if identification is difficult. There is less agreement about semicryptic and cryptic species. More than once at the Copenhagen HAB meeting in 2006, the opinion was expressed that there was no need to give names to every cryptic biological species, at least among protists. The reasoning seems to be that such species cannot be recognized during ecological studies or monitoring based on microscopy, so that names given to them are effectively useless. Likewise, one of the pioneers of research into cryptic speciation in protists, T.M. Sonneborn, refused to give formal nomenclatural status to the syngens of *Paramecium aurelia* until there was a reliable means (in this case, the method was non-visual, relying on isozyme characterization: Sonneborn 1975) to identify them without recourse to mating tests versus authenticated strains. So, people say, why not refer to cryptic or semicryptic species by an informal name or number attached to and qualifying the morphospecies name?

We are unconvinced, for two reasons: (1) molecular genetic methods are now well developed and increasingly inexpensive, they can be used to characterize biological species, and they will increasingly be developed to replace morphology-based identifications (especially where certainty is essential in a 'difficult' genus, e.g. Scholin *et al.* 2000); and (2) organisms lacking a formal name are generally ignored.

In relation to (1), molecular 'bar-coding' (Hebert *et al.* 2003; Savolainen *et al.* 2005) is an especially attractive idea for identifying protist species, even where morphological characters are available, because of the training and skill needed for accurate identification by light and electron microscopy, the high capital cost of good quality equipment, and the bottle-neck created by the instrumentation (only one organism can be identified at a time). The main problem with bar-coding is the inconstant relationship between variation in the chosen marker gene(s) and speciation. Although particular genes have been shown to be remarkably good for species identification in particular groups (e.g. *cox1* in Lepidoptera; Hajibabaei *et al.* 2006), to be 100 % effective they would have to show no variation within a species and always differ between spe-

cies; this typological requirement is not met in nature. However, although bar-coding will sometimes fail (at least as generally implemented, via a single gene), it offers a huge improvement over current practice in protists and other small organisms.

Turning to point (2), the need for formal names is shown well by considering the attached freshwater diatom *Cocconeis placentula*. This was the first diatom in which pseudocryptic variation was studied intensively, beginning in the mid 1920s (Geitler 1927). Geitler discovered several demes growing sympatrically, without intergradation, in a small river (the Lunzer Seebach) in Austria. The demes were reproductively isolated and differed subtly in the morphologies of the araphid valve and the chloroplast, the number of pyrenoids, and the mating system. Geitler (1927) recognized them as varieties (in his pre-Mayrian view, species were not inherently different from other taxonomic categories), calling them vars *klinoraphis* and *pseudolineata* (these were new taxa), and *lineata* (an existing taxon). All three were recognized and illustrated in Hustedt's diatom flora of 1930, which was the 'standard text' for identifying freshwater diatoms for 50 years. These varieties, together with var. *euglypta*, which was also illustrated by Hustedt (1930), were recorded frequently during the 20th century (as a Google search will quickly demonstrate). By contrast, var. *tenuistriata*, which was studied at Lunz by Geitler (1932) but not included by Hustedt (1930), has rarely been recorded since its description. Other varieties described but not illustrated by Hustedt (vars *rouxiii* and *intermedia*) and also var. *euglyptoides* (Geitler 1958) are likewise ignored. Geitler's work on the Lunz populations continued for nearly 60 years (Geitler 1982) and included some of the only reliable chromosome counts for diatoms (Geitler 1973). However, this classic study of pseudocryptic variation applied only to the Lunz region. Elsewhere in Austria, Geitler discovered nine other populations, differing from the Lunz 'varieties' in the same kinds of characteristics as the Lunz varieties differed from each other. The non-Lunz demes, which were never given formal names but simply referred to as 'var. II', var. *euglypta* 'Schladming', etc, have not been recorded by anyone else.

From this and similar examples (e.g. the variants described by Mann 1989), we conclude that: (A) even if groups are reproductively isolated, and well characterized (morphologically or molecularly), they will not generally be recorded if they have only an informal name (the *Pandorina morum* syngens discovered

by Coleman 1959 are widely known to exist but it is the morphospecies alone that is generally recorded); (B) taxa known only through the original validating descriptions, rather than via standard floras (increasingly, access will be made through Web biodiversity portals, which may 'democratize' taxonomic results), are likely to be ignored unless they have special features (e.g. toxicity) that attract attention. We therefore recommend that, once variation and species limits are well understood, cryptic and semicryptic species should be given the same status as any other species. Even though they may currently be difficult to detect, identification will undoubtedly become easier in the near future, as molecular methods improve still further.

At the same time, however, formalization should not be premature. Formal names can easily imply greater generality than is justified, as the *C. placentula* example shows: it is by no means certain that Austria contains the whole, or even a representative part, of *C. placentula* biodiversity.

### Acknowledgements

We have benefited from discussion with many people, but notably our friends and colleagues in the Protistology & Aquatic Ecology group, University of Gent and the Stazione Zoologica, Naples, and Prof A. Poulícková, Prof. L. Medlin and Dr R. Trobajo.

### References

- Amato, A., Kooistra, W.H.C.F., Levaldi Ghiron, J.H., Mann, D.G., Pröschold, T. & Montresor, M. (2007). *Protist* 158: 193–207.
- Baldwin, B.G. (2006). *Ann. Missouri Bot. Gard.* 93: 64–93.
- Barbosa, D., Font, E., Desfilis, E. & Carretero, M. A. (2006). *J. Chem. Ecol.* 32: 1587–1598.
- Behnke, A., Friedl, T., Chepurinov, V.A. & Mann, D.G. (2004). *J. Phycol.* 40: 193–208.
- Blackburn, S.I. & Tyler, P.A. (1987). *Br. Phycol. J.* 22: 277–298.
- Cepicka, I., Kutisova, K., Tachezy, J., Kulda, J. & Flegl, J. (2005). *Vet. Parasitol.* 128: 11–21.
- Coleman, A.W. (1959). *J. Protozool.* 6: 249–264.
- Coleman, A.W. (2005). *J. Euk. Microbiol.* 52: 68–77.
- Coyne, J.A. & Orr, H.A. (2004). *Speciation*. Sinauer, Sunderland, Ma.
- Darling, K.F., Kucera, M., Pudsey, C.J. & Wade, C.M. (2004). *PNAS* 101: 7657–7662.
- Droop, S.J.M., Mann, D.G. & Lokhorst, G.M. (2000).

- Phycologia 39: 527–546.
- Evans, K.M., Kühn, S.F. & Hayes, P.K. (2005). *J. Phycol.* 41: 506–514.
- Figueroa, R.I. & Rengefors, K. (2006). *J. Phycol.* 42: 859–871.
- Finlay, B.J., Esteban, G.F., Brown, S., Fenchel, T. & Hoef-Emden, K. (2006). *Protist* 157: 377–390.
- Geitler, L. (1927). *Arch. Protistenk.* 59: 506–549.
- Geitler, L. (1932). *Arch. Protistenk.* 78: 1–226.
- Geitler, L. (1958). *Öst. Bot. Z.* 105: 350–379.
- Geitler, L. (1973). *Öst. Bot. Z.* 123, 299–321.
- Geitler, L. (1982). *Arch. Hydrobiol., Suppl.* 63.1: 1–11.
- Gilmour, J.S.L. & Heslop-Harrison, J. (1954). *Genetica* 27: 147–161.
- Gottschling, M., Knop, R., Plötner, J., Kirsch, M., Willems, H. & Keupp, H. (2005). *Eur. J. Phycol.* 40: 207–220.
- Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. & Hebert, P.D.N. (2006). *PNAS* 103: 968–971.
- Harder, L.D. & Barrett, S.C.H. (2006). *Ecology and Evolution of Flowers*, Oxford Univ. Press.
- Hasle, G. R. (1965). *Skr. Norske Videnskaps-Akad., Mat.-Naturv. Klasse, Ny Serie*, 18: 1–45.
- Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003). *Proc. Roy. Soc. Lond., ser. B* 270: 313–21.
- Hori, M., Tomikawa, I., Przybos, E. & Fujishima, M. (2006). *Mol. Phyl. Evol.* 38: 697–704.
- Hubbell, S.P. (2001). *The Unified Neutral Theory of Biodiversity and Biogeography*, Princeton Univ. Press.
- Hustedt, F. (1930). *Bacillariophyta*. G. Fischer, Jena.
- Kelly, M.G., Bayer, M.M., Hürlimann, J. & Telford, R.J. (2002). *Automatic Diatom Identification*, Du Buf, J.M.H. & Bayer, M.M. (eds), World Scientific Publishing, Singapore, pp. 75–91.
- Kim, E., Wilcox, L., Graham, L. & Graham, J. (2004). *Microb. Ecol.* 48: 521–7.
- Kucera, M. & Darling, K. F. (2002). *Phil. Trans. Roy. Soc. Lond., ser. A*, 360: 695–718.
- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. & Miller, P. (2006). *J. Phycol.* 42: 464–481.
- Mann, D.G. (1984). *Ann. Bot.* 54: 429–438.
- Mann, D.G. (1989). *Pl. Syst. Evol.* 164: 215–237.
- Mann, D.G. (1999). *Phycologia* 38: 437–495.
- Mann, D.G. & Chepurnov, V.A. (2005). *Phycologia* 44: 335–350.
- Mann, D.G., McDonald, S.M., Bayer, M.M., Droop, S.J.M., Chepurnov, V.A., Loke, R.E., Ciobanu, A. & du Buf, J.M.H. (2004). *Phycologia* 43: 459–482.
- Montresor, M., Sgrosso, S., Procaccini, G. & Kooistra, W.H.C.F. (2003). *Phycologia* 42: 56–70.
- Nanney, D.L. (2004). *BioScience* 54: 720–721.
- Pouličková, A. & Mann, D.G. (2006). *J. Phycol.* 42: 872–886.
- Ramesh, M.A., Malik, S.-B. & Logsdon, J.M., Jr. (2005). *Curr. Biol.* 15: 185–191.
- Rynearson, T.A. & Armbrust, E.V. (2004). *J. Phycol.* 40: 34–43.
- Saint Martin, S. & Saint Martin, J.-P. (2005). *C. R. Palevol.* 4: 191–201.
- Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I. & Zingone, A. (2005). *J. Phycol.* 41: 151–176.
- Saunders, G.R. & Lehmkuhl, K.V. (2005). *Eur. J. Phycol.* 40: 293–312.
- Savolainen, V., Cowan, R.S., Vogler, A.P., Roderick, G.K. & Lane, R. (2005). *Phil. Trans. Roy. Soc. Lond., ser. B.* 360: 1805–1811.
- Scholin, C.A., Gulland, F., Doucette, G.J., *et al.* (2000). *Nature* 403: 80–84.
- Šlapeta, J., Lopez-Garcia, P. & Moreira, D. (2006). *Mol. Biol. Evol.* 23: 23–29.
- Sonneborn, T.M. (1975). *Trans. Am. Microsc. Soc.* 94: 155–178.
- Theriot, E.C., Fritz, S.C., Whitlock, C. & Conley, D.J. (2006). *Paleobiology* 32: 38–54.
- Zechman, F.W., Zimmer, E.A., & Theriot, E.C. (1994). *J. Phycol.* 30: 507–512.

## Taxonomic uncertainties concerning *Gambierdiscus toxicus*: proposed epitype

Patricia A. Tester<sup>1</sup>, Maria A. Faust<sup>2</sup>, Mark W. Vandersea<sup>1</sup>, Steven R. Kibler<sup>1</sup>, Mireille Chinain<sup>3</sup>,  
Michael Holmes<sup>4</sup>, William C. Holland<sup>1</sup> and R. Wayne Litaker<sup>1</sup>

<sup>1</sup>National Ocean Service, NOAA, 101 Pivers Island Road, Beaufort, North Carolina 28516.

Pat.Tester@noaa.gov, Mark.Vandersea@noaa.gov, Steve.Kibler@noaa.gov, Chris.Holland@noaa.gov, Wayne.Litaker@noaa.gov, <sup>2</sup>Department of Botany, United States National Herbarium, Smithsonian Institution, 4210 Silver Hill Road, Suitland, Maryland 20746. faustm@si.edu, <sup>3</sup>Laboratoire des Micro-Algues Toxiques Institut Louis Malardé, BP 30 98713 Papeete, Tahiti. MChinain@ilm.pf, <sup>4</sup>Tropical Marine Science Institute, 14 Kent Ridge Road, National University of Singapore, Singapore 119223 tmsmj@nus.edu.sg

### Abstract

Comparisons of new and extant *Gambierdiscus* species using morphological and molecular techniques have led to questions concerning the type species *Gambierdiscus toxicus*. The original description of *G. toxicus* was made from cells collected from the Gambier Islands that likely represent multiple species. This is indicated by the wide range of trans-diameter measurements which are greater than for all other described *Gambierdiscus* species combined. Unfortunately the number of species included in the original description cannot be resolved because the type material no longer exists. Given the need for definitive taxonomic identifications of this toxicologically important species, we propose: 1) that Fig. 1 in Adachi and Fukuyo (1979) be designated as lectotype for this species and 2) that the isolate *G. toxicus* GTT-91 described in Chinain *et al.* (1999), which has been well characterized both morphologically and genetically, be designated as the epitype under Article 9.7 of the International Code of Botanical Nomenclature.

### Introduction

Dinoflagellate species belonging to the genus *Gambierdiscus* have a pantropical distribution. An impetus for studying the genus is the production of toxins or precursors by some of the *Gambierdiscus* species that may lead to ciguatera fish poisoning (CFP). Worldwide more than 50,000 victims are stricken annually. The incidence of CFP is both spatially and temporally unpredictable. Whether this variability is due to differences in the relative abundance of species with varying toxicities, environmental induction of toxin production or other factors is not currently known. A major impediment to addressing these questions is the difficulty associated with accurately identifying *Gambierdiscus* species. *Gambierdiscus* cells observed using light microscopy have been typically referred to as either *Gambierdiscus toxicus* or *Gambierdiscus* sp., despite evidence that the genus is a multispecies complex with overlapping geographic distributions (Faust 1995; Holmes 1998; Chinain *et al.* 1999). This research was undertaken to better characterize *Gambierdiscus* species morphologically and molecularly as a prerequisite for developing species-specific molecular assays. During the course of this project we were able to establish many new isolates as well as obtain genomic DNA from four of the previously described species: *G. australes*, *G. pacificus*, *G. poly-*

*nesiensis*, and *G. yasumotoi* as well as isolates of *G. belizeanus*. The only species for which type material is not available is *G. toxicus*. Normally, this situation would be addressed by collecting new material from the type locality. However, despite an excellent description of the genus in the original report of *G. toxicus* (Adachi and Fukuyo 1979), the data presented below indicate that the original material almost certainly included multiple species obviating the utility of new collections from the type locality.

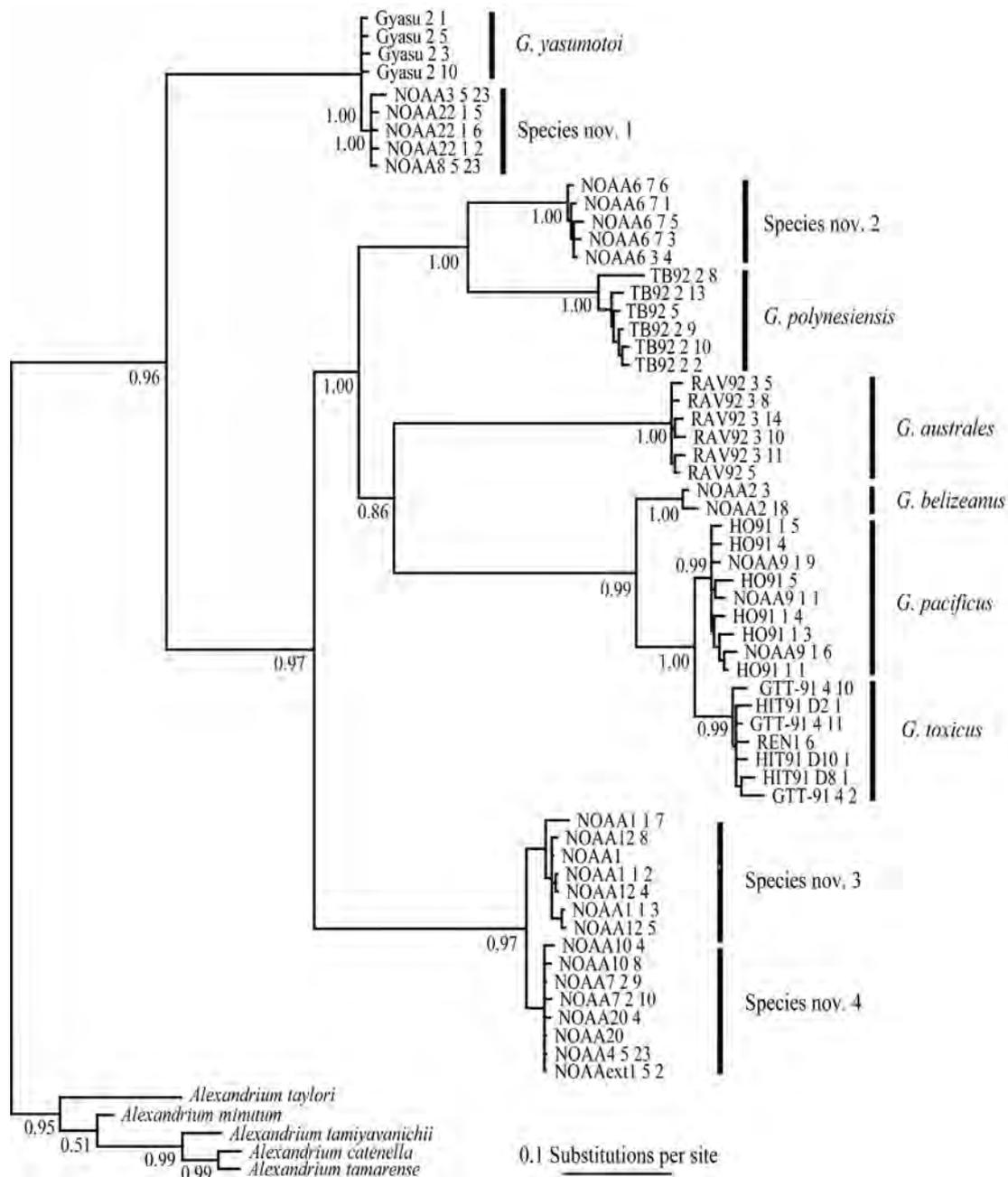
### Materials and Methods

Clonal cultures were made from field material collected in Belize, Central America and from Florida and North Carolina, USA. Single-cell isolates were also made from the *Gambierdiscus* cultures obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. Cells were prepared for detailed examination using scanning electron microscopy (SEM) as described in Faust (1995). Cell sizes were measured using SEM. Genomic DNA was extracted and the SSU through D1-D3 LSU ribosomal DNA (rDNA) region was PCR amplified, cloned and sequenced using the methods presented in Litaker *et al.* (2003). The resulting SSU sequences were aligned and phylogenetic relationships estimated using a Bayesian maximum likelihood analysis.

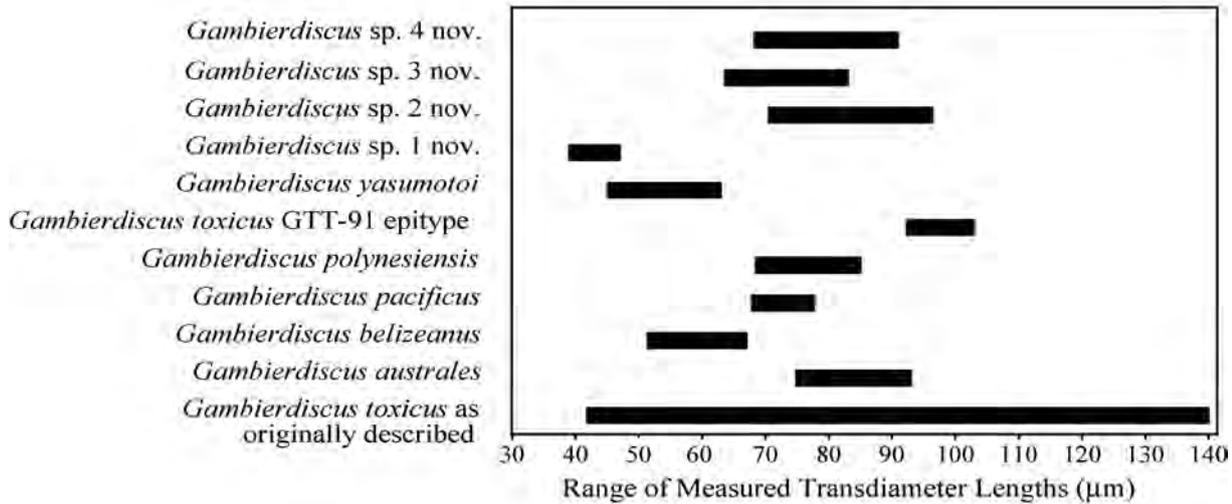
## Results

Phylogenetic analysis of the aligned sequence data indicated the existence of four new *Gambierdiscus* species (Litaker *et al.*, submitted) (Fig. 1). Morphological similarities in cell size and the shapes of thecal plates indicated that some species could not be differentiated without using SEM. When the detailed plate structures of the isolates examined in this study

were compared to those in the original description of *G. toxicus* (Adachi and Fukuyo 1979), we found the data in the original description to be insufficient to differentiate any of the new isolates from *G. toxicus*. For example, the trans-diameter reported for *G. toxicus* (42–140  $\mu\text{m}$ ) was much greater and more variable than the combined range for all of the other described *Gambierdiscus* species (Fig. 2; Faust 1995; Holmes 1998; Chinain *et al.* 1999).



**Figure 1.** Small subunit phylogeny showing the evolutionary relationships among the six known *Gambierdiscus* species and four newly proposed species (Litaker *et al.*, submitted). The NOAA designation for a sequence followed by a number denotes the unique number assigned to that particular single cell isolate. The additional numbers which follow the isolate designation indicate different copies of the SSU region sequenced from that particular isolate.



**Figure 2.** The range in size of *Gambierdiscus* cells reported in the original species description compared with the other known species, including the four new proposed species identified in this study.

## Discussion

The absence of type material, the lack of detailed SEM or calcofluor documentation of thecal plates, and the broad range in cell size reported for *G. toxicus* (Adachi and Fukuyo 1979) make it difficult to definitively identify cells as *G. toxicus*. Thus, despite the fact that the original *Gambierdiscus* genus description is excellent and has proven robust for subsequent species descriptions, there is currently no way to unambiguously identify cells in field samples or cultures as being "*G. toxicus*". These ambiguities further preclude the isolation of new *G. toxicus* isolates from the type location which could serve as a definitive epitype.

To resolve this situation, we propose 1) that Fig. 1 in Adachi and Fukuyo (1979) be designated as the lectotype for this species and 2) that the isolate *G. toxicus* GTT-91 described in Chinain *et al.* (1999) be designated as the epitype under Article 9.7. of the ICBN. Of all the isolates examined, GTT-91 matched the original description most closely. Further, the GTT-91 isolate is well-defined morphologically and genetically, and preserved material has been deposited in the U.S. National Herbarium, Smithsonian Institution, Washington DC. 20560. Establishing this well-defined epitype will help advance future studies on *Gambierdiscus* species.

It should also be noted that the overlapping size and geographic distributions, as well as the similar morphology of certain species observed in this study

complicate methods based on light microscopy for distinguishing *Gambierdiscus* species. In the future, routine quantitative detection of these cells will likely be based on quantitative PCR or in situ hybridization assays.

## Acknowledgements

We thank Dr. Klaus Rützler, NMNH, Smithsonian Institution for supporting long-term research in Belize. Special thanks to Michael Carpenter and Bertol Pfeiffer for logistic support. Øjvind Moestrup, Paul Silva and Antonio Calado provided advice on the epitype designation. This investigation was supported by grants from the NMNH Caribbean Coral Reef Ecosystem Program (CCRE) and The Center for Coastal Fisheries Habitat Research, National Ocean Service, NOAA. CCRE Publication no. 815.

## References

- Adachi, R. & Fukuyo, Y. (1979). Bull. Jpn. Soc. Sci. Fish. 45: 67-71.
- Chinain, M., Faust, M.A. & Pauillac, S. 1999. J. Phycol. 35: 1282-1296.
- Faust, M.A. (1995). J. Phycol. 31: 996-1003.
- Holmes, M.J. (1998). J. Phycol. 34: 661-668.
- Litaker, R.W., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Steidinger, K.A., Millie, D.F., Bendis, B.J., Pigg, R.J. & Tester, P.A. 2003. J. Phycol. 39: 754-761.



## 9. TOXICOLOGY



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Prolonged toxicity of *Scrobicularia plana* after a PSP event and its relation to *Gymnodinium catenatum* cyst consumption and toxin depuration

M.L. Artigas<sup>1,2</sup>, A. Amorim<sup>1</sup>, P. Vale<sup>2</sup>, S.S. Gomes<sup>2</sup>, M.J. Botelho<sup>2</sup> and S.M. Rodrigues<sup>2</sup>

<sup>1</sup>Instituto de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, 1749-016 Lisboa, Portugal

<sup>2</sup>Instituto Nacional de Investigação Agrária e das Pescas-IPIMAR, Av. Brasília, 1449-006 Lisboa, Portugal

### Abstract

In contrast to mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*), the clam *Scrobicularia plana* has been observed to maintain PSP toxicity for long periods after a bloom event. Because this species is a deposit feeder, consumption of *Gymnodinium catenatum* cysts from the sediments was suggested as a possible explanation. In the autumn of 2005, a bloom of *G. catenatum* was detected along the NW coast of Portugal. The gut contents of *S. plana* from affected areas were examined for dinoflagellate cysts during and after the bloom, and the PSP content in the bivalves was followed by HPLC. The presence of *G. catenatum* cysts in gut contents was maximal during the bloom, and four months later still represented over 40 % of the cyst assemblage. Depuration experiments with *S. plana* mussels and cockles revealed that toxins in *S. plana* were almost undetectable within 1 week, while during the same period, a reduction to half the toxin burden was observed in mussels and cockles. The prolonged PSP toxicity observed in *S. plana* may primarily be a result of a species-specific slow depuration rate. However, consumption of toxic *G. catenatum* cysts cannot be ruled out as an additional source of toxicity.

### Introduction

Portugal has a two-decade history of monitoring harmful phytoplankton and shellfish for marine biotoxins. *Gymnodinium catenatum* was identified as the major causative species for Paralytic Shellfish Poisoning (PSP) events along the coast of Portugal between 1986 and 1995 (Sampayo 1989; Moita *et al.* 1998). In the autumn 2005, blooms of *G. catenatum* were observed again along the NW coast (Moita *et al.* 2006). Blooms started in October 2005, and maximal cell concentrations were recorded in late November and December. *Scrobicularia plana*, a commercial clam exploited together with mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*), has been observed to maintain PSP toxicity for long periods, in contrast to other bivalves harvested in the area (Vale and Sampayo 2001). *S. plana* is a deposit feeder, and consumption of cysts of *G. catenatum* may explain the prolonged toxicity.

As part of its sexual life cycle, *G. catenatum* produces a fossilizable resting cyst (Anderson *et al.* 1988), which has been shown to be toxic when produced under laboratory conditions (Bravo *et al.* 1998). In 1995, a cyst monitoring programme was started along the Portuguese coast. Results have shown that immediately after bloom events, *G. catenatum* cysts may represent over 70 % of the total cyst assemblage. However, at any given time, the proportion of *G. cat-*

*enatum* cysts with viable cell contents is very low (Amorim *et al.* 2001). At the Atlantic coast of the Iberian Peninsula, cyst germination occurs as soon as the short mandatory resting period is completed and no extensive cyst beds are accumulated in the area. This finding is supported by laboratory results in which *G. catenatum* cysts a very short mandatory dormancy period have been found ( $\leq 12$  days) and also that the cysts do not require environmental conditioning for germination (Blackburn *et al.* 1989; Bravo and Anderson 1994).

In the present study we investigated PSP toxicity in *S. plana* after a bloom of *G. catenatum*, and its possible relation to the consumption of *G. catenatum* cysts.

### Materials and Methods

Samples of naturally contaminated bivalves including *M. galloprovincialis*, *C. edule* and *S. plana* were collected weekly from the Mondego estuary (Lat. 40° 8'N, Long. 8° 50'W, NW coast of Portugal) as part of the ongoing monitoring programme. Whole flesh was used for PSP toxin analysis by HPLC according to Vale and Taleb (2005). Samples of *S. plana* were collected monthly (except January) from natural beds in the Mondego estuary between November 2006 and May 2006 for analysis of gut contents. Guts were dissected from each specimen, and a sample was prepared

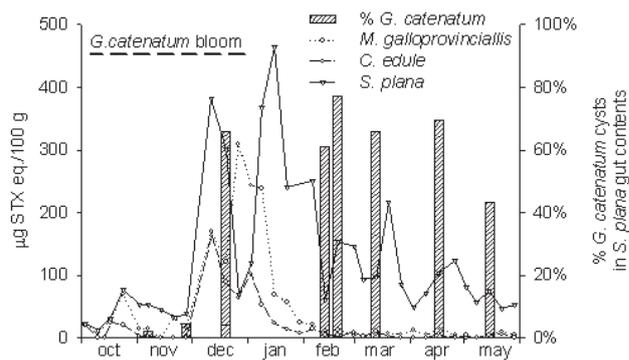
from a pool of 6 individuals in order to reduce variability and obtain a significant number of cysts. Samples were prepared on permanent microscope slides. No chemical treatments were applied in order to have the whole cyst assemblage represented (adapted from Amorim *et al.* 2001). A minimum of 300 cysts were counted in each sample, and results were expressed as percentage of the whole cyst assemblage. In December 2005 and April 2006, surface sediments were collected from the same sites as specimens of *S. plana* for description of cyst assemblages. Samples were collected at low tide using plastic corers (12 cm long; 5 cm i.d), from both non-vegetated (exposed) and vegetated beds (*Zostera noltii*). Cores were extruded and sectioned, and the top cm of sediment was prepared according to Amorim *et al.* (2001). Cyst assemblages were described from permanent slides under LM.

For the detoxification experiment, live specimens of naturally contaminated bivalves from the Mondego estuary were used: *M. galloprovincialis*, *C. edule*, and *S. plana*. Fifty to one hundred individuals, depending on the species, were maintained in tanks with recirculating filtered seawater and fed with non-toxic algae (*Thalassiosira* sp. and *Isochrysis* sp.) between 7 and 21 days. Every one or two days, 12 individuals were taken from each tank; the hepatopancreas was removed and analysed for PSP toxins by HPLC according to Vale and Taleb (2005).

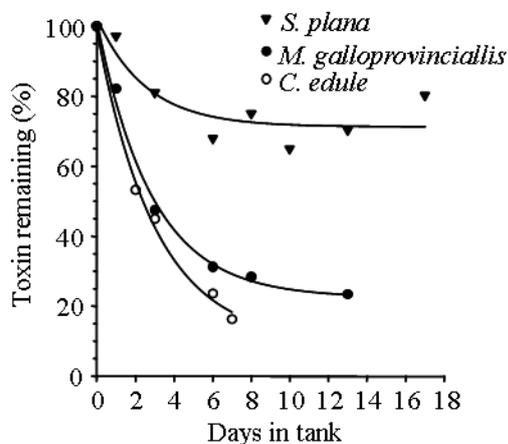
## Results and Discussion

The PSP toxin profile detected in all three bivalve species was representative of contamination by *G. catenatum* (Artigas *et al.*, unpubl. data). The pattern of PSP toxicity is plotted in Fig. 1. The increase in PSP toxicity in mussels and cockles coincided with the observed maximum concentration of *G. catenatum* on the NW coast (Moita *et al.* 2006). Several hypotheses may explain the time lag between *G. catenatum* maximum cell density and maximum toxicity in shellfish, from methodological aspects like vertical migration and the instantaneous nature of phytoplankton to changes in the accompanying species. However, the toxicity pattern of *S. plana* showed higher and prolonged toxicity in relation to mussels or cockles.

Cyst assemblages in sediments from *S. plana* beds were dominated by *G. catenatum* cysts (Table 1). Results indicate that several months after bloom decline cysts were still present in high relative abundance both in exposed sediments and in sea grass beds. However, potentially viable cysts (protoplast present) of *G. catenatum* were very rare, and declined with



**Figure 1.** PSP toxicity in bivalves (lines) from Mondego estuary between autumn 2005 and spring 2006, and *G. catenatum* cysts (bars) in *S. plana* gut contents as percentage of total cyst assemblage.



**Figure 2.** Laboratory controlled detoxification experiments with 3 different bivalve species, naturally contaminated with PSP.

time. These results agree with Amorim *et al.* (2001) in that at any given time, few non-germinated cysts are present in surface sediments. The high numbers of empty cysts is interpreted as being related to the species' short mandatory dormancy period and year round favourable environmental conditions for excystment in warm-temperate zones.

Dinoflagellate cysts were always recorded in the gut contents of *S. plana* (Fig. 1). In November 2005, cysts of *G. catenatum* represented only a small proportion. From December 2005 until May 2006, five months after the bloom declined, *G. catenatum* cysts represented more than 40 % of the gut cyst assemblage, reaching ca. 80 % in February.

Cysts of *G. catenatum* are very resistant to decay. However, not all dinoflagellate cysts have a resistant wall, and they can be affected by chemical processes such as the digestive system of grazers. Persson and

Rosenberg (2003) showed that with time grazing processes might affect cyst assemblages by positively selecting for resistant fossilizable cysts. This could explain the higher proportion of *G. catenatum* cysts in gut contents relative to cyst assemblages in shellfish beds, and the persistent high values recorded inside *S. plana*. Live *G. catenatum* cysts always represented less than 1% of the total gut assemblage. This value is lower than in the sediments, indicating that some of the grazed cysts were digested. Since *G. catenatum* cysts have been shown to be toxic (Bravo *et al.* 1998), we cannot rule out that part of the prolonged toxicity in *S. plana* might be due to cyst consumption, as reported by Persson *et al.* (2006) for *Alexandrium* cysts in oysters. To conclusively evaluate the role of *G. catenatum* cysts in shellfish toxification, further work based on controlled laboratory experiments is needed.

Figure 2 and Table 2 show the results obtained with laboratory detoxification experiments. The three species tested showed clear differences in PSP detoxification kinetics: *M. galloprovincialis* and *C. edule* lost half the toxicity within 3 days, while at the end of the experiment (17 days), *S. plana* still retained 80 % of the toxin. The persistence of PSP toxicity in bivalves may therefore depend on species-specific detoxification kinetics. Under the experimental conditions tested, *S. plana* showed the lowest depuration rates, in agreement with results obtained from natural populations (Fig. 1).

## Conclusions

Blooms of *G. catenatum* along the Portuguese coast have been responsible for the main PSP events during the past 20 years. There is indication that the initial PSP toxification process is related to the availability of cells in the plankton. Once established, toxicity depends on other factors, such as species-specific detoxification kinetics and possibly trophic mechanisms

(plankton vs benthic feeder) related to consumption of toxic resting cysts.

## References

- Amorim, A. Dale, B., Godinho, R. & Botas, V. (2001). *Phycologia* 40: 572-582.
- Anderson, D.M., Jacobsen, D.M., Bravo, I. & Wrenn, J.H. (1988) *J. Phycol.* 24: 255-262.
- Artigas, M.L., Vale, P., Gomes, S. S., Botelho, M.J., Rodrigues, S.M. & Amorim, A. (2007). Submitted to *J. Chromatography A*.
- Blackburn, S.I., Hallegraeff, G.M. & Bolch, C.J. (1989). *J. Phycol.* 25: 577-590.
- Bravo I. & Anderson D.M. (1994). *J. Plankton Res.* 16: 513-525.
- Bravo, I., Franco, J.M., & Reyero, M. I. (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M.L., and Wyatt, T., (eds), Xunta da Galicia and IOC of UNESCO, Spain, pp. 356-358.
- Moita, M.T., Vilarinho, M.G. & Palma, A.S., (1998). In: *Harmful Algae*, Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T. (eds), Xunta de Galicia and IOC of UNESCO, Spain, pp. 182-183.
- Moita, M.T., Palma, S., Oliveira, P.B., Vidal, T., Silva, A. & Vilarinho, M.G. (2006). PO.06-14, Programme and Abstracts, 12th International Conference on Harmful Algae, Denmark, p. 242.
- Persson, A. & Rosenberg, R. (2003). *Harmful Algae* 2: 43-50.
- Persson, A., Smith, B. C., Wikfors, G.H. & Quilliam, M. (2006). *Harmful Algae* 5: 678-684.
- Sampayo, M.A.M., (1989). In: *Red Tides: Biology, Environmental Science and Toxicology*, Okaichi, T., Anderson, D.M. & Nemoto, T. (eds), Elsevier, New York, pp. 89-92.
- Vale, P. & Sampayo, M.A.M. (2001). *Toxicon* 39: 561-571.
- Vale, P. & Taleb, H. (2005). *Food Additives and Contaminants* 22: 838-846.

## Evaluation of the toxicity of *Prorocentrum* species by liquid chromatography-mass-spectrometry and cell-based assay

A. Caillaud<sup>1,2\*</sup>, E. Cañete<sup>1,2</sup>, E. Mallat<sup>1,2</sup>, M. Fernández<sup>1,2</sup>, N. Mohammad-Noor<sup>3,5</sup>, Ø. Moestrup<sup>3</sup> and J.M. Franco<sup>4</sup>

<sup>1</sup> Centre d'Aqüicultura, IRTA, Ctra. Poble Nou s/n, 43540 Sant Carles de la Ràpita, Spain. <sup>2</sup> CRA, Centre de Referència en Aqüicultura, CIRIT-Generalitat de Catalunya, Spain. <sup>3</sup> Phycology Department, Biological Institute, University of Copenhagen, Øster Farimagsgade 2D, 1353 Copenhagen K, Denmark. <sup>4</sup> Fitoplàncton Tóxico, Instituto de Investigaciones Marinas de Vigo (CSIC), 36 200 Vigo, Spain. <sup>5</sup> School of Science and Technology, University Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia.

\* Author for correspondence : amandine.caillaud@irta.es

### Abstract

Dinoflagellates belonging to the genus *Prorocentrum* were studied for toxicological and toxin production properties by cell assays and liquid chromatographic methods, using fluorescence and mass-spectrometry detection (LC-FD, LC-MS). Cytotoxicity, characterised by IC<sub>50</sub> and morphological changes, was examined. IC<sub>50</sub> ranged from 2 · 10<sup>3</sup> cell equivalents mL<sup>-1</sup> for *P. cf. faustiae* to 133 · 10<sup>3</sup> for *P. rhathymum*. Morphological changes such as rounding and membrane blebbing, were observed in fibroblasts exposed to *Prorocentrum belizeanum* and all *Prorocentrum lima* from Malaysia, suggesting the presence of okadaic acid (OA). Analytical measurements confirmed the production of OA. However, toxicity not correlated with the presence of OA suggests the production of derivatives of OA or others toxins.

### Introduction

Some *Prorocentrum* spp. have been reported to produce okadaic acid (OA), a diarrhetic shellfish poisoning (DSP) toxin. Toxicological properties of eight species of *Prorocentrum*: *Prorocentrum rhathymum*, *Prorocentrum cf. faustiae*, four strains of *Prorocentrum lima* (NMN01, NMN04, NMN07 and Vgo 620), *Prorocentrum belizeanum* and *Prorocentrum* sp.1 were evaluated. Methanol extracts of each species were analyzed by liquid-chromatography mass-spectrometry for detection and quantification of some of the toxins produced, and the cytotoxicity was determined using a cell-based assay.

### Methods

#### Cytotoxicity assays

a) Toxicity quantification: determination of IC<sub>50</sub> for each extract

*In vitro* cultured fibroblasts BGM (Buffalo Green Monkey) were exposed to crude extracts of *Prorocentrum* and cell viability was determined using a cell viability assay (Neutral Red Uptake Test). IC<sub>50</sub> is expressed as cell equivalents mL<sup>-1</sup>. A standard of OA with an IC<sub>50</sub> of 250 · 10<sup>-3</sup> µM, was used as a positive control and *Tetraselmis chui* as a negative control.

#### b) Morphological alterations

To assess the presence of specific toxins, a first approach was observation by light microscopy of morphological damage caused by the algal ex-tracts on BGM fibroblasts.

#### LC-FD, LC-MS analysis: determination and quantification of toxin production

Cell cultures of each dinoflagellate were filtered, extracted twice in methanol and analyzed by liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography coupled to a fluorescence detector (LC-FD) to detect and quantify the presence of OA and its derivatives. LC-FD measurements were carried out according to a modification of Lee's derivatization and separation method for the detection of okadaic acid (Lee *et al.* 1987). Separation was performed on an octadecylsilica column, mobile phases consisted of acetonitrile/H<sub>2</sub>O (80:20), and fluorimetric detection was performed at λ<sub>exc</sub> 365 nm and λ<sub>e</sub> 412 nm. LC-MS measurements were carried out on a liquid chromatograph coupled to an ion-trap mass spectrometer (Thermo Finnigan LCQ Advantage) using an electrospray interface in the positive ion mode. Separation was conducted on an octadecylsilica column, and the mobile phase was 2 mM NH<sub>4</sub>AC pH 5.8/ MeOH (30:70).

## Results

*Toxicity quantification* (Table 1).

The 3 strains of *P. lima* from Malaysia together with *P. cf. faustiae* and *P. belizeanum* were the most toxic species for the BGM fibroblasts.

*Prorocentrum lima* NMN01 and NMN07 from Sipadan Island had similar effect on the fibroblasts as *P. lima* NMN04 from Sepangar Bay ( $p=0.194$ ). *Prorocentrum lima* from Vigo displayed very low cytotoxic activity, indicating a regional diversity of toxicity within this species. IC50 of *Prorocentrum* sp.1 and *P. rhathymum* were very high compared to the other *Prorocentrum* spp. An extract of *Tetraselmis chui* did not show any toxicity to the fibroblasts at concentrations up to  $159 \cdot 10^3$  *T. chui* cells mL<sup>-1</sup>, confirming that the cytotoxicity measured was not due to an effect of the algal matrix.

*Morphological alterations* (Fig. 1).

Fibroblasts showed time-dependent alterations of their morphology when exposed to the extracts of *Prorocentrum* spp. except *P. rhathymum* from Malaysia.

*P. rhathymum* did not cause morphological changes when tested at a concentration corresponding to the IC50 (Fig. 1, G).

Cells exposed to the three strains of *P. lima* from Malaysia NMN01, NMN04 and NMN07 (Fig. 1, B, C, D) and *P. belizeanum* (Fig. 1, I) became rounded, and detached from the support. Several blebs were produced. This type of alteration is typical of the presence of OA (Fig. 1, F).

Fibroblasts exposed to *P. cf. faustiae* (Fig. 1, H) showed different alterations: cells became thinner and remained attached to the support. Numerous blebs were produced. *P. lima* from Vigo (Fig. 1, E) and *Prorocentrum* sp. 1 (Fig. 1, J) produced large blebs on rounded cells which induced lysis. These very distinctive alterations indicate the presence of another type of toxin.

*LC-FD, LC-MS analysis: evaluation of toxin production*

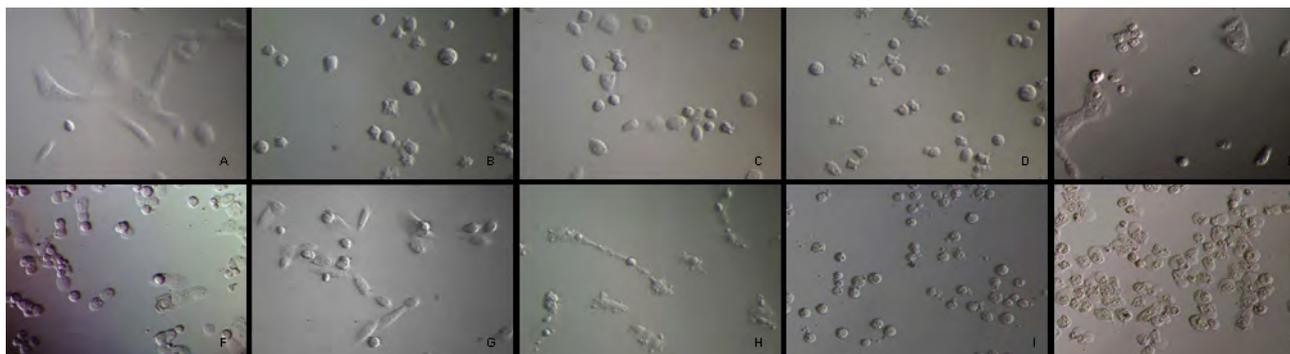
Okadaic acid, esterified forms of OA, and DTX-1 were detected in *P.lima* NMN01, NMN04 and NMN07

**Table 1.** IC50 of *Prorocentrum* spp. tested in cell eq. mL<sup>-1</sup>. CV, Coefficient of variation.

Origin	Species	IC <sub>10</sub>	
		Cells eq mL <sup>-1</sup>	CV %
Sipadan Island, East Malaysia	<i>Prorocentrum lima</i> NMN01	2097	6,8
	<i>Prorocentrum lima</i> NMN07	2383	25,9
Sepangar Bay, East Malaysia	<i>Prorocentrum lima</i> NMN04	3189	11,2
Sabah, East Malaysia	<i>Prorocentrum cf. faustiae</i>	2041	13,7
	<i>Prorocentrum rhathymum</i>	132595	23,8
Vigo, Spain	<i>Prorocentrum lima</i> V go620	68000	35,5
La Réunion, France	<i>Prorocentrum belizeanum</i>	8850	6,8
Alfacs Bay, Spain	<i>Prorocentrum</i> sp.1	35022	19,6

**Table 2.** Detection of okadaic acid, OA-D8 and DTX-1 by LC-MS, in *P. lima* NMN01, NMN04, NMN07; *P. rhathymum* and *P. cf. faustiae*. Detection of OA by LC-FD for *P. lima* Vgo620, *P. belizeanum* and *Prorocentrum* sp. 1. NT Not tested, - Not detected, + Detected

Detection method	Species	OA	OA-D8	DTX-1
LC-MS	<i>Prorocentrum lima</i> NMN01	+	+	+
	<i>Prorocentrum lima</i> NMN07	+	+	+
	<i>Prorocentrum lima</i> NMN04	+	-	+
	<i>Prorocentrum cf. faustiae</i>	-	-	-
	<i>Prorocentrum rhathymum</i>	+	+	
HPLC	<i>Prorocentrum lima</i> V go620	-	NT	NT
	<i>Prorocentrum belizeanum</i>	+	NT	NT
	<i>Prorocentrum</i> sp.1	-	NT	NT



**Figure 1.** Fibroblasts (BGM) exposed to extracts of *Prorocentrum* spp. (A) Control after 3 h of exposure; (B) *P. lima* NMN01 (7 401 cell eq. mL<sup>-1</sup> MEM) exposed for 3 h ; (C) *P. lima* NMN07 (7 839 cell eq. mL<sup>-1</sup> MEM) 3 h ; (D) *P. lima* NMN04 (3 370 cell eq. mL<sup>-1</sup> MEM) 3 h ; (E) *P. lima* Vgo620 (172 103 cell eq. mL<sup>-1</sup> MEM) exposed for 3 h ; (F) OA standard (150 ng mL<sup>-1</sup> MEM) exposed for 6 h ; (G) *P. rhathymum* (13 104 cell eq. mL<sup>-1</sup> MEM) exposed for 6 h ; (H) *P. cf. faustiae* (4 941 cell eq. mL<sup>-1</sup> MEM) exposed for 3 h ; (I) *P. belizeanum* (8 740 cell eq. mL<sup>-1</sup> MEM) exposed for 6 h ; (J) *Prorocentrum* sp. 1 (10 104 cell eq. mL<sup>-1</sup> MEM) exposed for 6 h. X200, inverted microscope Nikon Eclipse TE2000-S.

(Table 2). Okadaic acid was also found in extracts of *P. rhathymum* from Malaysia, *P. belizeanum* from the Indian Ocean and *P. lima* from Malaysia. On the other hand, *P. lima* Vgo 620 and *Prorocentrum* sp. 1 did not produce OA, but some indications of OA derivatives could be seen for *P. lima* Vgo620 and *Prorocentrum* sp. 1. Regarding *P. cf. faustiae*, no detectable concentrations were observed for OA, its derivatives or esterified forms.

### Discussion and Conclusion

Among the species of *Prorocentrum* successfully cultured and examined, morphological alterations of fibroblasts related to the presence of OA were confirmed in *P. lima* NMN01, NMN04, NMN07 and *P. belizeanum*. Other effects may have been associated with the presence of derivatives and esterified forms of OA. However, the lack of production of OA and DTX-1 by *P. cf. faustiae* is in contradiction with the literature (Morton 1998), and it suggests the presence of other OA derivatives or other toxins. However, identification of this species needs further study before final conclusions can be made.

This work showed variation in toxicity within the genus *Prorocentrum* and also within the species *P. lima*. Results obtained by LC-FD and LC-MS allow for identification of the toxin produced, and the effect obtained by cellular assay are used to evaluate the toxicity. Combination of the two methods is a

valuable tool for characterisation of the toxicological properties, which could be applied to a wide range of *Prorocentrum* species.

### Acknowledgements

We acknowledge support from the technical staff at IRTA-Sant Carles de la Ràpita. *Prorocentrum belizeanum* RN01 was originally isolated by J.P. Quod and J. Turquet from ARVAM. This project was funded by project ACU-02-005 - INIA / AGL2005-07924-CO4-CO2 2005-2008 - Ministry of Education and Science, Spanish Government/Centre de Referència en Aqüicultura, DURSI, Generalitat de Catalunya. We also acknowledge an INIA PhD grant to A. Caillaud. NM-N acknowledges financial support from the Malaysian Government.

### References

- Diogène, G., Fessard, V., Dubreuil, A. & Puiseux-Dao, S. (1995). Toxic. *in vitro* 1: 1-10.
- Lee, J. S., Yanagi, T., Kenma, R. & Yasumoto, T. (1987). Agric. Biol. Chem. 51: 877-881.
- Morton, S.L., Moeller, P.D., Young, K.A. & Lanoue, B. (1998). Toxicon 36: 201-206.
- Murakami, Y., Oshima, Y. & Yasumoto, T. (1982). Bull. Japan Soc. Sci. Fish. 48: 69-72.
- Heredia-Tapia, A.H., Arredondo-Vega, B. O., Nuñez-Vázquez, E.J., Yasumoto, T., Yasuda, M. & Ochoa, J.L. (2002). Toxicité 40: 1121-1127.

## *Dinophysis sacculus* from Alfacs Bay, NW Mediterranean. Toxin profiles and cytotoxic potential

Cañete E.<sup>1,2</sup>, Caillaud A.<sup>1,2</sup>, Fernández M.<sup>1,2</sup>, Mallat E.<sup>1,2</sup>, Blanco J.<sup>3</sup> and Diogène J.<sup>1</sup>

<sup>1</sup> Centre d'Aqüicultura, IRTA, Ctra. Poble Nou s/n, 43540 Sant Carles de la Ràpita, Spain, elisabeth.canete@irta.es, <sup>2</sup> CRA, Centre de Referència en Aqüicultura, CIRIT-Generalitat de Catalunya, Spain, <sup>3</sup> CIMA, Centro de Investigacions Mariñas, Galicia, Spain

### Abstract

A bloom of *Dinophysis sacculus* was detected in a small pond connected to Alfacs Bay (NW Mediterranean) during the fall of 2005, allowing estimation of the toxin profile of this species. In some samples collected, *D. sacculus* represented more than 90% of the phytoplankton, reaching concentrations of up to 81 600 cells L<sup>-1</sup>. Cytotoxicity assays and analytical procedures were used to identify the toxins and evaluate the toxic potential of this species. Toxicity was examined by cell viability estimation, and with evaluation of the morphological effect on neuroblastoma cells (N2a). Results were compared with okadaic acid (OA) and other diarrhetic shellfish poisoning (DSP) reference material. LC-MS analysis allowed the detection of OA and pectenotoxins (PTXs). OA equivalents per *D. sacculus* cell were estimated to be 17.75 pg cell<sup>-1</sup>.

### Introduction

*Dinophysis sacculus* is common in the Mediterranean as well as in Atlantic European waters (Reguera *et al.* 2003). It is the principal cause of diarrhetic shellfish poisoning (DSP) toxic episodes in seafood production areas in the Ebre Delta, Spain, and it has been found to produce okadaic acid (Delgado *et al.* 1996). As for other species of the genus *Dinophysis*, it is difficult to obtain cultures that would facilitate study of the toxins.

To investigate the toxin profile and toxicity of *Dinophysis sacculus* in the Ebre Delta Bays two strategies were selected: cytotoxicity assays and chromatographic analysis.

### Methods and Results

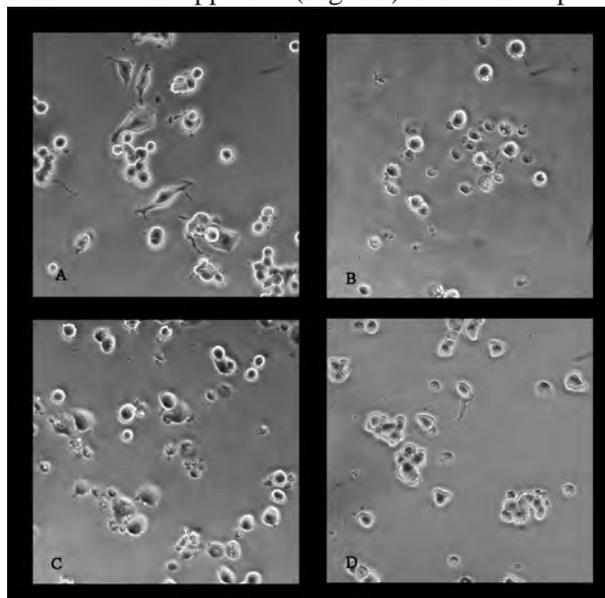
#### Material

Samples of filtered water (GF/F filters) were collected during a *D. sacculus* bloom in a small pond in Alfacs Bay from 29 October 2005 to 2 January 2006.

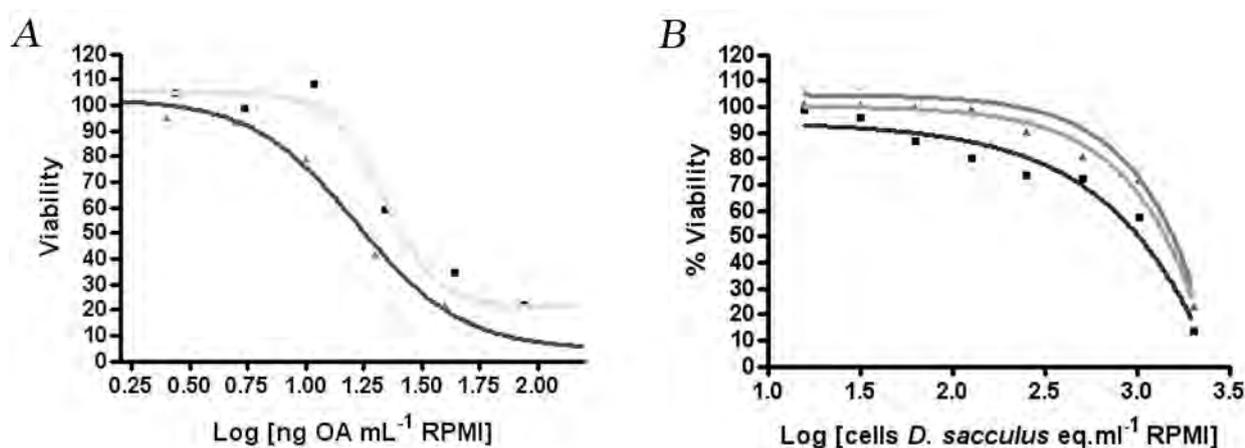
To expose mussels (*Mytilus galloprovincialis*) to *D. sacculus*, mussel samples (n = 210) were suspended close to the surface at the study site. Mussels were collected every week (n = 30) for six weeks following their introduction into the pond. During this period, temperature and salinity were 6.3-12.8 °C and 26.6-34.7 psu, respectively. Hepato-pancreas from mussels were processed to extract lipophilic toxins. The homogenised hepatopancreas were extracted by sonication with methanol:water (80:20) and extract supernatants filtered through a 0.45-µm nylon filter. The filtrate was used for further analysis by either LC-MS or cytotoxicity assay.

#### Cytotoxicity assays

Neuroblastoma (N2a) cells were used to conduct cytotoxicity studies. This cell type has been used previously to evaluate OA activity (R. Dickey, pers. comm.). N2a cells exposed to okadaic acid (OA) and pectenotoxin-2 (PTX2) showed different morphological alterations (Fig. 1). In the OA treatment, swelling of cells was observed (Fig. 1C), blebs appeared on the membrane surface (Fig. 1C, 1B), and the cells rounded up and detached from the bottom (Fig. 1C, 1B). At high doses, cells became thinner, and remains from dead cells appeared (Fig. 1B). N2a cells exposed



**Figure 1.** Effect of OA and PTX-2 on N2a cells: control (A), cells exposed to different doses (24 h) of OA (B: 49 ng mL<sup>-1</sup> RPMI; C: 24 ng mL<sup>-1</sup> RPMI) and PTX2 (D: 215 ng mL<sup>-1</sup> RPMI). Phase contrast 200x magnification; Nikon Eclipse TE2000-S inverted microscope.



**Figure 2.** Cytotoxicity evaluation. Dose-response curves of N2a cells exposed to okadaic acid (OA) and for sample F221205.

**Table 1.** Sensitivity of N2a cells to OA, *D. sacculus* extract, and estimation of OA equivalent in *D. sacculus*.

	EXP 1	EXP 2	EXP 3	$\bar{x}$	sd	CV%
OA IC <sub>50</sub> : ng mL <sup>-1</sup> RPMI	18.05	26.31	62.31			
IC <sub>50</sub> : <i>D. sacculus</i> cell equivalent/ml RPMI	1018.47	1412.18	1557.10	1329.25	278.73	4,89
pg OA equivalent/ <i>D. sacculus</i> cell	17.73	18.63	16.90	17.75	0.87	4.89

to PTX2 (Fig. 1D) aggregated into clusters. Further work is required to establish the relationship between morphological effects and toxin exposure in N2a cells.

#### *N2a* viability assay

To study the toxic potency of *D. sacculus*, we examined several samples. We focused our work on one water sample (F221205) in which 99% of the phytoplankton cells were *D. sacculus* and on a hepatopancreas extract (DM050106) from a mussel that had been submerged for 24 d in the presence of *D. sacculus*.

We used the MTT cytotoxicity assay to evaluate N2a cell viability (Manger *et al.* 1993). According to dose-response curves of three experiments (Fig. 2B), the IC<sub>50</sub> for *D. sacculus* in extract F221205 was estimated at 1329 cell equivalents mL<sup>-1</sup> RPMI medium. OA IC<sub>50</sub> evaluation (Fig. 2A) on the same day of the experiment allowed to estimate the potency of the extracts. In extract F221205 we estimated 17.75 pg of OA equivalents per cell of *D. sacculus* in each experiment (Table 1). The response of N2a cells exposed to mussel extract (DM050106, 43,8 mg of mussel tissue mL<sup>-1</sup> RPMI) allowed to obtain a preliminary estimation of 2771 ng of OA equivalents in 1 g of mussel hepatopancreas.

#### Cytotoxin profile

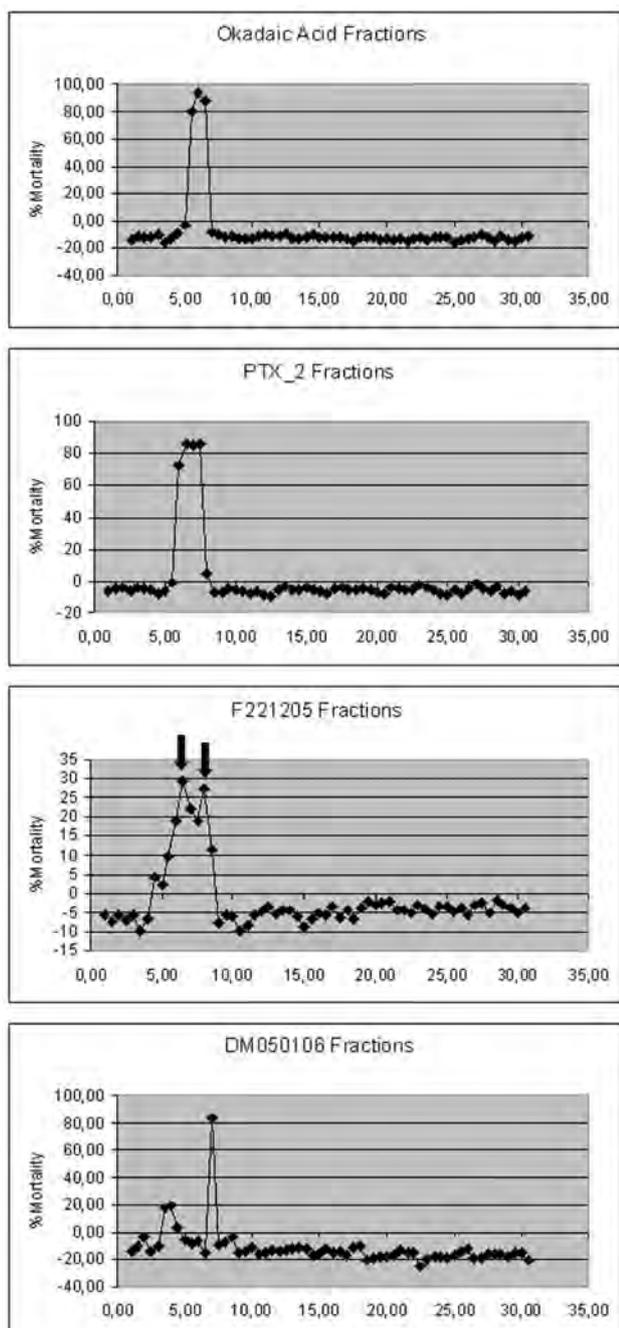
To conduct cytotoxicity studies in semi-purified extracts, crude extracts were injected into an HPLC (mobile phase: 65 % ACN, 35 % water; 0.3 mL min<sup>-1</sup>

- Luna C18 column; 30 min run, 30 °C) and fractions of the eluate were collected every 30 s. Approximately 60 fractions were obtained for each crude extract. N2a cells were exposed to each fraction of the extracts and cell viability was evaluated after 24 h (Fig. 3). Standards and extracts fractionated included: OA (800 ng), PTX2 (430 ng), and the extracts from samples F221205 (6936 equiv. cells of *D. sacculus*) and DM050106 (0.0476 g equiv. of mussel hepatopancreas). Similar retention times were obtained for OA (from 5.5 to 6.5 min) and PTX-2 (from 6 to 8 min). In F221205, cell viability was reduced by fractions obtained between 4.5 and 8.5 min. It could not be determined whether the two small peaks (arrows at 6.5 and 8 min; Fig. 3) corresponded to two different toxins. In DM050106 at least two types of toxins were present, the first eluting from 3.5 to 4.5 min and the second eluting at 7 min.

#### Chromatographic Analysis

Samples of filtered water and mussel extracts from the *D. sacculus* episode were analyzed by LC-MS using a Thermo Deca XP plus (spray voltage 4.5 kV, capillary T 250 °C, capillary voltage: 5V) with a BDS HypersilC8, 50 x 2 mm, 3 μm column (20 °C), a mobile phase of 50% H<sub>2</sub>O-ACN, and a flow of 0.2 mL min<sup>-1</sup> (Suzuki *et al.* 2003).

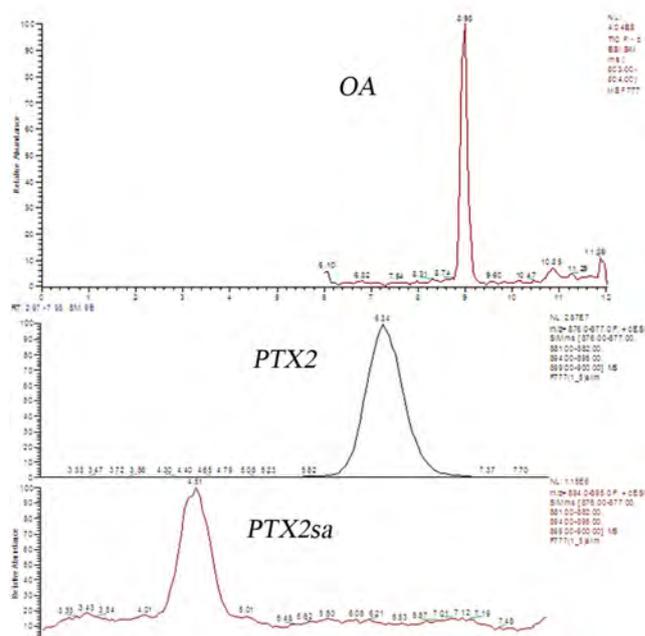
Chromatographic analysis of filtered water samples confirmed the presence of OA, OA esters, PTX-2 and PTX-2sa (Fig. 4). Chromatographic analysis of mussel extract samples collected during the episode confirmed the presence of OA, PTX-2, and PTX-2sa.



**Figure 3.** N2a cell mortality exposed (24 h) to 60 HPLC fractions of OA standard, PTX-2 standard, and crude extracts of *D. sacculus* and *M. galloprovincialis*.

### Conclusions

The Monitoring Programme for Harmful Algae enabled us to identify the toxic episode of *Dinophysis sacculus* and allowed study of its toxins. Research on the toxic profile of *D. sacculus* using chemical analysis and cytotoxic assays provided conclusive results regarding its toxicity, toxin content, and on toxin accumulation in mussels. Further work is needed to describe the relationship between OA content in mussels and cytotoxicity response.



**Figure 4.** LC-MS chromatograms corresponding to the selected m/z 803.5 (OA), m/z 876.5 (PTX2, m/z corresponding to the ammonium adduct), and m/z 894.5 (PTX2sa, m/z corresponding to the ammonium adduct), of the sample F221205.

LC-MS analyses demonstrated the presence of PTX-2 and OA in mussels and *D. sacculus* samples. The presence of OA in *D. sacculus* from Ebre Delta Bays were reported by Delgado *et al.* (5 pg cell<sup>-1</sup>), but this is the first evidence of PTX2 in *D. sacculus*. Other authors have found PTX2 in *Dinophysis acuta*, *D. acuminata* and *D. caudata* (Reguera 2003).

Linking HPLC analysis with extract fractionation and cytotoxicity evaluation is a valuable approach for obtaining information on the presence of toxins.

### Acknowledgements

We acknowledge support received by technical staff from IRTA-Sant Carles de la Rapita.

This study was funded by: project ACU-02-005, INIA, Ministry of Education and Science, Spanish Government/Centre de Referència en Aquicultura, DURSI, Generalitat de Catalunya.

### References

- Delgado, M., Garcès, E. & Camp, J. (1996). In: Harmful and Toxic Algal Blooms, Yasumoto, T. & Oshima, Y. (eds.), UNESCO, Paris, pp. 261-264.
- Manger, R.L., Leja, L.S. & Lee, S.Y., Hungerford J.M. & Wekell, M.M. (1993). *Anal. Biochem.* 214: 190-194.
- Reguera, B. (2003). PhD thesis. University of Barcelona.
- Suzuki, T, Beuzenberg, V., Mackenzie, L. & Quilliam, M.A. (2003). *J. Chromatogr. A* 992: 141-150.

## Cytotoxic and genotoxic effects of microcystins in mammalian cell lines

E. Dias<sup>1,2</sup>, P. Pereira<sup>2</sup>, M.C.C. Batoreu<sup>3</sup>, P. Jordan<sup>1</sup> and M.J. Silva<sup>1</sup>

<sup>1</sup>Centro de Qualidade Hídrica, <sup>2</sup> Centro de Genética Humana, Instituto Nacional de Saúde, Av. Padre Cruz 1649-016 Lisboa, Portugal, [elsa.dias@insa.min-saude.pt](mailto:elsa.dias@insa.min-saude.pt),

<sup>3</sup>Centro de Estudos de Ciências Farmacêuticas, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-043 Lisboa, Portugal

### Abstract

Microcystin-LR (MCLR) has been recognized as a tumour promoter, but its carcinogenic mechanisms remain largely unknown. In this work we evaluated the genotoxic potential of microcystins (extracted from *M. aeruginosa* strains) in a mammalian cell line by the micronucleus assay (5-40 µg/mL). Cytotoxicity tests (MTT reduction, LDH release) were used to determine the sensitivity of several cell lines (Vero, HepG2 and AML12) to MCLR (1-175 µg/mL). Although all MCLR-treated cell lines presented some cytotoxic response, Vero cells were the most sensitive, showing more than 80 % decrease in viability when exposed to 22 µg/mL MCLR for 72 h. Preliminary results revealed an aneugenic or clastogenic activity of MCLR (≥ 20 µg/mL) in Vero cells. In summary, we identified a permanent mammalian cell line as a useful model system for microcystin toxicity assessment and we show that MCLR has genotoxic properties.

### Introduction

The chronic effects of human exposure to low doses of microcystins (MC) are poorly understood. Microcystin-LR (MCLR) is considered a tumour promoter (Nishiwaki-Matsushima *et al.* 1992), probably through the inhibition of protein phosphatases. However, it is not clear if MCLR can also act as tumour initiator by a genotoxic mechanism. Several tests with bacteria (Grabow *et al.* 1982; Repavich *et al.* 1990; Tsuji *et al.* 1995; Ding *et al.* 1999) and human cell lines (Susuki *et al.* 1998; Zhan *et al.* 2004) have been performed to evaluate the mutagenic potential of MC. However, results are somehow contradictory and inconclusive. On the other hand, results from Comet (Rao and Battacharya 1996; Rao *et al.* 1998; Ding *et al.* 1999; Mankiewicz *et al.* 2002; Zegura *et al.* 2002, 2003; Lankoff *et al.* 2004) and Micronucleus (Ding *et al.* 1999; Zhan *et al.* 2004) assays suggest that MCs may produce DNA and chromosome breaks. Nonetheless, it remains unclear whether these effects are due to a cytotoxic or genotoxic activity and, if genotoxic, what the mechanisms are. This paper reports our first results on the analysis of cytotoxic and genotoxic effects of MC in mammalian cell lines.

### Methods

#### 1. Microcystin production from *Microcystis aeruginosa* cultures

Two strains of *M. aeruginosa* isolated from cyanobacterial blooms were cultured under laboratory-con-

trolled conditions. The LMECYA7 strain is a MCLR producer (Pereira *et al.* 2001) and the LMECYA 127 is a non-toxicogenic strain used to exclude eventual cyanobacterial matrix effects. Biomass from both strains was extracted with 75 % methanol and the resulting aqueous extracts were semi-purified by size exclusion followed by reverse phase preparative chromatography. The extracts were freeze dried, dissolved in the respective cell lines culture medium, and sterilized by filtration. Toxin analysis was performed for both extracts by HPLC-DAD (Watanabe *et al.* 1996).

#### 2. Mammalian cell line maintenance

HepG2 (human hepatocellular carcinoma), AML12 (mouse hepatocytes) and Vero (African green monkey kidney) cells were obtained from the American Type Culture Collection. AML12 cells were maintained in DMEM:Ham's F12 (1:1) with 10 % foetal bovine serum (FBS), 1 % insulin-transferin-selenium mixture, and penicillin (100 U/mL)/streptomycin (100 µg/mL). HepG2 and Vero cells were maintained in MEM with 10 % FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and penicillin (100 U/mL)/streptomycin (100 µg/mL). All cells were cultured at 37 °C in a 5 % CO<sub>2</sub> humidified incubator. All media and supplements were purchased from Gibco BRL (Paisley, UK).

#### 3. Cytotoxicity assays

HepG2, AML12, and Vero cells were cultured in 96-well plates (5000 cells/well) in triplicate and exposed to serial dilutions of *M. aeruginosa* extracts after cell

adherence. The extract from the LMECYA7 strain contained MCLR ranging from 1 to 175  $\mu\text{g/mL}$ . In parallel, the non-toxicogenic extract from LMECYA127 was applied at the same dilutions. Cells were exposed for 24, 48, 72, and 96 h to both extracts. The negative control consisted of cells plus culture medium. After each incubation period, LDH release to the growth medium (Legrand *et al.* 1992) and MTT reduction by adherent cells (Mossmann 1983) were determined spectrophotometrically.

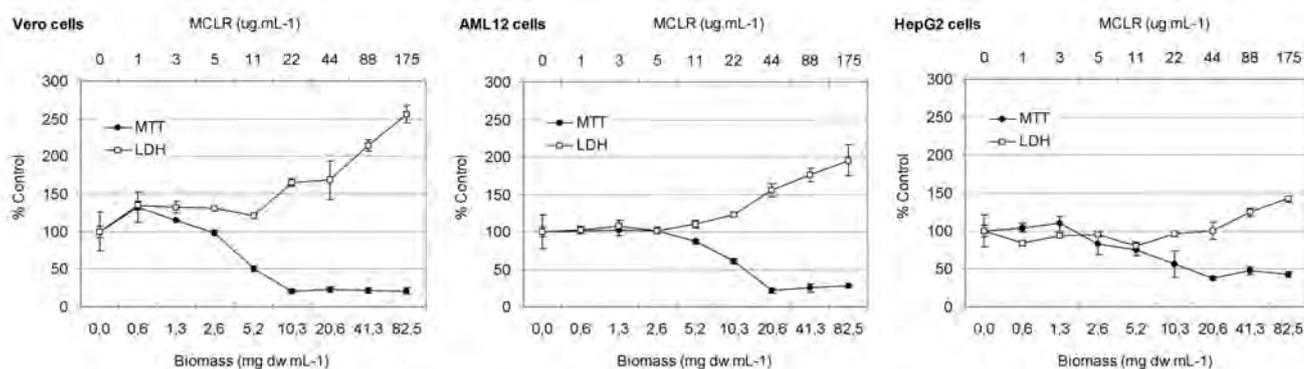
#### 4. Cytogenetic assay

The Cytokinesis-Blocked Micronucleus Assay (Fenech and Morley 1986) was used to evaluate the genotoxic potential of MCLR in Vero cells. Cultured cells were exposed for 24 h to LMECYA7 extract containing MCLR ranging from 5 to 40  $\mu\text{g/mL}$ . Cytokinesis was blocked with cytochalasin B (6  $\mu\text{g/mL}$ ) for 24 h. Cells were fixed, spread onto glass slides, and stained according to standard protocols. For each treatment condition, 1000 binucleated cells were scored to determine the frequency of micronucleated cells.

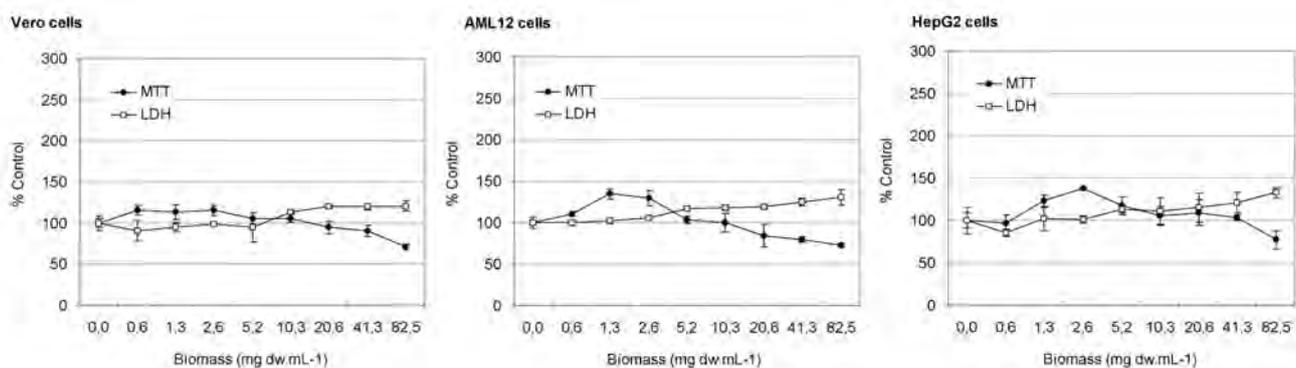
## Results

### 1. Cytotoxic effects

All cell lines showed a cytotoxic response when exposed to the LMECYA7 extract containing MCLR, revealed by an increase of LDH release and a decrease of MTT reduction throughout all incubation times. Fig. 1 shows the results obtained after cells were exposed for 72 h, with values corresponding to the maximum responses. In the MTT assay, Vero cells showed a 50 % decrease in viability after exposure to 11  $\mu\text{g/mL}$  of MCLR. Exposure to higher doses of MCLR (22-175  $\mu\text{g/mL}$ ) produced an 80 % reduction in cell viability. A pronounced cytotoxicity was also observed in AML12 cells, but at a higher MCLR concentration (44  $\mu\text{g/mL}$ ). For HepG2 cells the viability decrease never exceeded 50 %. LDH results were inversely related to those of the MTT assay. Again, the highest cytotoxicity was obtained in Vero cells (156 % increase in LDH release at 175  $\mu\text{g/mL}$ ). Some variation was observed in cells exposed to the non-toxicogenic extract, but they never exceeded 35 % of the control (Fig. 2). Based on these data, Vero cells were



**Figure 1.** LDH release and MTT reduction by Vero, AML12 and HepG2 cells after exposure for 72 h to a serial dilution of LMECYA7 extract containing MCLR (1-175  $\mu\text{g/mL}$ ). Results are expressed as mean %  $\pm$  SD of three replicates relative to the control.

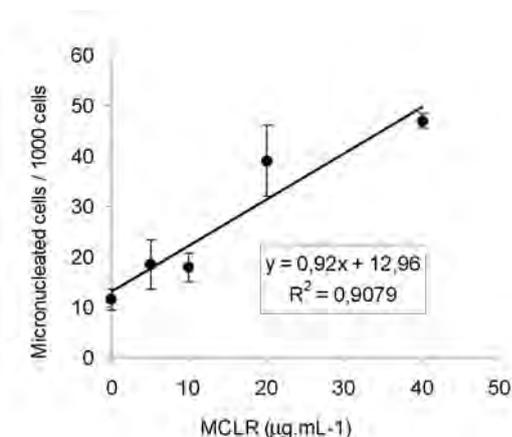


**Figure 2.** LDH release and MTT reduction by Vero, AML12 and HepG2 cells after exposure for 72 h to a serial dilution of non-toxicogenic LMECYA127 extract. Results are expressed as mean %  $\pm$  SD of three replicates relative to the control.

the most sensitive and were selected to evaluate the potential genotoxicity of MCLR.

## 2. Genotoxic effects

In a pilot experiment the frequency of micronuclei (MN) was analyzed in Vero cells after 24 h of exposure to a *M. aeruginosa* extract containing MCLR (Fig. 3). MCLR concentrations of 20 and 40 µg/mL increased 3.4- and 4.1-fold the number of cells with MN, respectively, compared to the control. A linear dose-response relationship between the frequency of micronucleated cells and the MCLR concentration was obtained (t test for a Pearson correl. coeff.,  $p = 2,81E-4$ ).



**Figure 3.** Induction of Micronuclei in Vero cells exposed to a *M. aeruginosa* extract (LMECYA7) containing 5, 10, 20 and 40 µg/mL of MCLR for 24 h. Data are expressed as mean ± SD of two replicates.

## Discussion

The organotropism of MC has been attributed to the mechanism of their uptake by cells. MC enters the cells through the Organic Anion Transporting Polypeptides (OATP) (Fisher *et al.* 2005), present mainly in the liver and to a much lesser extent in other organs. It has been assumed that primary hepatocytes preserve these transporters, but that permanent cell lines tend to lose them. This assumption and the failure of some authors to obtain toxic responses in mammalian cell lines *in vitro* may explain, at least in part, the relatively low number of toxicological studies on microcystins using permanent cell lines as the experimental model.

In this work we showed that *M. aeruginosa* extracts containing MCLR induced strong cytotoxic responses in human (HepG2), monkey (Vero), and mouse (AML12) cell lines. The failure of a non-toxicogenic *M. aeruginosa* extract to cause a similar effect lead us to conclude that microcystin-LR was responsible for the cytotoxicity. Interestingly, and contrary

to what we expected, the most sensitive cells were the non-liver derived Vero cells. No previous data have been published regarding the cytotoxic effects of microcystins in AML12 cells. However, Boaru *et al.* (2006) and Chong *et al.* (2000) reported that HepG2 and Vero cells are insensitive to MCLR. These contradictions might be explained by the use of different experimental set-ups. Those authors used pure toxin, whereas we used semi-purified toxin. We did not detect other microcystins besides MCLR in the LMECYA7 extract. Thus, our results can not be justified by a synergistic effect of different toxin variants. However, the complex and unidentified cyanobacterial matrix may somehow influence MCLR toxicity. The present work points to the usefulness of Vero cells as a model to study microcystin toxicity and suggests that this permanent cell line might preserve their OATP, or that MCLR uptake/toxicity might be triggered by another unknown mechanism. On the other hand, the observed induction of micronuclei in Vero cells by the toxic *M. aeruginosa* extract suggests that MCLR has a genotoxic activity. This raises the question of whether MCLR acts as a clastogenic or an aneugenic agent and supports the previous suggestion that MC can act as tumour initiators. Further studies using centromere labelling by fluorescence *in situ* hybridization will clarify the mechanism behind the genotoxic effect.

## Acknowledgements

This work was funded by the Fundação para a Ciência e Tecnologia, Portugal (PhD grant to E. Dias SFRH/BD10585/2002 and Project POCI/AMB/60351/2004).

## References

- Boaru, D.A., Dragos, N. & Schirman, K. (2006). *Toxicology* 218: 134-148.
- Chong, M., Gu, K., Lam, P., Yang, M. & Fong, W. (2000). *Chemosphere* 41: 143-147.
- Fenech, M. & Morley, A.A. (1986). *Mut. Res.* 161: 193-198.
- Fischer, W.L., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R. & Hagenbuch, B. (2005). *Toxicol. Appl. Pharmacol.* 203: 257-263.
- Grabow, W., Randt, W., Prozesky, O. & Scott, W. (1982). *Appl. Environ. Microbiol.* 43: 1425-1433.
- Lankoff, A., Krzowski, L., Glab, J., Banasik, A., Lisowska, H., Kuszowski, T., Gozdz, S. & Wojcik, A. (2004). *Mut. Res.* 559: 131-142.
- Legrand, C., Bour, J.M., Jacob, C., Capiaumont, J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G.,

- Demangel, C. & Duval, D. (1992). *J. Biotechnol.* 25: 231-243.
- Mankiewicz, J., Walter, Z., Tarczynska, M., Palyvoda, O., Wojtysiak, S.M. & Zalewski, M. (2002). *Environ. Toxicol.* 17: 341-350.
- Mossmann, T. (1983). *J. Immunol. Meth.* 65: 53-63.
- Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Ishikawa, T., Carmichael, W.W. & Fujiki, H. (1992). *J. Cancer Res. Clin. Oncol.* 118: 420-424.
- Pereira, P., Onodera, H., Andrinolo, D., Franca, S., Araújo, F., Lagos, N. & Oshima, Y. (2001). In: *Harmful Algal Blooms 2000*, Hallegraef, G.M., Blackburn, S.I., Bolch C.J., and Lewis, R.J. (eds), UNESCO, Paris, pp. 108-111.
- Rao, P.V. & Bhattacharya, R. (1996). *Toxicology* 114: 29-36.
- Rao, P. V., Bhattacharya, R., Parida, M.M., Jana, A.M. & Bhaskar, A.S. B. (1998). *Environ. Toxicol. Pharmacol.* 5: 1-6.
- Repavich, W.N., Sonzogni, J.H., Standridge, R.E., Wedphohl, L.F. & Meisner, L.F. (1990). *Water Res.* 24: 225-231.
- Suzuki, H., Watanabe, M.F., Wu, Y., Sugita, T., Kita, K., Sato, T., Wang, X., Tanzawa, H., Sekiya, S. & Suzuki, N. (1998). *Int. J. Mol. Med.* 2: 109-112.
- Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Suzuki, S., Nakazawa, H., Suzuki, M., Uchida, H. & Harada, K. (1995). *Toxicon* 33: 1619-1631.
- Watanabe, M., Harada, K., Carmichael, W.W. & Fujiki, H. (1996). *Toxic Microcystis*. CRC Press, New York.
- Zhang, Z., Yu, S. & Chen, C. (2001). *Zhonghua Yu Fang Yi Xue Za Zhi* 35: 75-78.
- Zhan, L., Sakamoto, H., Sakuraba, M., Wu, D.S., Zhang, L. S., Suzuki, T., Hayashi, M. & Honma, M. (2004). *Mut. Res.* 557: 1-6.
- Zegura, B., Sedmak, B. & Filipic, M. (2003). *Toxicon* 41: 41-48.
- Zegura, B., Filipic, M., Suput, D., Lah, T. & Sedmak, B. (2002). *Radiol. Oncol.* 36: 159-161.

## The dinoflagellate *Gyrodinium fissum*: harmful species or potential biotechnological object?

E. Gol'din

Southern Branch of the National Agrarian University-Crimean Agricultural and Technological University; Crimean State Medical University; 122/89, Kievskaya Street, P.B. 2223, Simferopol, Crimea, Ukraine 95043, Evgeny\_goldin@mail.ru; oblako@home.cris.net

### Abstract

The dinoflagellate *Gyrodinium fissum* is a poorly studied alga. Some authors regard it as a red tide organism; others as a non-toxic diet for crustaceans. Biocidal activity of *G. fissum* has not been properly studied. We exposed lackey and brown-tale moths, fall webworm and Colorado potato beetle to *G. fissum*. Leaves were treated with *G. fissum* culture and fed to insects. *G. fissum* showed various inhibitory effects: (1) Repellent action: lackey moth larvae did not feed and gathered in the substratum and jar walls; (2) Long-term deterrent action with residual consequences: larval nutrition fell considerably (3.0-5.0 % of control); (3) Short-term deterrent action: brown-tale moth larvae; (4) Inhibition of growth: developmental lag of larvae of lackey moth (50.0 %), fall webworm (29.2-68.0 %), the larvae and imago in Colorado potato beetle (48.5-63.5 % and 15.4-16.1 %); (5) Dysfunction of metamorphosis: defects of pupation and imago formation; treatment of eggs of Colorado potato beetle caused elimination of eggs (56.3 %) and hatching larvae (32.6 %); (6) Mortality during 10-20 d in lackey moth (95.0 %), fall webworm (100.0 %) and Colorado potato beetle (84.4-100.0 %). Histological examination revealed the degradation of midgut and fat body in Colorado potato beetle. *G. fissum* can be propagated in controlled culture; its toxicity is selective. This species is not toxic to non-target objects and it could potentially be used in biological pest control.

### Introduction

The widespread dinoflagellate *Gyrodinium fissum* (Levander) Kofoid & Swezy 1921 is poorly known. Some authors regard it as a harmful red tide organism responsible for algal blooms in coastal waters of Korea and China (Kim *et al.* 1993); others identify it as a non-toxic diet for crustaceans (Jeschke 2002). Its biocidal activity has received little attention. As part of our investigations on a group of microalgae suppressing the vital functions of herbivorous insects (Gol'din 1997, 1999, 2004), we studied the inhibitory activity of *G. fissum*.

### Methods and Materials

*Gyrodinium fissum* (strain IBSS) was isolated into culture from the Black Sea in Sevastopol Bay and obtained from the collection at Institute of Biology of the Southern Seas (Sevastopol). We exposed eggs, larvae and imago of lackey moth *Malacosoma neustria*, brown-tale moth *Euproctis chrysorrhoea*, fall webworm *Hyphantria cunea* and Colorado potato beetle *Leptinotarsa decemlineata* to a strain of *G. fissum* maintained in modified Goldberg medium (salinity psu) (Lanskaya 1971; Poryvkina *et al.* 2000) and its autolysate. In other tests the leaves of plant hosts (potato, ash-leaved maple, apricot) were treated with

microalgal culture via a laboratory syringe and fed to insects. *G. fissum* was applied in different ways to observe the specific inhibitory action. Insects were kept in 1-liter glass containers, 10-12 specimens in each. Each experiment included 3-10 replicates, and seawater from 10-mile offshore, salinity 17 psu, or Goldberg medium served as controls. In some experiments, a culture of the unicellular haptophyte *Pavlova (Nephrochloris) salina*, was used for comparison. It is known to possess high biological and insecticidal effect (Gol'din 1997). The observations included feeding behaviour, growth, metamorphosis, survival, and histological pathology of the test objects. The histological structure of tested and control insects were inspected using standard methods (Gol'din 2004).

### Results and Discussion

Different stages of *G. fissum* cultures (from stationary to autolysate) demonstrated various inhibitory effects on insect vital functions:

- (1) Repellent action: lackey moth larvae stopped feeding during 3-5 days and collected in the substratum and along the jar walls
- (2) Long-term deterrent action with residual consequences: larval feeding was highly reduced in Colorado potato beetle and lepidopterous insects (3.0-5.0 % of control; fall webworm was the most susceptible)

(3) Short-term deterrent action: suppression of trophic function during 24 h (brown-tale moth larvae)

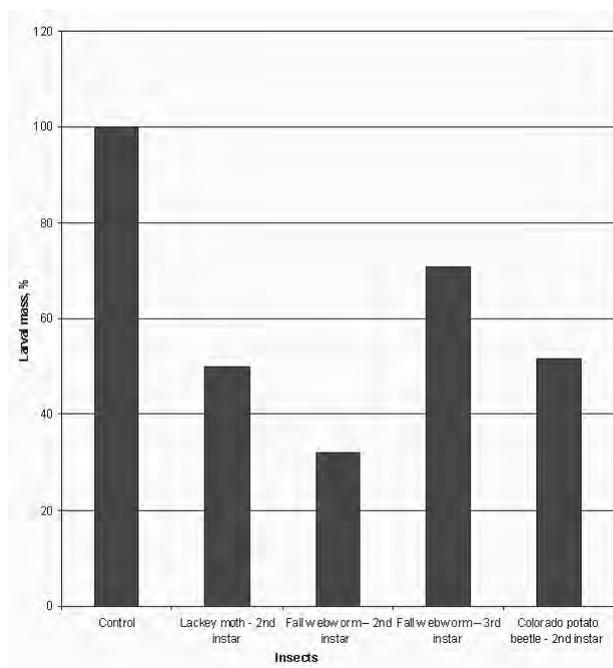
(4) Inhibition of fat synthesis and growth: developmental lag of test larvae in lackey moth (50.0 %), fall webworm (29.2-68.0 %), larvae and imago in Colorado potato beetle (larval indices - 48.5 %; some individuals 63.5 %; imago - 15.4-16.1 %) (Fig. 1, Table 1)

(5) Dysfunction of metamorphosis: reduction in number of pupae and imago in all insects; treatment of eggs of Colorado potato beetle caused the elimination of eggs (56.3 %) and hatching larvae (32.6 %) (Table 2)

(6) Mortality: lethal effects were seen during 10-20 d in lackey moth (95.0 %) (Table 3), fall webworm (100.0 %) (Table 4) and Colorado potato beetle (84.4-100.0 %) (Table 5). Prolongation of the effect of treated feeding increased mortality in fall webworm (Table 4).

Histological examination revealed that feeding of treated leaves by the 2nd instar larvae of Colorado potato beetle correlated with destructive changes in midgut (Fig. 3) and fat body (Fig. 4) and led to the degradation of these organs and tissues during 7 d.

Our study demonstrated a biocidal effect due to the ingestion of treated diet by herbivorous insects or due to a direct effect (Fig. 2) (combined with possible massive growth of *G. fissum* in controlled culture or experimental ponds). Its selectivity to non-target objects (crustaceans) may be applied in biological pest control.



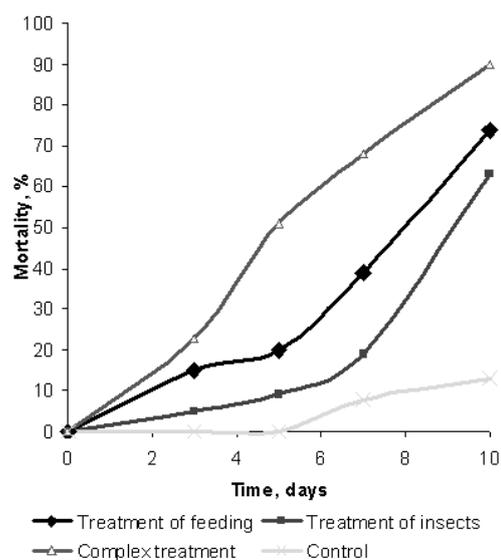
**Figure 1.** Larval biomass after 3-day feeding with treated leaves (on the 10th day of observation).

**Table 1.** Influence of microalgae on development of imago of Colorado potato beetle (the 10th day of testing: potato leaves were treated with microalgal suspension) (30 individuals). M – mean; m – standard error.

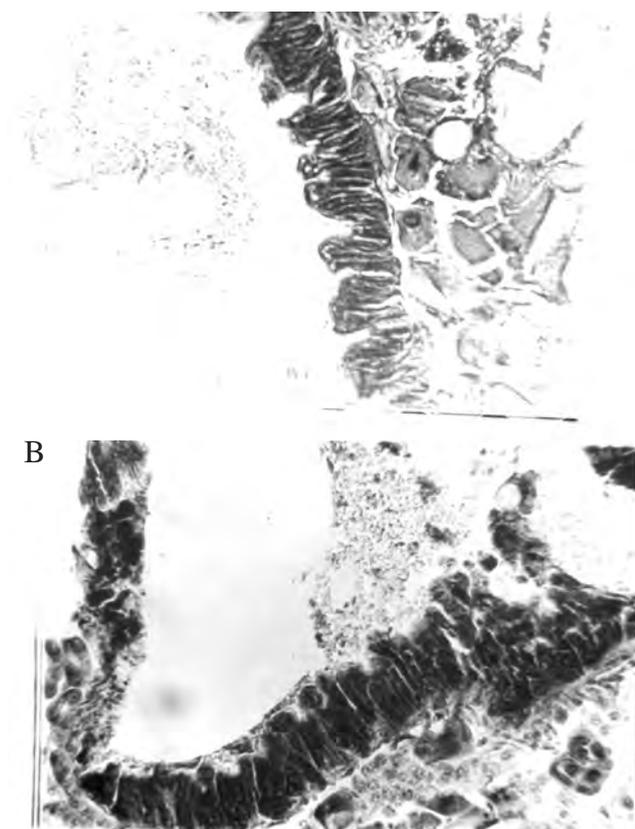
Variant	Age of culture, days	Average imago mass, mg M ± m	Comparison with control, %
Experiment: <i>Gyrodinium fissum</i>	90	135.8 ± 3.8	83.9
	60	137.0 ± 5.5	84.6
Comparison standard: <i>Pavlova salina</i>	90	133.5 ± 4.8	82.5
Control: Sea Water	-	161.8 ± 18.4	100.0

**Table 2.** Mortality of eggs and hatched larvae of Colorado potato beetle after treatment of eggs with microalgal suspension.

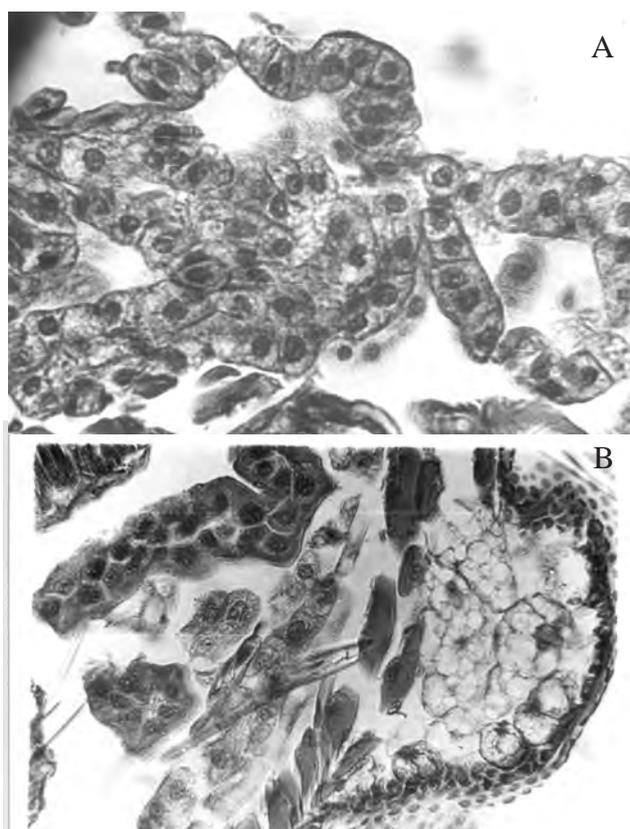
Microalgal culture	Number of eggs	Age of culture, days	Mortality, %		
			Eggs	Larvae	Total
<i>Gyrodinium fissum</i>	257	90	56.3 ± 6.9	32.6 ± 6.9	88.9 ± 3.4
	372	60	13.6 ± 6.4	56.1 ± 8.6	69.7 ± 4.8
Control: Goldberg medium	-	-	0	4.2	4.2



**Figure 2.** Correlation between insect mortality and type of treatment (2nd instar larvae of fall webworm, 75 specimens in each experiment).



**Figure 3.** Midgut of Colorado potato beetle (2nd instar larva on the 7th day of experiment). A - normal structure, 16 x 6.3, Van Gieson stain. B - pathological degradation: desquamation of epithelium, uncovering of muscle layer and rupture of intestinal wall; 16 x 6.3, Van Gieson stain.



**Figure 4.** Fat body of Colorado potato beetle (2nd instar larva on the 7th day of experiment). A - normal structure, 16 x 6.3, haematoxylin-eosin stain. B - pathological situation: vacuolization of cytoplasm, lysis of nuclei, areas deprived of nuclei, seats of structural degradation; 16 x 6.3, haematoxylin-eosin.

**Table 3.** Larval mortality of lackey moth after 3-day feeding of the 2nd instar stage on treated leaves (120 individuals).

Version	Age of culture, days	Mortality, %		
		10th day	15th day	20th day
<i>Gyrodinium fissum</i>	90	39.2 ±5.5	58.3 ±8.9	95.1 ±1.4
Control: Goldberg medium	-	0	0	0

**Table 5.** Larval mortality of Colorado potato beetle after 3-day feeding of the 2nd instar larval stage on treated leaves (120 individuals).

Version	Age of culture, days	Mortality, %		
		10th day	15th day	20th day
<i>Gyrodinium fissum</i>	90	84.4±2.3	84.4±2.3	90.2±2.3
	60	93.3±4.5	100.0	
Control: Goldberg medium	-	0	0	0

**Table 4.** Larval mortality of fall webworm in relation to length of feeding on treated leaves (70 individuals).

Version	Time of feeding with treated leaves, days	Mortality, %						
		2nd instar			3rd instar			
		7th day	10th day	15th day	7th day	10th day	15th day	20th day
<i>Gyrodinium fissum</i>	3	82.2±6.8	97.8±6.8	100.0	52.9±11.3	77.1±16.2	88.6±9.7	100.0
	15	82.2±11.4	100.0					
Control: Goldberg medium	-	4.4±2.8	6.7	15.6±2.3	0	0	2.2	2.2

### Acknowledgements

Our sincere thanks to the colleagues from the Institute of Biology of the Southern Seas of the National Academy of Sciences of Ukraine (Sevastopol) for the culture of *G. fissum*.

### References

- Gol'din, E.B. (1997). *Phycologia* 36, Suppl.: 35.
- Gol'din, E.B. (1999). In: Proc. Joint Conf. MED-COAST 99 & EMECS 99, E.Ozhan (ed.), MED-COAST, Ankara, pp. 133-146.
- Gol'din, E.B. (2004). In: Xth Int. HAB Conf. , K.A. Steidinger, J.H. Landsberg, C.R. Tomas, G.A. Vargo, (eds), Florida Marine Research Institute, Florida Fish and Wildlife Commission, Florida Institute of Oceanography, IOC of UNESCO, pp. 476-478.
- Jeschke, J.M. (2002). Funktionelle Reaktionen von Konsumenten: die SSS Gleichung und ihre Anwendung. Dissertation Ludwig-Maximilians-Universität München, Mai 2002, pp. 1-192.
- Kim, H.G., Park, J.S., Fukuyo, Y., Takayama, H, An, K.H. & Shim, J. M. (1993). In: P. Lassus, G. Arzul, E. Erard, P. Gentien & C. Marcaillou (Eds), Harmful Marine Algal Blooms, Lavoisier, Intercept Limited, Paris, New York, pp. 59-63.
- Lanskaya, L.A. (1971). The Cultivation of Algae. In: K. Khailov (ed.), Ecological Physiology of Marine Planktonic Algae (in cultural conditions), Naukova Dumka, Kiev, pp. 5-21.
- Poryvkina, L, Babichenko, S. & Leeben, A. (2000). In: Proc. EARSeL-SIG-Workshop LIDAR, Dresden/FRG, June 16- 17, 2000, 1, pp. 224-233.

## ELISA screening for yessotoxins in Portuguese shellfish

S.S. Gomes<sup>1</sup>, P. Vale<sup>1</sup>, M.J. Botelho<sup>1</sup>, S.M. Rodrigues<sup>1</sup>, M. Cerejo and M.G. Vilarinho<sup>2</sup>

<sup>1</sup>Instituto Nacional de Investigação Agrária e das Pescas, IPIMAR, Av. Brasília, s/n, 1449-006, Lisboa, Portugal, biotoxinas@ipimar.pt and <sup>2</sup>vilarinh@ipimar.pt

### Abstract

Several Portuguese shellfish species were screened for the presence of yessotoxin (YTX) using a newly developed immunoassay kit, sensitive to a wide variety of YTX analogues (YTXs). Shellfish samples were collected from the Aveiro and Formosa lagoons and the Algarve offshore during the summer/autumn 2005. In lagoon species, YTXs were detected in the following order: *Mytilus galloprovincialis* >> *Cerastoderma edule* > *Ruditapes decussatus* ≈ *Venerupis pullastra* ≈ *Solen marginatus*, but not detected in *Crassostrea* spp. In offshore species, YTXs were detected with decreasing concentrations from *Spisula solida* > *Donax* spp ≈ *Chamelea gallina*. Some of the mussel samples collected in northern Portugal (Aveiro), as well as the south (Formosa Lagoon), had levels near the current EU regulatory limit of 1 mg kg<sup>-1</sup> shellfish meat. Prolonged persistence of YTXs was observed in all three areas studied. In the Aveiro Lagoon, it was possible to associate temporary increases in YTX contamination with *Protoceratium* spp. and *Gonyaulax spinifera* in the plankton, but not with *Lingulodinium polyedrum*. This is the first report of YTX contamination of bivalve molluscs from Portugal.

### Introduction

Yessotoxin (YTX) and its analogues (YTXs) are lipophilic disulphated polyether toxins produced by the marine microalgae *Protoceratium reticulatum* and *Lingulodinium polyedrum*, species identified in culture as primarily responsible for production of YTX analogues in Japan, New Zealand, Italy and Norway (Samdal *et al.* 2005). More recently the YTXs detected in New Zealand were produced by *Gonyaulax spinifera* (Rhodes *et al.* 2006).

Initially YTXs were included in the diarrhetic shellfish poisons (DSP). However, not only the YTXs chemistry but also their toxicology differ distinctly from the DSP toxins (Aune *et al.* 2002). In Europe, since 2002, YTX and three analogues are regulated separately from DSP, at 1 mg YTX equivalents per kg shellfish meat intended for human consumption (Regulation (EC) no 853/2004).

An antibody towards YTX developed by AgResearch (Hamilton, New Zealand) (Briggs *et al.* 2004) was recently made available in kit format by Biosense Laboratories (Bergen, Norway), and detects all the yessotoxin analogues required by EU regulation. By using this kit prototype, it was possible to detect the presence of YTXs in several shellfish species collected in northwestern (Aveiro Lagoon) and southern (Algarve offshore and Formosa Lagoon) areas of the Portuguese coastline.

This is the first report of YTXs contamination of bivalve molluscs from Portugal.

### Material and Methods

As part of the Portuguese shellfish monitoring programme, plankton and shellfish samples were harvested at Aveiro and Formosa lagoons and the Algarve offshore during the summer/autumn 2005.

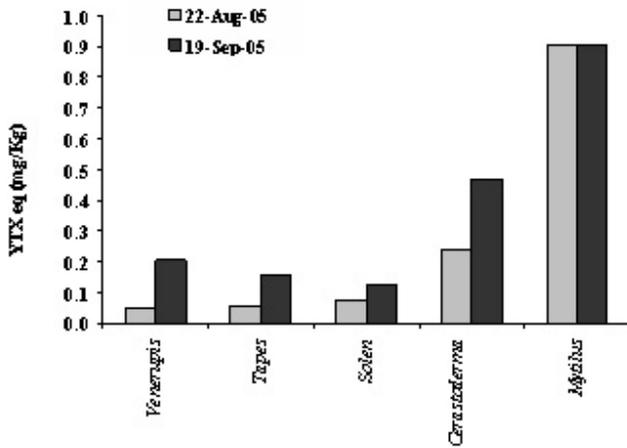
Plankton was collected from the surface, preserved with acidified formalin solution and concentrated by centrifugation for 30 min at 2500 rpm. Cell counts were performed in a standard microscope using a Palmer-Maloney chamber.

Toxins were extracted from a 2-g aliquot of whole flesh homogenates with 18 ml 90 % aqueous methanol. ELISA assays were conducted in duplicate according to the Biosense Laboratories protocol, and calibrated with the YTX standard supplied in the kit. Plate readings were performed on an ELX-808i Bio-Tek Instruments reader with a 450 nm filter. Norwegian mussels contaminated with YTXs were tested as a positive control.

### Results

#### *Aveiro Lagoon*

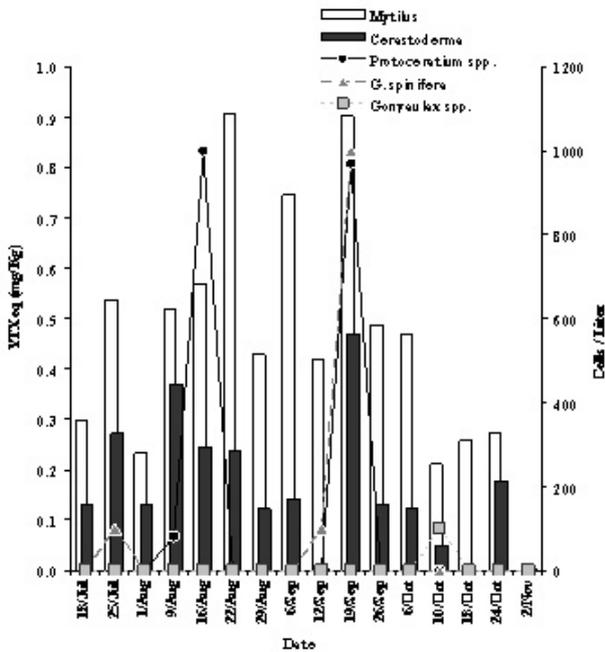
In the shellfish species collected in Aveiro Lagoon, YTXs were detected in the following taxa in decreas-



**Figure 1.** Concentration of YTXs in several shellfish species collected in Aveiro Lagoon on 22 August and 19 September 2005.

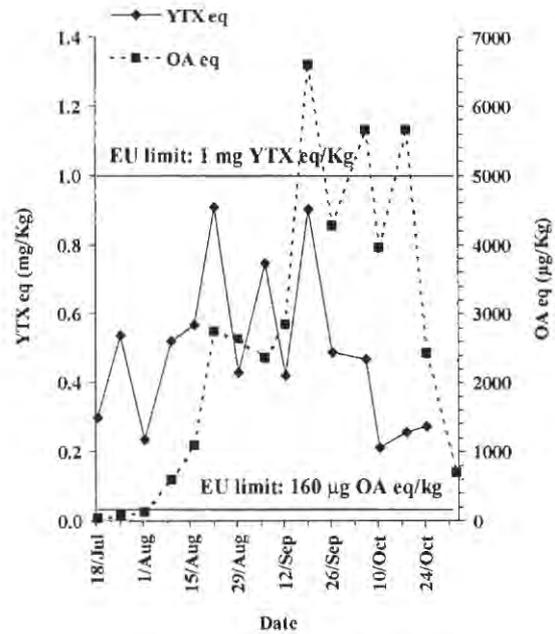
ing concentrations: *Mytilus galloprovincialis* >> *Cerastoderma edule* > *Tapes decussatus* ≈ *Venerupis pullastra* ≈ *Solen marginatus* (Fig. 1).

In two shellfish species (*M. galloprovincialis* and *C. edule*) analysed throughout summer/autumn 2005, contamination with YTXs was very persistent (Fig. 2).



**Figure 2.** Temporal variability of YTXs in *Mytilus galloprovincialis* and *Cerastoderma edule* and the occurrence of several microalgae in Aveiro Lagoon in summer/autumn 2005.

The occurrence of *Gonyaulax spinifera* was coincident with the increase in concentrations of YTXs in both mussels and cockles at the end of July and mid-September. The detection of *Protoceratium* spp. in mid-August and mid-September was also coincident with the observed increase of YTXs in mussels.

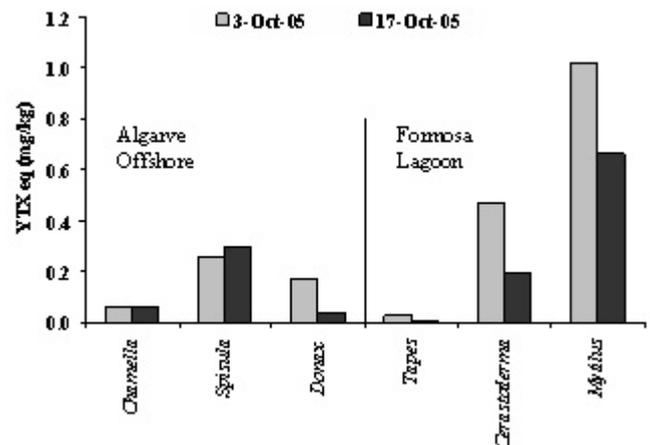


**Figure 3.** Temporal variability of YTX and OA equivalents in *M. galloprovincialis* from Aveiro Lagoon.

Aveiro Lagoon is a known endemic area for contamination by okadaic acid analogues. Contamination with YTXs and DSP toxins can be coincident in time (Fig. 3). However, their maxima may occur independently. While the EU limit for OA toxins ( $160 \mu\text{g OA equiv. kg}^{-1}$ ) was largely surpassed for several weeks in mussels, the EU limit for YTX ( $1 \text{ mg YTX equiv. kg}^{-1}$ ) was not.

*Algarve Offshore and Formosa Lagoon*

In offshore species from the Algarve coast, YTXs were detected in the following order of decreasing concentrations: *Spisula solida* > *Donax* spp ≈ *Chamelea gallina* (Fig. 4).



**Figure 4.** Concentration of YTXs in several shellfish species collected in the Algarve offshore and Formosa Lagoon on 3 and 17 October 2005.

In the Formosa Lagoon, YTXs were detected in the invertebrates at decreasing concentrations: *M. galloprovincialis* >> *C. edule* > *Tapes decussatus*.

Although high concentrations were observed in *M. galloprovincialis*, the regulatory limit of 1 mg YTX equiv. kg<sup>-1</sup> was only slightly exceeded. In off-shore species, the levels observed during the same period were lower and did not exceed the maximal allowable limit.

### Conclusions

In two lagoon systems in opposing locations of the coast (northwest and south), the species most contaminated with YTXs were always mussels, followed by cockles. Clams and razor clams always had the lowest levels.

Only *M. galloprovincialis* samples contained levels near the EU regulatory limit of 1 mg kg<sup>-1</sup> shellfish meat. However, this level might be overestimated due to broad antibody specificity. It has been reported that analysis of Norwegian mussels by this ELISA assay largely overestimated the sum of the legislated toxins derived by LC-MS analysis (Samdal *et al.* 2005).

Prolonged persistence of YTXs was observed in animals in all areas studied. When comparing mussels and cockles, mussels seem to depurate slower than cockles.

Despite the prolonged persistence of YTXs in shellfish tissues, it was possible to associate tempo-

ral increase in YTXs contamination with the sporadic presence of *Protoceratium* spp. and *Gonyaulax spinifera*, but not with *Lingulodinium polyedrum* in the plankton. Contamination with YTXs occurs with low levels of suspect toxic algae, probably due to slow depuration.

### Acknowledgements

The programme "Safety, Surveillance and Quality of Bivalve Molluscs" (QCAIII/med.4/MARE Programme) supported this work. We thank T. Aune from the Norwegian NRL for supplying mussels contaminated with YTXs.

### References

- Samdal, I.A., Aasen, J.A.B., Briggs, L.R., Dahl, E. & Miles, C.O. (2005). *Toxicon* 46: 7-15.
- Rhodes, L., McNabb, P., Salas, M., Briggs, L., Beuzenberg & V., Gladstone (2006). *Harmful Algae* 5: 148-155.
- Aune, T., Sorby R., Yasumoto T., Ramstad H. & Landsverk, T. (2002). *Toxicon* 40: 77-82.
- Regulation (EC) no 853/2004, OJ, L-139, 30.04.2004, p. 55.
- Briggs, L.R., Miles, C.O., Fitzgerald, J.M., Ross, K.M., Garthwaite, I. & Towers, N.R. (2004). *J. Agric. Food Chem.* 52: 5836-5842.
- YTX ELISA pilot kit protocol. Biosense Laboratories.

## ***In vivo* exposure to microcystins induced DNA damage in haemocytes of the zebra mussel, as measured with the Comet assay**

G. Juhel<sup>1\*</sup>, J. O'Halloran<sup>1</sup>, S.C. Culloty<sup>1</sup>, R.M. O'Riordan<sup>1</sup>, J. Davenport<sup>1</sup>, N.M. O'Brien<sup>2</sup>, K.J. James<sup>3</sup>, A. Furey<sup>3</sup> and O. Allis<sup>3</sup>

<sup>1</sup>Department of Zoology, Ecology & Plant Science, and Environmental Research Institute, University College Cork, Distillery Fields, North Mall, Cork, Ireland.

<sup>2</sup>Department of Food Science, Food Technology and Nutrition, University College Cork, Ireland.

<sup>3</sup>PROTEOBIO, Mass Spectrometry Centre for Proteomics and Biotxin Research, Department of Chemistry, Cork Institute of Technology, Cork, Ireland.

\*Corresponding author: g.juhel@ucc.ie

### **Abstract**

The Comet assay was used to investigate the potential of microcystins to induce DNA damage in the haemocytes of the freshwater zebra mussel, *Dreissena polymorpha*. Laboratory *in vivo* exposure experiments were conducted over a 21-d period with three strains of the cyanobacterium *Microcystis aeruginosa*, with different toxicities and toxic profiles and one non-toxic strain. Mussels were sampled at 0, 7, 14 and 21 d. A positive control was performed with CdCl<sub>2</sub>-spiked water. Cell viabilities were high throughout the study. A clear dose-response in the DNA damage was observed following exposure to CdCl<sub>2</sub>. DNA damage, measured as percentage tail DNA was observed with the three toxic *Microcystis* strains but not with the non-toxic strain. Toxin analysis of the cyanobacterial cultures revealed the presence of two MC variants, MC-LF and MC-LR. The DNA damage appeared to be strain-specific and increased with increasing MC concentration. This study is the first to demonstrate that *in vivo* exposure to microcystins induces DNA damage in the haemocytes of zebra mussels.

### **Introduction**

Microcystins (MCs) are hepatotoxins produced by HAB-forming cyanobacteria such as *Microcystis aeruginosa*. MCs are primarily liver-specific toxins (Rao *et al.* 1995) acting as inhibitors of serine/threonine phosphatases 1 and 2A (Yoshizawa *et al.* 1990). MCs are tumour initiators (Žegura *et al.* 2003). Furthermore, *in vitro* studies have shown that MCs are genotoxic (Ding *et al.* 1999). However, there is little information on the genotoxicity of MCs, particularly using *in vivo* models.

The zebra mussel, *Dreissena polymorpha*, is an invasive species that has recently colonised Ireland after being restricted to Europe and North America (Astanei *et al.* 2005). Because of its high density in nature, it has had serious ecological impacts (Reeders *et al.* 1989), such as promoting blooms of toxic blooms of *Microcystis aeruginosa* through selective filtration (Juhel *et al.* 2006a). MCs also proved to affect the feeding behaviour and to depress the Scope for Growth (Juhel *et al.* 2006b) of zebra mussels in feeding experiments. The zebra mussel is therefore a good candidate to study the potential *in vivo* genotoxicity of MCs. The Comet assay is a sensitive method to measure DNA damage in individual cells (Collins *et al.* 1997) and has been used to detect DNA dam-

age in zebra mussels exposed to organic contaminants (Bolognesi *et al.* 2004). The objective of this study was to determine if DNA damage could be detected in *D. polymorpha* fed several MC-containing cyanobacterial culture strains.

### **Material and Methods**

#### *Algal strains*

Four strains of *Microcystis aeruginosa* were cultured under controlled conditions (20 °C; 12 h L:D cycle): CCAP 1450/06; CCAP 1450/10 from the Culture Collection of Algae and Protozoa (CCAP, Argyll, UK) and SAG 17.85 and SAG 48.80 from Sammlung von Algenkulturen Göttingen (SAG, Göttingen, Germany).

#### *Toxin analysis*

The general procedure of Ortea *et al.* (2004) was followed to determine the toxin profile of each of the four strains of *Microcystis aeruginosa*. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of MCs were carried out to determine the quantity of each of the following MC variants standards: MC-LR, MC-RR, MC-YR and MC-LF.

#### *Zebra mussel collection and handling*

Mussels were collected at Dromineer Marina, County Tipperary, Ireland in October 2004, transported to the

laboratory and acclimated for 48 h prior to the experiments. (Average mussel size:  $25.1 \pm 1.66$  mm (mean  $\pm$  SD)).

#### *Exposure to microcystins*

Mussels were fed the 4 strains daily at approximately  $10^4$  cells  $\text{ml}^{-1}$  for 3 weeks in a static system. Three replicate tanks were sampled per treatment. 15 mussels were sampled at time  $t_0$  and 5 mussels per tank were sampled at 7-day intervals over the following three weeks, used for the Comet assay and replaced by marked mussels.

#### *Positive control*

Zebra mussels were exposed to 0, 4, 40, and 100  $\mu\text{M}$   $\text{CdCl}_2$ -spiked freshwater for a week without feeding. 5 mussels were placed in each tank, with 3 replicate tanks used per treatment.

#### *Haemolymph collection*

Haemolymph was collected from the posterior adductor muscle sinus of the zebra mussels using a 1 ml hypodermic syringe and kept on ice for analysis with the cell viability test and the Comet assay.

#### *Cell viability test*

All samples were tested with the Fluorescein Diacetate/Ethidium Bromide (FDA/EtBr) assay as described by Anderson *et al.* (1994).

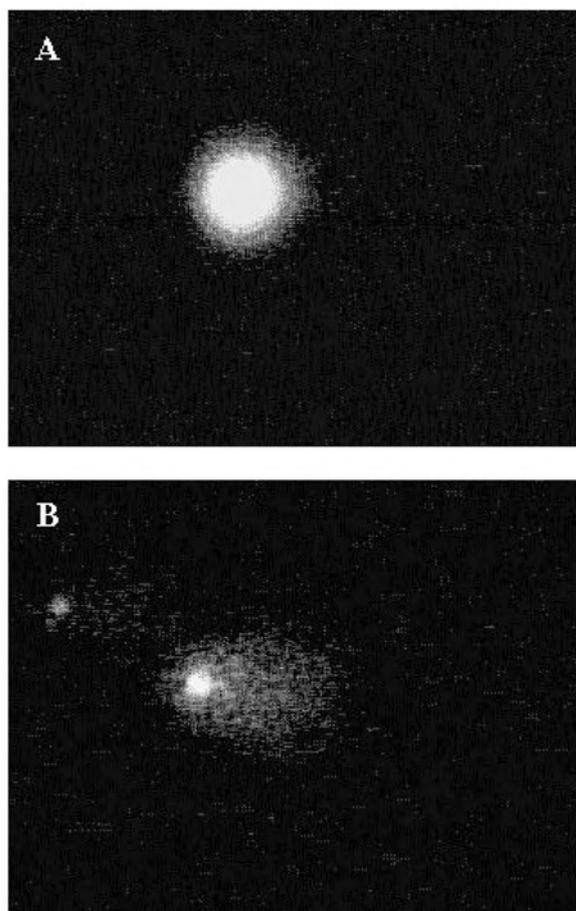
#### *Comet assay*

The Comet assay procedure was adapted from Woods *et al.* (1999). A triple-layered agar 'sandwich' containing zebra mussel's haemocytes was prepared on microscopic slides, lysed, placed into an electrophoresis tank with an alkaline solution, subjected to electrophoresis and stained with ethidium bromide. DNA damage was estimated with a Nikon EFD-3 epifluorescence microscope. Fifty randomly chosen nucleoids per replicate slide were scored. Fig. 1A shows a healthy haemocyte with no visible DNA damage. Fig. 1B shows haemocytes of zebra mussels with visible DNA strand breakage as seen by the greatly reduced nucleus core and a large 'cloud' of DNA fragments. DNA damage was expressed as percent tail DNA (% tDNA) (Olive *et al.* 1990).

## Results

#### *Toxin analysis*

Strain SAG 48.80 contained no MCs and served as reference strain. Strain CCAP 1450/06 contained only one variant, MC-LR, in relatively low concentration ( $7.4 \mu\text{g l}^{-1}$ ), whereas strain CCAP 1450/10 contained relatively high concentrations of two of the toxin vari-



**Figure 1.** (A) The nucleus of a healthy haemocyte from a zebra mussel exposed to non-toxic cyanobacteria. (B) The nuclei of two haemocytes of zebra mussels exposed to MCs.

ants,  $23.8 \mu\text{g l}^{-1}$  MC-LR and  $82.9 \mu\text{g l}^{-1}$  MC-LF. Strain SAG 17.85 had the highest concentration of toxins with  $19.3 \mu\text{g l}^{-1}$  MC-LR and  $95.8 \mu\text{g l}^{-1}$  MC-LF.

#### *Cell viability*

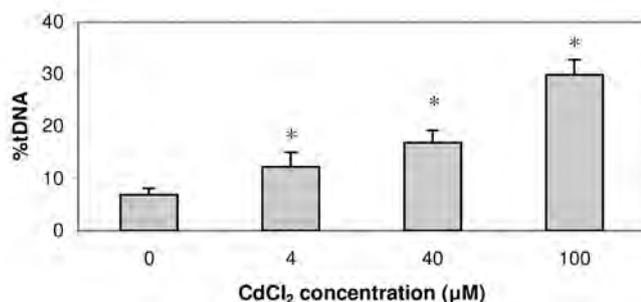
Throughout the MC study, cell viabilities were consistently above 80 % and ranged from 74 % to 92 % when mussels were exposed to  $\text{CdCl}_2$ .

#### *Positive control*

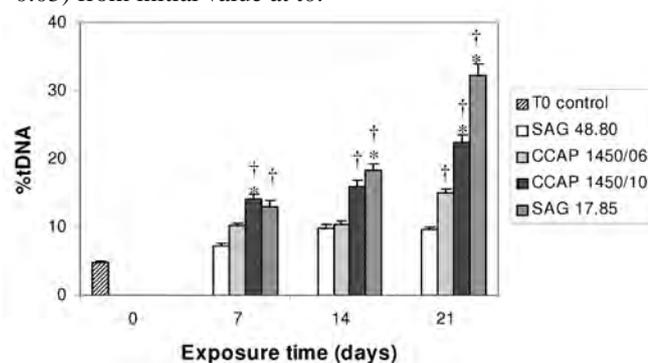
A significant difference (one-way ANOVA,  $P < 0.001$ ) was observed between the control mussels and those exposed to  $\text{CdCl}_2$ . Furthermore, a clear dose-related response was observed for the haemocytes of mussels exposed to the  $\text{CdCl}_2$  (Fig. 2).

#### *Mussels exposed to microcystins*

% tDNA did not differ significantly from  $t_0$  values for the mussels exposed to the reference cyanobacterial strain (strain SAG 48.80) at any stage during the 21-d exposure period (ANOVA on Ranks;  $P > 0.05$ ). % tDNA increased significantly over  $t_0$  values in haemocytes from animals exposed to strains CCAP 1450/10



**Figure 2.** CdCl<sub>2</sub>-induced DNA damage in mussel haemocytes detected with the Comet assay. DNA damage expressed as percentage of tail DNA (% tDNA) was measured in zebra mussels after one week of exposure. Data expressed as mean ± SEM; n = 3 replicates of 5 mussels for each CdCl<sub>2</sub> concentration. (\*): significant difference (p < 0.05) from initial value at t0.



**Figure 3.** Degree of DNA damage, expressed as percentage of tail DNA (% tDNA), in haemocytes of zebra mussels (*Dreissena polymorpha*) exposed to different strains of the cyanobacterium *Microcystis aeruginosa*. Data are expressed as mean ± SEM; n = 3 replicates of 5 mussels for each treatment at each time; (†): significant difference (p < 0.05) from initial value at t0; (\*): significant difference (p < 0.05) from the SAG 48.80 (non-toxic reference strain) response at the corresponding sampling time.

and SAG 17.85 for 7 d of exposure and thereafter until 21 d of exposure (Figure 3). % tDNA increased significantly only after 21 d of exposure to CCAP 1450/06.

There was evidence of temporal DNA damage for the haemocytes of the zebra mussels fed the two most toxic strains, CCAP 1450/10 and SAG 17.85 (Fig. 3).

## Discussion

MCs derive their toxic and tumour-promoting activities directly from their mechanism of action (protein phosphatase inhibition), and may secondly induce DNA damage *in vitro* following the formation of reactive oxygen species (Žegura *et al.* 2003). Cell viability tests showed that MC exposures had little effect on the viability of haemocytes throughout the study peri-

od. These observations indicate that the mussels were exposed to non-cytotoxic concentrations of MCs, and that the DNA damage measured was not the result of cell death. The experiments also confirm that exposure of mussels to standard freshwater containing Cd-Cl<sub>2</sub>, a known genotoxicant (Pruski and Dixon 2002), induced DNA damage. Furthermore, our results indicate that feeding MC-containing cyanobacteria to zebra mussels significantly affects the integrity of the DNA in their haemocytes; the response observed was strain-specific. It also appears that the more MCs present in the strain, the more genotoxic that strain is, as observed by the increase in haemocyte DNA damage. Although no conclusions can be drawn about the relative toxicity of MC-LF and MC-LR variants, the experiments suggest that the strains producing MC-LF may be both more toxic and more genotoxic to zebra mussels than the strain containing MC-LR. The detection of DNA damage using the Comet assay is a result of complex interactions between the production of DNA damage and the repair of that damage (Collins *et al.* 1997). The temporal response in DNA damage observed in the study suggests that the damage caused by a high concentration of MC-LF may be more persistent than the damage induced by a low level of MC-LR. This may be the result of differences in the rates of DNA repair between treatments, and it may reflect the potential inhibitory effect of MC-LF on such repair. The observed temporal response may also be explained by an increase in *Microcystis* consumption over time by mussels who are adapting to this new food source or by an accumulation of free toxins in the mussels in the tank.

The results thus demonstrate another sublethal adverse effect of MCs on zebra mussels.

## References

- Anderson, D., Yu, T.W., Philips, B.J. & Schmerzer, P. (1994). *Mutation Res.* 307: 261-271.
- Astanei, I., Gosling, E., Wilson, J. & Powell E. (2005). *Mol. Ecol.* 14: 1655-1666.
- Bolognesi, C., Buschini, A., Branchi, E., Carboni, P., Furlini, M., Martino, A., Monteverde, M., Poli, P. & Rossi, C. (2004). *J. Total Env.* 333: 127-136.
- Collins, A.R., Dobson, V.L., Dušinska, M., Kennedy, G. & Ština, R. (1997). *Mutation Res.* 375: 183-193.
- Ding, W.X., Shen, H.M., Zhu, H.G., Lee, B.L. & Ong, C.N. (1999). *Mutation Res.* 442: 69-77.
- Juhel, G., Davenport, J., O'Halloran, J., Culloty, S.C., Ramsay, R.M., James, K.J., Furey, A. & Allis, O.

- (2006). *J. Exp. Biol.* 209: 810-816.
- Juhel, G., Davenport, J., O'Halloran, J., Culloty, S.C., O'Riordan, R.M., James, K.J., Furey, A. & Allis, O. (2006). *Aquat. Toxicol.* 79: 391-400.
- Olive, P.L., Banath, J.P. & Durand, R.E. (1990). *Radiol. Res.* 122: 86-94.
- Ortea, P.M., Allis, O., Healy, B.M., Lehane, M., Ní Shuilleabháin, A., Furey, A. & James, K.J. (2004). *Chemosphere* 55: 1395-1402.
- Pruski, A.M. & Dixon, D.R. 2002. *Aquat. Toxicol.* 57: 127-137.
- Rao, P.V., Bhattacharaya, R., Parida, M.M., Jana, A.M. & Bhaskar, A.S. (1998). *Env. Toxicol. Pharmacol.* 5: 1-6.
- Reeders, H.H., Bij de Vaate, A. & Slim, F.J. (1989). *Freshw. Biol.* 22: 133-141.
- Woods, J.A., O'Leary, K.A., Mc Carthy, R.P. & O'Brien, M.N. (1999). *Mutation Res.* 429: 181-187.
- Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K.I., Carmichael, W.W. & Fujiki, H. (1990). *J. Cancer Res. Clin. Oncol.* 116: 609-614.
- Žegura, B., Sedmak, B. & Filipič, M. (2003). *Toxicon* 41: 41-48.

## Aerosolized red tide toxins (brevetoxins) and asthma: a 10 day follow up after 1 hour acute beach exposure

Barbara Kirkpatrick<sup>1</sup>, Judy A. Bean<sup>2</sup>, Lora E. Fleming<sup>3,5</sup>, Lorraine C. Backer<sup>4</sup>, Rachel Akers<sup>2</sup>, Adam Wanner<sup>5</sup>, Dana Dalpra<sup>1</sup>, Kate Nierenberg<sup>1</sup>, Andrew Reich<sup>6</sup> and Daniel G. Baden<sup>7</sup>

<sup>1</sup>Mote Marine Laboratory, Sarasota, Florida, 34236, bkirkpat@mote.org; <sup>2</sup>Children's Hospital Medical Center and University of Cincinnati, Cincinnati, Ohio, 04524; <sup>3</sup>NSF AND NIEHS Oceans and Human Health Center and the NIEHS Marine and Freshwater Biomedical Sciences Center, University of Miami Rosenstiel School of Marine and Atmospheric Sciences, Miami, Florida, 33149; <sup>4</sup>National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia, 30341; <sup>5</sup>University of Miami School of Medicine, Miami, Florida, 33136; <sup>6</sup>Florida Department of Health, Tallahassee, Florida, 32399, <sup>7</sup>Center for Marine Science, University of North Carolina at Wilmington, Wilmington, NC, 28409

### Abstract

Blooms of the toxic dinoflagellate *Karenia brevis* occur annually around the Gulf of Mexico. A unique feature of this organism is the incorporation of its brevetoxins into the marine aerosol. Animals, including humans, then inhale the toxins which cause respiratory irritation. Recent studies have demonstrated acute changes in both symptoms and spirometry in asthmatics after a 1-hour exposure to the aerosols. This study investigated if there were latent or sustained effects after the initial beach exposure during a documented Florida red tide. Asthmatics who participated in the 1-hour exposure study were asked to keep a symptom diary and to measure their peak flow daily for 10 days after exposure. Environmental air samplers were placed on an inland transect line to document continuing toxic marine aerosols. Although there was no statistical change in the peak flow measurements over the 10 days, when the number of symptoms were scored, an increase in symptoms occurred over the 10-day exposure period compared to the non-exposure period. These findings suggest that asthmatics exposed to *K. brevis* aerosols may continue to have symptoms after their initial beach exposure.

### Introduction

An estimated 10.6 % of non-institutionalized adults and 12.5 % of children have been diagnosed with asthma in the US (Gold and Wright 2005). Environmental exposures can trigger asthma flares and contribute to an increased need for medical intervention and missed days of work or school. Recently, environmental exposure to the Florida red tide aerosols has been demonstrated to cause a significant increase in reported symptoms and a decrease in pulmonary function after a 1-hour beach exposure in asthmatics (Fleming 2005, 2007). This may have important implications for asthmatics that live and/or work at or near the beach. With the population explosion occurring around the US coastline including Florida, more people are being impacted by the toxic aerosols. In addition, there is concern that these Florida red tides are increasing in intensity and duration.

### Methods and Materials

This study was a preliminary companion study to the ongoing evaluation of the exposure to aerosolized *K. brevis* red tide brevetoxins and their possible acute and chronic adverse health effects in humans and ani-

mals by an interdisciplinary team of researchers from federal, state, private, and local organizations. The methods and results for environmental monitoring of the water (for organisms and toxins) and the air (for toxins and particulate) during the 1-hour beach exposure during a Florida red tide are described in detail in Fleming (2005) and Fleming *et al.* (2007). All studies have been approved by the institutional review boards of the University of Miami School of Medicine and the Florida Department of Health.

The three questions to be investigated by this study were, for asthmatics during a 1-hour acute Florida red tide toxin exposure at the beach compared to a non-exposure period:

- 1) Are the effects (as measured by reported respiratory symptoms and peak flow) highest immediately after people leave the beach?
- 2) Are there greater effects with delayed onset hours to days after this acute exposure that have not been monitored or detected?
- 3) Are these effects temporary or do they persist for some time after exposure?

To address the questions, a subset of the asthmatic subjects participating in the NIEHS PO1 study

of aerosolized Florida red tide toxin exposures and health effects (Fleming *et al.* 2005) were asked to participate in a Follow-Up Study. Two Florida red tide exposure studies (February and March 2005) and 1 non-exposure study (May 2004) were conducted.

After study enrolment, participants were instructed in the proper techniques for peak flow measurement (Spiroflow© Adult Peak Flowmeter) and the completion of a daily respiratory symptom log. Subjects were instructed to monitor their peak flow and symptoms at approximately the same time every day, preferably first thing in the morning prior to use of any regular asthma medications. At the conclusion of the 10 days, the subjects mailed back the symptom/peak flow diary in a pre-addressed, stamped envelope. In addition, to assure participant compliance, each participant was called on Day 1 and Day 10 of the study, and a brief telephone questionnaire with more extensive symptom information was collected.

#### Statistical methods

The symptoms consisted of 7 respiratory symptoms (cough, wheeze, throat irritation, shortness of breath, chest tightness, nasal congestion, and eye irritation); for the purposes of an analysis, a symptom score was created by adding all the reported symptoms at a given time. All calculations were performed using SAS, version 9.1. Prior to any analyses, descriptive statistics, means, standard deviations and frequencies were run and examined. The next step was to identify the exposed period and unexposed period for each person who recorded data for the following 10 days after walking on the beach for each time. Individuals that had either an unexposed period or an exposed period but not both were not included these analyses. After the matching was performed, the data were analyzed using a mixed model approach. Bonferroni's adjustment was done when comparing the various means. For the main analyses, a p-value less than 0.05 was considered significant and for the multiple comparisons a p-value of less than 0.05 was said to be significant.

#### Results

With 100 % compliance in returning the completed 59 diaries occurring in all three studies, a total of 36 (59 %) were female with a mean age of 38 (+/- 19 SD) (Table 1). Of these asthmatics, 34 (58 %) were considered severe asthmatics since they regularly used their medications within 12 h of coming to the beach. With regards to residence location, 19 (32 %) lived in

coastal areas within 1 mile of the coast while 40 (68 %) lived inland > 1 mile from the coast.

**Table 1.** Participant Demographics

N	59
N Female (%)	36 (59%)
Mean Age + SD	38 + 19
Race –Caucasian	57 (97%)
N (%) Meds in Last 12 h	34 (58%)
N (%) Coastal Resident	19 (32%)
N (%) Inland Resident	40 (68%)

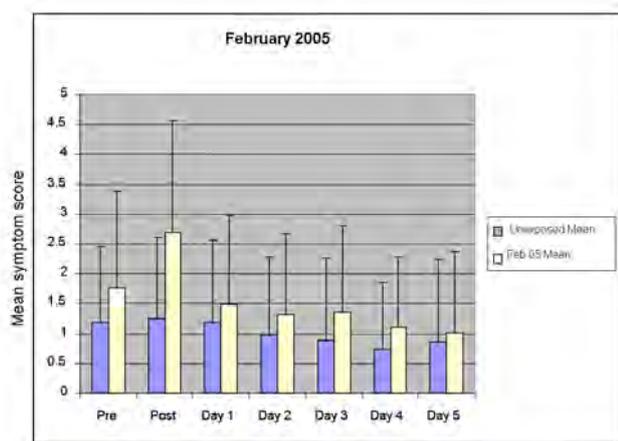
The highest respiratory symptom scores were seen after 1 h acute red tide exposure at the beach during the 2 exposure periods (Figs 1, 2). There was no evidence of any delayed onset of increased reported symptoms within days of the 1-h beach exposure. Of note, particularly in March 2005 when there had been a continuous red tide for several months, the asthmatic subjects came to the beach already reporting relatively high symptom scores presumably from ongoing environmental exposures. There was no increased symptom score after 1 h of beach exposure during a non Florida red tide exposure period.

The reported symptoms continued to be elevated for up to 5 d after the initial 1-h acute Florida red tide exposure. By Day 5, the reported respiratory symptom score had returned to the pre-exposure baseline and there was no further elevation of symptoms reported through Day 10 (data not shown). During the non-exposure study, the reported symptom score remained at baseline throughout the 10-d period.

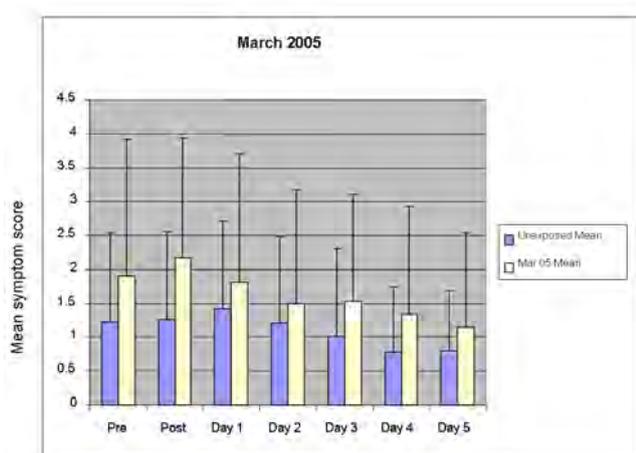
There were no significant differences in the peak flow measurements throughout the follow-up study; however, there were significant changes in FEV1 and other spirometry measures using more sensitive instrumentation after only 1 h of beach exposure during the Florida red tide but not during the non-exposure period (Fleming *et al.* 2005, 2007).

#### Conclusions

This 10-day Follow-Up Study was performed based on anecdotal reports by study participants of delayed and/or prolonged effects after Florida red tide toxin exposures. In addition, occupational asthma studies have reported delayed onset of asthma from occupational and environmental exposures (Reed 1981; Mapp *et al.* 2006). The study demonstrated that just 1 h of Florida red tide toxin exposure can cause the greatest effect in reported respiratory symptoms, and



**Figure 1.** Mean symptom scores versus non exposure February 2005.



**Figure 2.** Mean symptom scores versus non exposure March 2005.

that these symptoms can last for several days. However, this study did not demonstrate any delayed onset of symptoms over a 10-day period of follow up.

There were a number of limitations to this study. First, the primary measure of effect was reported symptoms which were subjective but also highly clinically relevant to the study of asthma. Asthma is defined as reversible airway; therefore objective measurement using pulmonary function measures can be difficult particularly with relatively inaccurate peak flow meters and with a small study population. In addition, it is possible that people were exposed after the 1-h study exposure to the Florida red tide toxins due to occupation as well as residence locations.

However, this study also provided important information for people with asthma living near coastal areas with regular Florida red tide events. In conjunction with the other acute studies performed by these investigators, this study suggested that even 1 h of exposure during an active red tide may result in increased reported respiratory symptoms that can last for up to 5 days. Furthermore, given that these investigators have also demonstrated increased admissions to the emergency room for pneumonia, bronchitis, asthma and upper airway complaints particularly for coastal residents during active Florida red tides, it would seem that asthmatics should avoid beach areas during onshore winds with active Florida red tides.

### Acknowledgements

This research was supported by the Centers for Disease Control and Prevention and the Florida Department of Health (Cooperative Agreement: U50/CCU423360-02) as well as by the P01 ES 10594 DHHS NIH of the National Institute of Environmental Health Sciences. The authors thank Sharon Ketchen, JoEllen DeThomas, (FL Department of Health), Anays Lopez, (University of Miami), and Peg Kutlewski (Mote Marine Lab) for their support of this project. We also thank the dedicated study participants and their families for support of this project.

### References

- Fleming, L.E., Kirkpatrick, B., Backer, L.C., Bean, J.A., Wanner, A., Alpra, D., Tanner, R., Zaias, J., Yung Sung, C., Pierce, R., Naar, J., Abraham, W.M. Clark, R., Yue, Z., Henry, M.S., Johnson, D., van de Bogart, G., Bossart, G.D., Harrington, M. & Baden, D.G. (2005). *Env. Health Perspect.* 113: 650-657.
- Fleming, L.E., Kirkpatrick, B., Backer, L.C., Backer, L.C., Bean, J.A., Wanner, A., Reich, A., Zaias, J., Yung Sung, C., Pierce, R., Naar, J., Abraham, W.M. & Baden, D.G. (2007). *Chest* 131: 187-194.
- Gold, D.R. & Wright, R. (2005). *Ann. Rev. Public Health* 26: 89-113.
- Mapp, C.E., Miotto, D. & Boschetto, P. (2006). *Med. Lav.* 97: 404-409.
- Reed, C.E. (1981). *Postgrad. Med.* Aug 70: 140-153.

## ASP toxins of pennate diatoms and bacterial effects on the variation in toxin composition

Y. Kotaki<sup>1</sup>, N. Lundholm<sup>2</sup>, T. Katayama<sup>1</sup>, E. F. Furio<sup>3</sup>, M.L. Romero<sup>4</sup>, J.R. Relox<sup>4</sup>, T. Yasumoto<sup>5</sup>, H. Naoki<sup>5</sup>, M.Y. Hirose<sup>5</sup>, T.D. Thanh<sup>6</sup>, C.V. Thuoc<sup>6</sup>, N.T.M. Huyen<sup>6</sup>, P.T. Thu<sup>6</sup>, Y. Takata<sup>1</sup>, M. Kodama<sup>1</sup> and Y. Fukuyo<sup>7</sup>

<sup>1</sup>Kitasato University, Sanriku, Iwate 022-0101, Japan, E-mail: kotaki@kitasato-u.ac.jp

<sup>2</sup>University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark; <sup>3</sup>NFRDI, 940 KPI Bldg., Quezon Avenue, Quezon City 1100, Philippines; <sup>4</sup>BFAR, 860 Arcadia Bldg., Quezon Avenue, Quezon City 1100, Philippines; <sup>5</sup>Okinawa CREATE, JST, Suzaki, Uruma, Okinawa 904-2234, Japan; <sup>6</sup>IMER, 246 Da Nang Street, Haiphong City, Vietnam; <sup>7</sup>Tokyo University, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657 Japan

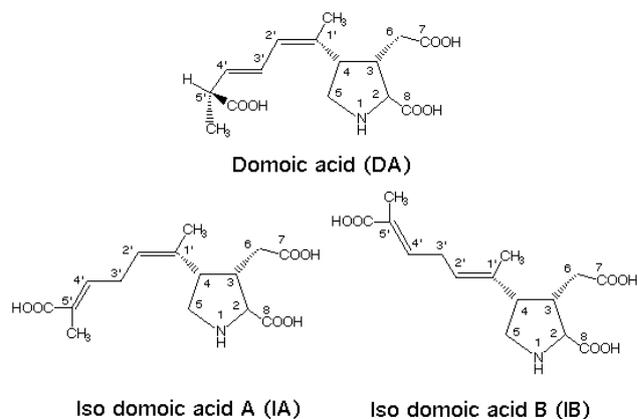
### Abstract

Two types of Philippine strains of *Nitzschia navis-varingica* were recognised in previous studies based on toxin composition: DA-IB type produces domoic acid (DA) and isodomoic acid B (IB), whereas IA-IB type produces isodomoic acid A (IA) and IB. We have extended the studies of toxin composition to 392 strains isolated from Japan, Vietnam and the Philippines. A total of 214 of the 392 strains produced ASP toxins. The IA-IB type was restricted to 29 strains from three areas in the northern Philippines, whereas the other 185 strains belonged to the DA-IB type. Non-axenic substrains kept the same toxin type as the parental strain. However, one out of three axenic substrains changed from the DA-IB type to the IA-IB type. It returned to the original DA-IB type when the culture medium was replaced with the cell-free but non-axenic medium of the parental strain, suggesting a bacterial role in controlling the toxin type. Axenic substrains of the IA-IB type did not change toxin composition. In *P. multiseriis* and *P. delicatissima*, IA and IB were detected along with DA, and IA and IB made up 5-12 % of the total cellular toxin amount.

### Introduction

Domoic acid (DA) is well known as a toxin that causes amnesic shellfish poisoning (ASP) (Wright *et al.* 1989). DA was originally isolated from a red alga (*Chondria armata*) as an insecticidal compound (Takemoto and Daigo 1958). Several DA derivatives, including isodomoic acids A (IA), B (IB) (Fig. 1), C (IC) (Maeda *et al.* 1986), domoilactones A and B (Maeda *et al.* 1987) and isodomoic acids G and H, have also been isolated from *C. armata* (Zaman *et al.* 1997). Small amounts of isodomoic acids D, E, F and 5'-epi-domoic acid have been isolated from shellfish and/or *Pseudo-nitzschia multiseriis*, a species known as a vector for DA to shellfish (Wright *et al.* 1990). IC was also found in *P. australis* (Holland *et al.* 2005). Several different *Pseudo-nitzschia* species produce DA (Bates 2000). Recently, the benthic pennate diatom *Nitzschia navis-varingica* isolated from Do Son in Vietnam was found to be a major producer of DA (Kotaki *et al.* 2000). This species is distributed widely in Asian countries (Kotaki *et al.* 2004). Strains of *N. navis-varingica* subsequently isolated from a limited area in the Philippines produced IA and IB instead of DA, whereas strains from other areas in the Philip-

ippines produced DA and IB (Kotaki *et al.* 2005). This suggests that *N. navis-varingica* strains outside the Philippines also produce a complex of ASP toxins. In this paper, we investigated the toxin composition of several strains from Japan and Vietnam and compared their toxin composition with those from the Philippine strains. Bacterial effects on the variation in toxin composition were investigated by comparing axenic and non-axenic substrains. The toxin composition of two species of *Pseudo-nitzschia* was also examined.



**Figure 1.** Structure of domoic acid and isodomoic acids A and B.

## Materials and Methods

### Collection of diatoms

*Nitzschia*-like diatoms were collected in estuarine areas in Japan (Okinawa Islands in July, and east coast of Tohoku district in September 2005) and in Vietnam (Haiphong in October 2005). Strains of *N. navis-varingica* previously isolated from the Philippines (San Roque, Manila in March 2003 and Iba in March 2005) (Kotaki *et al.* 2005; Bajarias *et al.* 2006) were used for comparison of toxin composition among strains from different countries.

### Isolation and culture of diatoms

Unialgal cultures of *Nitzschia*-like diatoms were established and cultured in a tissue culture tube at 25 °C under an irradiance level of 60  $\mu\text{mol m}^{-2}\text{s}^{-1}$  with a 16:8 L:D cycle. Growth was monitored by *in vivo* Chl. *a* fluorescence.

### Analysis of ASP toxins

Cultures were harvested for toxin analysis 10 d after growth reached stationary phase. Toxin composition of the ultra-sonicated samples was analyzed for DA, IA and IB by HPLC (Kotaki *et al.* 2005). Standard DA from a *P. multiseriis* culture and IA and IB from a *N. navis-varingica* culture were prepared according to Kotaki *et al.* (2005) and calibrated by purchased Canadian standard DACS-1D (DA and IA) and by weight (IB). Toxin composition was expressed as the cellular amount of ASP toxins.

### Toxin composition of *N. navis-varingica* substrains

Substrains of the Philippine strains were established by the capillary washing method and analyzed for toxin composition.

### Bacterial effects on toxin composition variation of *N. navis-varingica*

In order to examine potential bacterial effects on the toxin composition of *N. navis-varingica*, the toxin composition of axenic and non-axenic strains was compared. Axenic strains were prepared by exposure to an antibiotic mixture (gentamicin, penicillin G, streptomycin [0.05, 1.6, 0.8 mg mL<sup>-1</sup>]), followed by capillary washing. The axenic conditions were confirmed by inoculating each culture into three kinds of agar mediums and by DAPI staining (Kotaki *et al.* 2000). The one axenic strain that changed its toxin profile relative to the non-axenic parental strain had its medium replaced with filtered (3  $\mu\text{m}$ ) cell-free medium of the non-axenic parental strain. A small amount of the replaced culture was inoculated into new f/2 medium and cultured for 3 weeks under the

same conditions as above. This process was repeated twice and the newly inoculated culture was analyzed for toxin composition every week for a month.

### Toxin composition of *Pseudo-nitzschia* cultures

*Pseudo-nitzschia multiseriis*, *P. cf. delicatissima* and *P. pungens* isolated from Ofunato Bay, Japan were cultured for three months and analyzed for ASP toxins weekly for the first month and monthly thereafter.

### Species identification of diatoms

Species identification of the *Nitzschia*-like diatom was performed according to Lundholm and Moestrup (2000). *Pseudo-nitzschia* spp. were identified according to Hasle and Syvertsen (1997).

## Results and Discussion

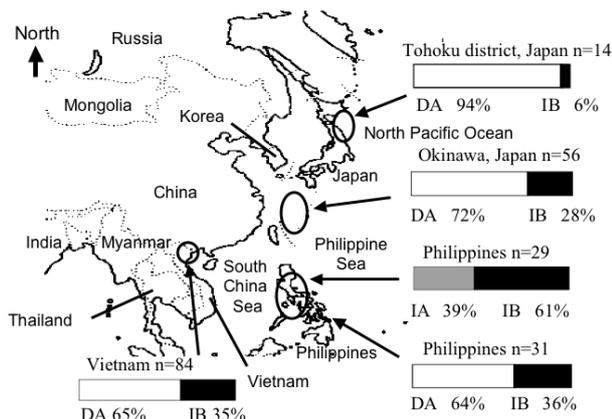
### Diatom strains

Eighty-five isolates of *Nitzschia*-like diatoms were obtained from eight areas of Tohoku district (Japan), 148 isolates were obtained from eight areas of Okinawa Islands and 99 isolates were obtained from 16 areas near Haiphong. Sixty isolates of *N. navis-varingica* previously established from seven areas of the Philippines were included. Representative *Nitzschia*-like strains positive for toxin production were identified as *N. navis-varingica*.

### Toxin composition of *N. navis-varingica*

Fourteen strains from one estuary in Tohoku district (Japan), 56 strains from six locations in Okinawa (Japan) and 84 strains from 16 locations in northern Vietnam showed production of ASP toxins, with a composition of DA and IB. The total toxin content (pg cell<sup>-1</sup>) were 0.4-2.9 (Av. 1.5), 0.1-9.8 (Av. 2.1) and 0.5-11.3 (Av. 3.1), respectively. All 60 Philippine strains produced ASP toxins. Of these, only 29 strains from three areas in the northern part of the Philippines showed a toxin composition of IA and IB, without DA, in which the total toxin content was 0.5-11.1 (Av. 7.3) pg cell<sup>-1</sup>, whereas the other 31 strains had a DA-IB type toxin composition with a total toxin content of 2.3-12.1 (Av. 5.3) pg cell<sup>-1</sup>. The average toxin compositions of the positive strains in each country are shown in Fig. 2. The most frequent ASP toxin composition type of *N. navis-varingica* was DA-IB, but the ratio of the two toxins varied among the sampling areas. The ratio of IB to DA in the DA-IB type strains was 6 % in Tohoku district (Japan), 28 % in Okinawa (Japan), 35 % in Haiphong (Vietnam) and 36 % in the southern part of the sampling sites in the Philippines. This indicates a tendency for the IB to DA ratio to be higher in the

isolates from the southernmost areas. No peaks other than DA, IA and IB were observed in the HPLC chromatograms.



**Figure 2.** ASP toxin composition of *N. navis-varingica* in coastal waters of eastern Asia. N: Number of strains tested

#### Toxin composition of *N. navis-varingica* substrains

Twenty and 16 substrains, respectively, were established from two Philippine parental strains that had a DA-IB toxin composition. Ten substrains were established from another parental strain with an IA-IB toxin composition. All substrains showed the same toxin composition as the parental strains, indicating that toxin composition is stable within a strain.

#### Bacterial effects on compositional variations of *N. navis-varingica* toxins

Three axenic substrains were established from three Philippine parental strains in which the toxin composition of two of the parental strains was DA-IB and that of the third was IA-IB. In the axenic substrains of the parental type IA-IB, the toxin composition did not change. However, one of the axenic substrains of the parental DA-IB type changed its toxin composition to the IA-IB type, whereas the other sub-strain did not change toxin type. When the medium of the axenic culture that changed its composition was replaced with cell-free non-axenic medium of the parental strain, the toxin composition reverted to DA-IB. These results suggest that the toxin composition of *N. navis-varingica* might be affected by one or more species of bacteria.

#### Toxin composition of *Pseudo-nitzschia* cultures

In a 1-month old culture of *P. multiseriis*, DA (5.0 pg cell<sup>-1</sup>), IA (0.1 pg cell<sup>-1</sup>) and IB (0.1 pg cell<sup>-1</sup>) were detected. In a 3-month old culture, DA (82.2 pg cell<sup>-1</sup>), IA (4.1 pg cell<sup>-1</sup>) and IB (5.3 pg cell<sup>-1</sup>) were detected. Hence, IA and IB make up ~5-6% of the total cellular toxin amount. In a 2-week old culture of *P. cf.*

*delicatissima*, only DA (0.06 pg cell<sup>-1</sup>) was detected, whereas DA (0.40 pg cell<sup>-1</sup>), IA (0.02 pg cell<sup>-1</sup>) and IB (0.06 pg cell<sup>-1</sup>) were detected in a 3-week old culture. In this species, IA and IB make up ~4-12 % of the total cellular toxin amount. The data show that small amounts of IA and IB are detected along with DA in cultures of both *P. multiseriis* and *P. cf. delicatissima*. However, it is uncertain whether IA and IB are synthesized *de novo*. No ASP toxins were detected in the *P. pungens* culture during the entire culture period.

## References

- Bajarias, F.A., Kotaki, Y., Relox, J.R., Jr., Romero, M.L.J., Furio, E.F., Lundholm, N., Koike, K., Fukuyo, Y. & Kodama, M. (2006). *Coastal Mar. Sci.* 30: 121-129.
- Bates, S. S. (2000). *J. Phycol.* 36: 978-985.
- Hasle, G.R. & Syvertsen, E.E. (1997). In: *Identifying Marine Phytoplankton*, Tomas, C. R. (ed.), Academic Press, San Diego, pp. 5-385.
- Holland, P.T., Selwood, A.I., Mountfort, D.O., Wilkins, A.L., McNabb, P., Rhodes, L.L., Doucette, G.J., Mikulski, C.M. & King, K.L. (2005). *Chem. Res. Toxicol.* 18: 814-816.
- Kotaki, Y., Furio, E.F. & 11 others. (2005). *Toxicon* 46: 946-953.
- Kotaki, Y., Koike, K., Yoshida, M., Thuoc, C.V., Huynen, N.T.M., Hoi, N.C., Fukuyo, Y. & Kodama, M. (2000). *J. Phycol.* 36: 1057-1060.
- Kotaki, Y., Lundholm, N., Onodera, H., Kobayashi, K., Bajarias, F.A., Furio, E.F., Iwataki, M., Fukuyo, Y. & Kodama, M. (2004). *Fisheries Sci.* 70: 28-32.
- Lundholm, N. & Moestrup, Ø. (2000). *J. Phycol.* 36: 1162-1174.
- Maeda, M., Kodama, T., Tanaka, T., Yoshizumi, H., Takemoto, T., Nomoto, K. & Fujita, T. (1986). *Chem. Pharm. Bull.* 34: 4892-4895.
- Maeda, M., Kodama, T., Tanaka, T., Yoshizumi, H., Takemoto, T., Nomoto, K. & Fujita, T. (1987). *Tetrahedron Lett.* 28: 633-636.
- Takemoto, T. & Daigo, K. (1958). *Chem. Pharm. Bull.* 6: 578-580.
- Wright, J.L.C., Boyd, R.K. & 17 others. (1989). *Can. J. Chem.* 67: 481-490.
- Wright, J.L.C., Falk, M., McInnes, A. & Walter, J.A. (1990). *Can. J. Chem.* 68: 22-25.
- Zaman, L., Arakawa, O., Shimosu, A., Onoue, Y., Nishio, S., Shida, Y. & Noguchi, T. (1997). *Toxicon* 35: 205-212.

## Yessotoxin profiles of the marine dinoflagellates *Protoceratium reticulatum* and *Gonyaulax spinifera*

Bernd Krock<sup>1</sup>, Tilman Alpermann<sup>1</sup>, Urban Tillmann<sup>1</sup>, Grant C. Pitcher<sup>2</sup> and Allan D. Cembella<sup>1</sup>

<sup>1</sup>Alfred Wegener Institute, Am Handelshafen 12, 27570 Bremerhaven, Germany, bkrock@awi-bremerhaven.de, <sup>2</sup>Marine and Coastal Management, Private Bag X, Rogge Bay, 8012 Cape Town, South Africa, gpitcher@deat.gov.za

### Abstract

Yessotoxin (YTX) profiles were established for *Protoceratium reticulatum* isolated from the North Sea (Scottish east coast) and the Benguela Current (South Africa). The profiles were compared to those of plankton field samples from both regions. The presence of yessotoxins produced by *P. reticulatum* in South African waters is reported here for the first time. YTX was the predominant compound in isolates of *P. reticulatum* from both areas. Arabinosyl-YTX was found in both the South African isolate and field samples, but not in the material from the North Sea. An isolate of *Gonyaulax spinifera* from the North Sea was also analysed for yessotoxins, but none were found. The abundance of oxidized YTX-derivatives in the North Sea field samples containing *P. reticulatum* was unexpected and requires further investigation.

### Introduction

Yessotoxins (YTXs) are a large group of ladder-frame disulphated polyethers, first shown to be produced by the marine dinoflagellate *P. reticulatum* in coastal waters of New Zealand (Yasumoto 1997). The three known primary sources of YTXs are all marine gonyaulacoid dinoflagellates, specifically *P. reticulatum*, *Lingulodinium polyedrum* (Paz 2004) and *G. spinifera* (Rhodes 2006). Yessotoxin and an array of derivatives can accumulate in suspension-feeding shellfish, leading to positive responses in the mouse bioassay for lipophilic marine biotoxins. Yessotoxins are globally distributed in coastal and shelf waters, having been reported from Japan, Norway, Chile, New Zealand, Italy and the North Sea. These compounds were originally classed as diarrhetic shellfish poisoning (DSP) toxins, but are now regarded as distinct, as they are not diarrheagenic.

We established the YTX composition of a *P. reticulatum* isolate from the Benguela Current off the west coast of South Africa and of isolates of *P. reticulatum* and *G. spinifera* from the North Sea along the east coast of Scotland. Analysis was performed by HPLC coupled with tandem mass spectrometry (LC-MS/MS). YTX profiles from the cultured isolates were compared to the profiles found in natural phytoplankton assemblages from both locations. These results are the first reports of YTXs in South African coastal waters.

### Methods

*Protoceratium reticulatum* and *G. spinifera* were isolated from the Scottish east coast in June 2004. Clonal

isolates were grown in f/2-enriched seawater growth medium at 16 °C on a 16:8 h L:D photocycle and harvested in late exponential phase. Samples of plankton assemblages, collected from the Scottish east coast at the same time as the clonal isolates, were harvested by centrifugation (15 min, 3220 x g) and the dry cell pellets were stored at -80 °C prior to extraction.

*Protoceratium reticulatum* was isolated from the west coast of South Africa in March 2000. The culture was maintained in K-medium at 17 °C on a 12:12 h L:D photocycle. Field samples were collected daily off Lambert's Bay on the west coast 21 March-6 April 2005. Samples of 200 ml were collected from 0 and 5 m depth and harvested by filtration through glass fibre filters. Cell pellets and filters were homogenized in a FastPrep instrument. Samples were centrifuged, supernatants were filtered and filtrates were analyzed by LC-MS/MS.

Mass spectral measurements were carried out on a triple quadrupole mass spectrometer (API 4000 QTrap, ABI-Sciex) with turbo spray ionization in negative ion mode. Mass spectrometric analyses for YTXs were performed on a Hypersil BDS C8 column (50 × 2 mm, 3 µm, 120 Å) at a flow rate of 0.3 ml min<sup>-1</sup> using an elution gradient with two eluents (A: water and B: 95 % acetonitrile/methanol (1:2 v/v) and 5 % water, both eluents containing 2.0 mM ammonium formate and 50 mM formic acid). Initial composition was 40% B with a linear gradient to 100 % B at 6 min, isocratic 100 % B until 15 min, then returning to initial conditions. Selected transitions (precursor ion > fragment ion) are given in Table 1. Yessotoxin was identified by comparing retention times and MS3 spectra of

**Table 1.** Mass transitions used for LC-MS/MS sample analysis. a other YTX analogs with identical molecular mass are known.

Q1 Mass (amu)	Q3 Mass (amu)	Putative yessotoxin
991	911	#17 (Miles 2005)a
1047	967	41-ketoYTX a
1049	969	no proposed structure
1061	981	no proposed structure
1085	1005	#16 (Miles 2005)
1101	1021	TrinorYTX
1117	1037	no proposed structure
1131	1051	no proposed structure
1141	1061	YTX
1143	1063	no proposed structure
1155	1075	1-homoYTX a
1157	1077	45-hydroxyYTX
1159	1079	no proposed structure
1169	1089	9-Me-41a-homoYTX
1171	1091	45-hydroxy-1a-homoYTX
1173	1093	CarboxyYTX
1175	1095	44,55-dihydroxyYTX
1187	1107	no proposed structure
1189	1109	44,55-dihydroxy-41a-homoYTX a
1195	1115	no proposed structure
1203	1123	44,55-dihydroxy-9-Me-41a-homoYTX
1273	1193	32-O-arabinofuranosyl-YTX
1290	1210	#16 (Finch 2005)
1304	1224	#17 (Finch 2005)
1405	1325	32-O-[arabinofuranosyl-(5'->1'')]-arabinofuranosyl-YTX

samples and a reference standard (IMB-NRC, Halifax, Canada). Compounds with (M-H) masses of 1047, 1175 and 1273 were identified as YTX analogs by characteristic fragments (m/z 173, 855, 925) in MS3 experiments. Other compounds were not further characterised and have to be regarded as putative YTXs. Relative abundances are based on peak area comparisons, identical response factors for all transitions are assumed.

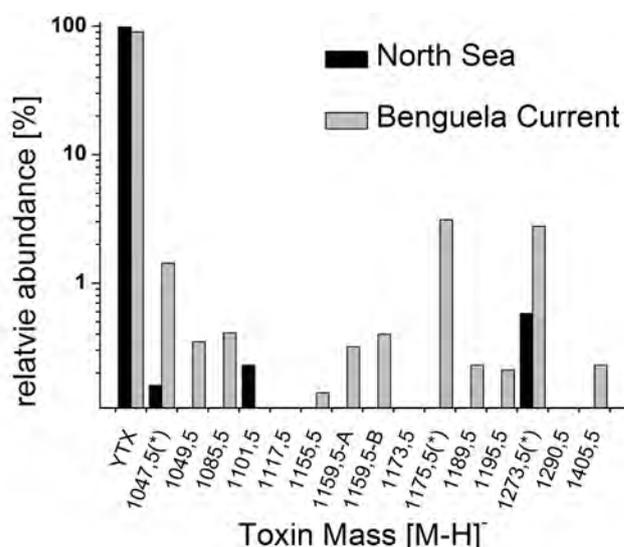
## Results

The yessotoxin profiles of isolates from the Scottish east coast and the Benguela current consisted prima-

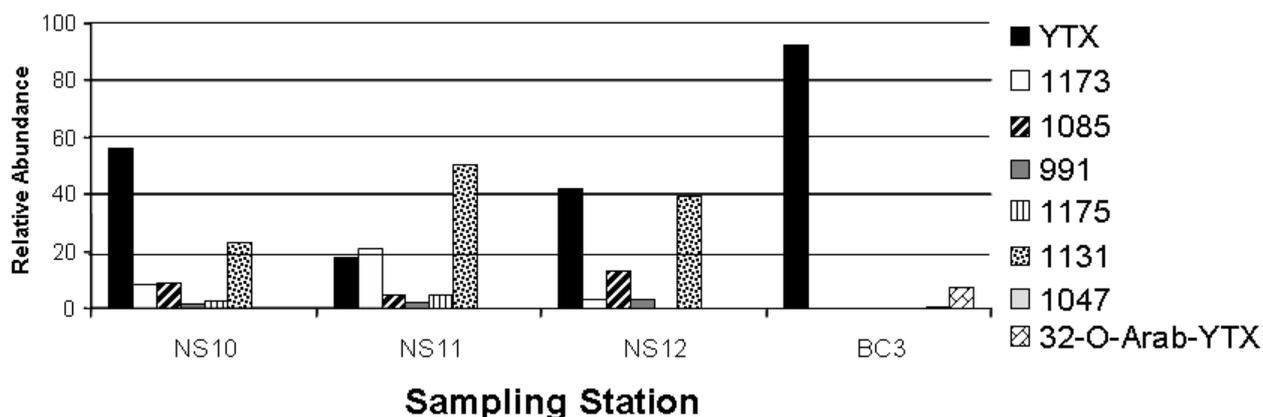
rily of YTX, 99 % in the Scottish isolate and 90 % in the South African isolate. Cell quotas were 100 and 75 fg/cell for YTX, respectively. The South African isolate also contained 3 % arabinofuranosyl-YTX and 3 % putative 44,55-dihydroxyYTX. Other yessotoxins were present only in trace amounts. Arabinofuranosyl-YTX has previously been reported from Japanese (Konishi 2004) and European and New Zealand isolates (Miles 2006). This compound was detected in both the South African isolate of *P. reticulatum* and in field samples from the Benguela.

Whereas the profiles of YTXs of *P. reticulatum* batch cultures and field samples from South Africa showed similar patterns, large differences were seen between the Scottish cultures and field samples from three different North Sea sampling sites. For example, the toxin profile of the North Sea isolate of *P. reticulatum* consisted almost entirely of YTX, with all other YTX derivatives detectable only at trace levels. However, field samples from the same geographical area demonstrated rather different composition. In particular, compounds with the masses of oxidized YTX derivatives, such as carboxyYTX (previously detected only in shellfish), 45-hydroxydinorYTX and 44,55-dihydroxyYTX, were present in higher proportions than in the cultured isolate.

Owing to the recent report by Rhodes (2006) that *G. spinifera* isolates from New Zealand were a source of YTXs, we carefully analyzed for these compounds in an isolate of *G. spinifera* from the Scottish east coast. No YTXs were detected, which suggests that



**Figure 1.** Yessotoxin profile of *P. reticulatum* isolates from the Scottish east coast and the Benguela Current. Compounds marked with (\*) have been identified as YTXs by characteristic mass fragments (m/z 713, 855, 925) in MS3 experiments.



**Figure 2.** YTX profiles of field samples from the North Sea (NS) and the Benguela Current (BC).

*G. spinifera* did not contribute to the YTX profile in the mixed plankton assemblages collected from the North Sea.

### Discussion

Yessotoxin profiles of the *P. reticulatum* isolate and phytoplankton field samples from the Benguela current were in good agreement, although minor differences were observed. For example a compound with the mass of 44, 55-dihydroxyYTX comprised up to 3 % of the toxin complement of the isolate, but could not be detected in the field samples. On the other hand arabinofuranosyl-YTX was more abundant in field samples than in the cultured isolate. These small discrepancies may be a consequence of the different environmental and harvest conditions to which the cultured material and field samples are subjected. In the North Sea, however, the yessotoxin profiles of cultures and field samples differed notably, and differences were also observed among field samples from different stations. This may reflect an underlying intra-specific genetic diversity in field populations. If this is the case, the toxin profile of an individual clonal isolate may not be strictly representative of the mixed toxin phenotypes present in natural assemblages. The presence of alternative sources of YTXs is an alternative explanation for this discrepancy, although *G. spinifera* is an unlikely source of YTXs along the Scottish coast because these compounds were not detected in the cultured isolate from this region. *L. polyedrum*, another potential source of YTXs, was not detected in the field samples. The field samples contained relatively high ratios of compounds with the masses of oxidized YTX-derivatives, such as 44, 55-dihydroxyYTX and carboxyYTX, the latter having previously been detected only in shellfish. This may be an indication of metabolic activity in the lower food web. In this scenario, the YTX metabolites may

be present in fecal pellets and thus captured in mixed plankton samples. It is unknown whether zooplankton such as copepods metabolize YTXs.

The results presented here are derived from mass spectral data alone and only the identity of YTX has otherwise been confirmed. Identification of yessotoxins by mass spectrometry is based on SO<sub>3</sub> loss after collision-induced fragmentation and for some compounds by MS<sub>3</sub> experiments. At this point, we cannot exclude the (unlikely) possibility that sulphated compounds other than YTXs, but with the same range in molecular weight, have been misinterpreted. Further research is needed to confirm spectral identifications and to understand the fate of these marine biotoxins in the food web.

### References

- Finch, S.C., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., MacKenzie, L., Beuzenberg, V., Quilliam, M.A., Olseng, C.D., Samdal, I.A., Aasen, J.A., Selwood, A.I., Cooney, J.M., Sandvik, M. & Miles, C.O. (2005). *Toxicon* 46: 160-170.
- Konishi, M., Yang, X., Li, B., Fairchild, C. R. & Shimizu, Y. (2004). *J. Nat. Prod.* 67: 1309-1313.
- Miles, C.O., Samdal, I.A., Aasen, J.A., Jensen, D.J., Quilliam, M.A., Petersen, D., Brigg, L.M., Wilkins, A.L., Rise, F., Cooney, J.M. & MacKenzie, L. (2005). *Harmful Algae* 4: 1075-1091.
- Miles, C.O., Wilkins, A.L., Selwood, A.I., Hawkes, A.D., Jensen, D.J., Cooney, J.M., Beuzenberg, V. & MacKenzie, L. (2006). *Toxicon* 47: 510-516.
- Paz, B., Riobó, P., Fernández, M.L., Fraga, S. & Franco, J.M. (2004). *Toxicon* 44: 251-258.
- Rhodes, L., McNabb, P., de Salas, M., Briggs, L., Beuzenberg, V. & Gladstone, M. (2006). *Harmful Algae* 5: 148-155.
- Yasumoto, T. & Takizawa, A. (1997). *Biosci. Biotechn. Biochem.* 61: 1175-1177.

## Involvement of cyanobacteria in ciguatera fish poisoning

D. Laurent<sup>1</sup>, A.S. Kerbrat<sup>1</sup>, I. De Fremicourt<sup>2</sup>, H.T. Darius<sup>3</sup>, S. Golubic<sup>4</sup>, M. Chinain<sup>3</sup> and S. Pauillac<sup>5</sup>

<sup>1</sup>Laboratoire de Pharmacochimie des Substances Naturelles et Pharmacophores Redox, UMR152 IRD–

Université Paul Sabatier Toulouse III, centre IRD de Nouméa, BPA5, 98848 Nouméa, New Caledonia, laurentd@noumea.ird.nc; <sup>2</sup>Dispensaire de Chepenehe, BP12, 98884 Lifou, New Caledonia, idef@canl.nc;

<sup>3</sup>Laboratoire des Micro-algues Toxiques, Institut Louis Malardé, BP30, 98713 Papeete, Tahiti, French Polynesia, mchinain@ilm.pf; <sup>4</sup>Biological Science Center, Boston University, <sup>5</sup> Kummington Street, Boston, MA

02215, USA, golubic@bu.edu; <sup>5</sup>Laboratoire des Biotoxines, Institut Pasteur de Nouvelle-Calédonie, BP61, 98845 Nouméa, New Caledonia, spauillac@pasteur.nc

### Abstract

In 2005, a marine benthic cyanobacterial bloom (*Hydrocoleum lyngbyaceum*) was associated with severe ciguatera outbreaks on the island of Lifou (Loyalty Island Province, New Caledonia). Observations outlined by the epidemiological data (severity and rapid onset of clinical symptoms, implication of giant clams in the intoxication, apparent inefficacy of commonly accepted traditional remedies) and the absence of *Gambierdiscus* cells in the area did not favour classical ciguatera fish poisoning (CFP). Results from mouse bioassay and competitive receptor-binding assay using tritiated brevetoxin-3, suggested the presence of ciguatoxin-like compounds both in field samples of cyanobacteria and lipid-soluble extracts from giant clams. Mice intraperitoneally injected with water-soluble extracts exhibited signs and symptoms similar to those of Paralytic Shellfish Poisoning (PSP). This is the first report of the simultaneous association of ciguatera-like toxins and PSP toxins in field samples of benthic marine cyanobacteria. Chemical identification of the toxins is underway. Although long-term observations are necessary to decipher the trends in waxing and waning of dinoflagellate and cyanobacterial populations as well as environmental parameters favourable for their development, the findings suggest that ciguatera risk assessment programmes should also include monitoring of cyanobacteria along with screening of dinoflagellates.

### Introduction

Ciguatera fish poisoning (CFP) is a form of human poisoning resulting from the ingestion of some species of tropical marine fish, that have accumulated potent naturally occurring neurotoxins (ciguatoxins, CTXs) during their diet (Lehane and Lewis 2000; Laurent *et al.* 2005; Lewis 2006). The primary causative agents of CFP are benthic dinoflagellates of the genus *Gambierdiscus* (Yasumoto *et al.* 1977; Chinain *et al.* 1999). CTXs enter the food chain via herbivorous fish that graze and browse on the filamentous or calcareous macroalgae that serve as food for these toxic algae. By bioaccumulation and biotransformation in the food chain, the toxins concentrate in fish to reach levels capable of intoxicating human consumers.

Environmental conditions appear to be of prime importance in promoting CFP outbreaks. These have been correlated with impacts of natural phenomena (storms, tsunamis, hurricanes, submarine volcanoes, coral bleaching, etc) and man-made disturbances of coral reefs (shipwrecks, construction of docks and piers, dredging and filling, chemical pollution, eutrophication, etc.). Following such disturbances,

changes in nutrient flux and increase in dead coral surfaces provide new substrates for macro- and micro-algae (Banner 1976).

### Epidemiology

During 2005, a severe CFP outbreak in Lifou (Loyalty Islands, New Caledonia) led to a public health alert. The epidemiological survey began by interviews of 35 natives from the Hunëtë tribe (about 300 inhabitants), who have been affected by CFP in recent years. In addition, other inhabitants, notably the chief of the fisher clan, were questioned about their fishing habits, the existence of high-risk areas, and the occurrence of natural and anthropogenic environmental changes during the last 10 years. Among the 35 recorded cases of intoxication, 33 were due to the consumption of fish (23 cases by herbivorous grazers mainly Scaridae, 6 cases by molluscivorous Lethridinae and 4 cases by carnivorous fish) or giant clams caught in the area facing the destroyed embarkation ramp. The implication of grazing and molluscivorous fish in the poisoning events is particularly disturbing because in New Caledonia fish belonging to families Scaridae

and Lethrinidae have a reputation of being safe. Despite some similarities with symptoms of CFP (gastrointestinal disorders, general fatigue, pain in the limbs and joints, reversal of hot and cold sensations, tingling sensation upon contact with water), the severity, diversity and rapidity of the onset of clinical symptoms in one third of the patients (those admitted to hospital) suggested the occurrence of toxins other than CTXs. The immediate burning sensation in the mouth, the uncommon nature of the ciguateric seafood (giant clams, herbivorous and molluscivorous fishes) and finally the apparent inefficacy of commonly accepted traditional remedies against CFP (Bourdy *et al.* 1992) were additional indications of the unusual nature of this intoxication event.

### Observations

Following intoxications, the natives identified an area as “high-risk”, where they no longer fish (Fig. 1). The area was inspected by our investigators in the search for possible causative agents of the toxins.

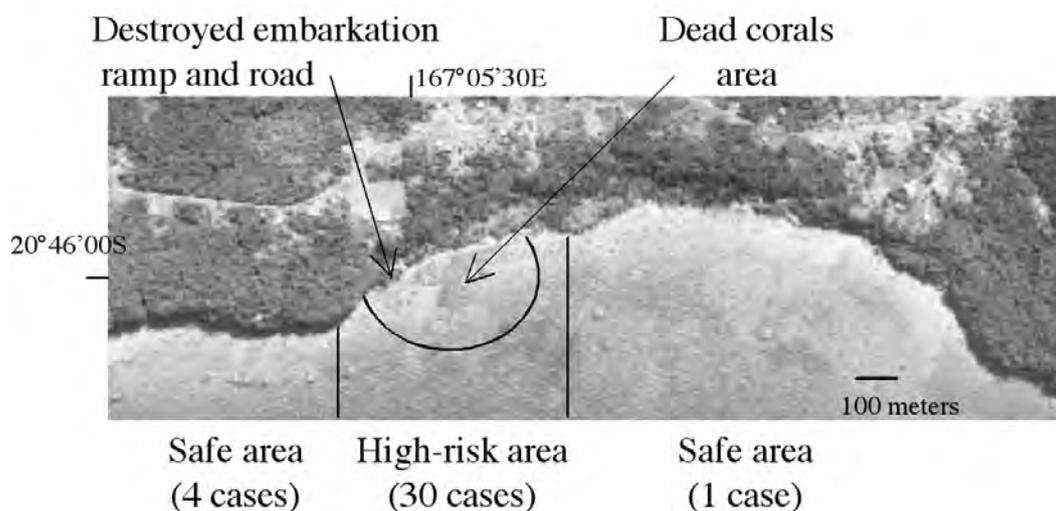
The under-water observations revealed a heavily damaged reef at the end of a road and embarkation ramp construction, covered by broken branched corals and skeletal rubble, overgrown with turfs of various algae. Large masses of dark-coloured filamentous cyanobacteria, later identified by microscopic examination as *Hydrocoleum lyngbyaceum* Kützing coated the coral rubble and the macroalgae. No dinoflagellates were found. Besides the absence of dinoflagellates as evidenced by applying the procedure of Chinain *et al.* (1999) in several surveys, the most striking features of this area were the absence of echinoderms and

brown algae of the genus *Turbinaria*, although some fish were present. In contrast, the regions reckoned as “safe areas” by the tribesmen, on either side of the “high-risk area”, were paved with live corals and characterized by the presence of *Turbinaria* spp. and a great number of sea cucumbers (*Stichopus chloronotus*). They were free of cyanobacteria. Approximately 100 m from the damaged zone, the seabed became light between the blocks of fringing reef flat, while the bed of turf gradually disappeared.

Masses of *Hydrocoleum* were collected and bagged during the dives and later transported to the laboratory for identification and toxicological analyses.

### Toxicology

Lipid-soluble extracts obtained from field samples of cyanobacteria and giant clams collected in the high-risk area were prepared as previously described (Chinain *et al.* 1999) and tested for toxicity using the mouse bioassay and the receptor-binding assay (RBA). Mouse bioassay results such as signs of quiescence, diarrhoea, reduced reflexes, hind-limb paralysis and death with doses up to 1 mg/g body weight, strongly suggest the presence of CTX-like compounds in these extracts: approx. 10 ng equivalent P-CTX-3C/mg of extract as assessed by RBA. Concurrently, signs observed upon injection of water-soluble extracts of both samples in mice (e.g. ataxia, laboured breathing, frequently convulsive spasms and paralysis with death within 1-2 h at doses up to 5 mg/g body weight, or ending with complete spontaneous recovery), suggested the presence of paralytic toxins, possibly similar to cyanobacterial toxins responsible for Paralytic



**Figure 1.** High-risk ciguatoxin area location on the coast facing the Hunëtë tribe (Lifou).

Shellfish Poisoning (PSP). The chemical identification of these toxins is underway by liquid chromatography coupled with mass spectrometry (LC/MS).

The occurrence of ciguatera usually follows a succession of events such as ecological disturbances promoting bleaching and death of coral reefs, thus offering new surfaces for colonization of macroalgae which are used as substrate for toxic dinoflagellates. In Hunëtë, however, the damaged reef surfaces and broken branched corals amassed between the blocks of fringing reef flat supported benthic cyanobacterial blooms, possibly new progenitors of ciguatoxins. Our results indicate a second trophic pathway of CTXs through the marine food chain via molluscs such as giant clams (Fig. 3). Benthic cyanobacteria spread by motile trichomes which become suspended by water movement caused by wind, currents and feeding fish. When suspended, the cyanobacteria enter the food chain of filter-feeding animals including clams.

Benthic cyanobacteria of the genus *Hydrocoleum* are common in New Caledonian and Polynesian lagoons. They are morphologically and genetically related to the pelagic genus *Trichodesmium* (Abed *et al.* 2006). *Trichodesmium erythraeum* has recently been identified as a source of ciguatera-like toxins transferred via food chains and causing poisoning by the Spanish mackerel *Scomberomorus commersoni* (Hahn *et al.* 1992; Endean *et al.* 2003).

This report is the first evidence to suggest simultaneous occurrence of PSP toxins and CTX-like toxins in benthic marine cyanobacterial extracts, and their implication in poisoning of humans. Although long-term observations will be necessary to decipher the trends in dinoflagellate and cyanobacteria populations as well as the environmental parameters suitable for their development, the new findings suggest that

CFP risk assessment and management programmes should include monitoring of cyanobacteria in addition to screening for dinoflagellates.

### Acknowledgements

We are much indebted to the tribe of Hunëtë, especially to B. Ijezie (the small chief), M. Ehnyimane (the chief of the fisher clan), and A. Holue (our laboratory technician originating from Lifou).

### References

- Abed, R.M.M., Palinska, K.A., Camoin, G. & Golubic, S. (2006). *FEMS Microbiol. Lett.* 260: 171-177.
- Banner, A.H. (1976). In: *Biology and Geology of Coral Reefs*, N.A. Jones & R. Endean (eds), Academic Press, New York, 3, pp. 177-213.
- Bourdy, G., Cabalion, P., Amade, P. & Laurent, D. (1992). *J. Ethnopharmacol.* 36: 163-174.
- Chinain, M., Germain, M., Deparis, X., Pauillac, S. & Legrand, A.-M. (1999). *Mar. Biol.* 135: 259-267.
- Endean, R., Monks, S.A., Griffith, J.K. & Llewellyn, L.E. (1993). *Toxicon* 31: 1155-1165.
- Hahn, S.T. & Capra, M.F. (1992). *Food Add. Contam.* 9: 351-355.
- Laurent, D., Yetting, B., Labrosse, P. & Gaudechoux, J.-P., (2005). SPC and IRD, Nouméa.
- Lehane, L. & Lewis, R.J. (2000). *Int. J. Food Microbiol.* 61: 91-125.
- Lewis R.J. (2006). *Toxicon* 48: 799-809.
- Yasumoto, T., Nakajima, I., Bagnis, R. & Adachi, R. (1977). *Bull. Jap. Soc. Scient. Fish.* 43: 1021-1026.

## Production of spirolides in single cells of *Alexandrium ostenfeldii* throughout the diurnal cycle

N. Lewis, C. Garnett, C. Leggiadro, C. Rafuse and M. Quilliam

National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford St, Halifax, NS, Canada, B3H 3Z, Nancy.Lewis@nrc-cnrc.gc.ca

### Abstract

The production of toxic metabolites by dinoflagellates can be affected by both environmental and genetic factors. Changes in toxin content during the cell cycle have been observed in several toxic species including *Alexandrium* spp. In this study, a microcolumn LC-MS method was used to quantify toxin concentration within a single cell over the diurnal cycle. This allowed accurate determination of spirolide quota in single cells of AOSH1, an isolate of *Alexandrium ostenfeldii*. Digital image analysis was used to quantify the volume of individual cells, and facilitated accurate determination of 13-desmethyl spirolide C concentration following LC-MS analysis. In addition, changes in mean cell size in the culture were monitored using a Coulter Multisizer. A distinct advantage of this method was that both the toxin profile and concentration could be determined for individual cells undergoing division. These methods allowed further examination of differences in the bio-volume of live and lugol-preserved cells.

### Introduction

Spirolides are a group of cyclic imine toxins produced by the gonyaulacoid dinoflagellate *Alexandrium ostenfeldii*. These compounds cause a characteristic rapid death in mice after intraperitoneal injections of lipophyllin extracts (Richard *et al.* 2001). Variability in toxin profile and concentration has been observed for this species in field samples (Cembella *et al.* 1999) and in cultures (unpublished data). Some of this variation can be attributed to changes in environmental conditions and/or genetic variability within a population. Maclean *et al.* (2003) demonstrated that cell quota of spirolides was more closely related to growth rate than to the effects of extrinsic factors on toxin production. Life history phase has been shown to influence toxin cell quota in this species (John *et al.* 2000). Likewise, preliminary data from our laboratory have shown a strong correlation between cell volume and spirolide concentration of single cells from a clonal isolate of *A. ostenfeldii*. In this paper spirolide concentration and cell volume were analysed in single and multiple cell samples taken from a culture of *A. ostenfeldii* isolate AOSH1 at regular intervals over the diurnal cycle. The effect on spirolide cell quota in *A. ostenfeldii* was assessed.

### Experimental

Cells in exponential growth phase were inoculated into 20-L Bellco glass carboys with L1 medium (Guillard & Hargraves 1993) and incubated at 14 °C under a photon flux density of 50-60  $\mu\text{mol photons}$

$\text{m}^{-2} \text{s}^{-1}$  on a 14:10 L:D cycle. The culture was mixed by stirring gently at minimum speed with a motorized paddle before samples were taken to monitor growth by microscopy. When cell concentration reached 1500 cells  $\text{mL}^{-1}$ , the lights were turned off for a period of 88 h to induce synchronisation of the cell cycle. Samples were taken immediately following resumption of the normal light regime and then every four hours after mixing. Cells were preserved in Lugol's solution for enumeration and volume measurements by microscope and with a Beckman Coulter Multisizer 3 particle counter. The volume of preserved cells was compared to that of live motile cells picked from the same sample for toxin analysis every four hours. Samples were also taken for DNA, extracted chlorophyll, and multiple cell toxin analysis. After 24 hours, sampling (except for cell picking) was increased to every two hours. Chlorophyll samples were filtered onto Whatman GF/C filters and extracted in the dark in 90 % acetone for 24 – 36 h prior to measurement with a fluorometer (Turner Designs). Cellular DNA was stained with PicoGreen (Molecular Probes) and analysed using a Becton Dickinson flow cytometer (Pan & Cembella 1998). Toxin samples (10 mL culture) were pelleted by centrifugation at 4000 x g and analysed as described by John *et al.* (2001).

Micropipette isolations of live cells were made using a Wild dissecting microscope. A video sequence of swimming cells was acquired at 100x magnification using a Leica DMRE light microscope. Each frame was examined to find a clear image of a cell in a ventral orientation for determination of volume. Quanti-

fication of volume was performed using the Simple PCI Compix image analysis software (Compix Inc. Imaging Systems, Sewickley, PA).

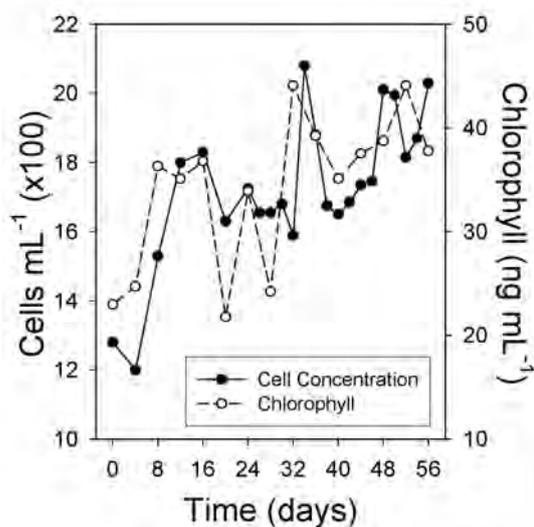
After an image of each cell was acquired, it was transferred by micropipette to 4-mm Millex-GV4 filters that had been presoaked in MeOH and rinsed in filtered seawater. After removing excess seawater by centrifugation, each cell was extracted for 5 min in 75  $\mu\text{L}$  of 80 % MeOH with 0.1 % formic acid. LC-LC separation was performed using an Agilent 1100 series HPLC (Palo Alto, CA) equipped with an OASIS-HLB (10  $\mu\text{m}$ , 20  $\times$  2 mm i.d.) and a Hypersil-BDS-C8 (10  $\mu\text{m}$ , 150  $\times$  1 mm i.d.) column connected through a switching valve. Mobile phases were (A)  $\text{H}_2\text{O}$  and (B) 95 % acetonitrile, both with 50 mM formic acid and 2 mM ammonium formate. Selected reaction monitoring (SRM) of major transition ions was performed in positive ion mode with an API 4000 triple quadrupole MS system (PE-SCIEX, Concord, ON) equipped with a Turbo IonSpray source (SCIEX, Streetville, ON, Canada). Further details on this method can be found in Quilliam *et al.* (this volume).

Statistical comparisons of means were performed using the Mann-Whitney Rank Sum test for non-parametric data (SigmaStat, Systat). Data are reported as the mean  $\pm$  SD.

## Results and Discussion

The prolonged dark period induced the formation of pellicular or temporary cysts and resulted in a decrease in cell number to 1200 cells  $\text{mL}^{-1}$ . When the lights were turned on, cell division resumed and cell concentration increased to 2000 cells  $\text{mL}^{-1}$  over the experimental period. Concentration of cells and of chlorophyll *a* decreased during dark periods (Fig. 1), but flow cytometer analysis of cellular DNA confirmed that synchronisation of cell division was not achieved by dark adaptation (data not shown).

Changes in cell size after chemical preservation have been described previously (Menden-Deuer *et al.* 2001). However, the methods used have been generally less precise because of the problems associated with measuring motile cells. Using the video sequencing technique, the volume of large numbers of live cells can be determined, permitting accurate comparisons with that of preserved cells. Cells preserved in Lugol's solution had a mean volume of 17  $\times 10^3 \mu\text{m}^3$  ( $n = 199$ ) and were 24 % smaller than live cells (23  $\times 10^3 \mu\text{m}^3$ ,  $n = 180$ ), a significant reduction in size ( $p < 0.001$ ). Measurements made using fixed samples can seriously underestimate cell volume. The

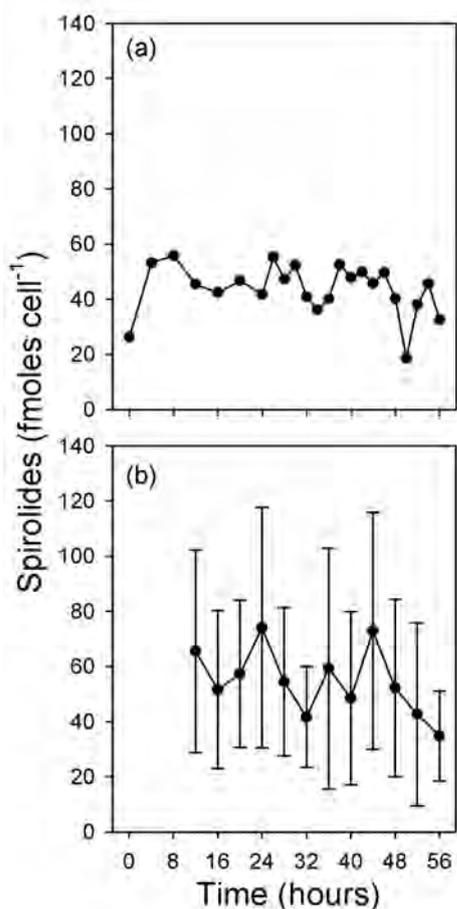


**Figure 1.** Variation in cell concentration and chlorophyll *a* in a culture of *A. ostensfeldii* (AOSH1) over the diurnal cycle. Periods of darkness are indicated with dark bars.

considerable amount of debris from lost thecae in the culture interfered with measurements of cell number and volume using the Coulter Multisizer, thus preventing comparisons with single cells.

All known spirolide analogues produced by this isolate were quantified in multiple cell samples. The toxin profile of AOSH1 is dominated by SPX-dmC (87 %), with relatively smaller quantities of the other known analogues (2 % SPX-C2, 3 % SPX-dmD, and 8 % SPX-C) present. Data showed that although this profile was stable, total spirolide concentration fluctuated throughout the diurnal cycle (Fig. 2a). In single cells, only SPX-dmC and SPX-C2 were within the range of quantification, while only trace amounts of other analogues were evident (Fig. 2b). Spirolide concentration in single cells was greater during the dark ( $69 \pm 39 \text{ fmol cell}^{-1}$ ,  $n = 95$ ) than during the light ( $56 \pm 36 \text{ fmol cell}^{-1}$ ,  $n = 87$ ,  $p = 0.003$ ). When the same comparison was performed with pelleted samples, no significant difference was observed ( $p = 0.202$ ). Multiple cell samples are typically used to estimate cell toxin quota, but intercellular variability is overlooked. The ability of a cell to produce toxic compounds may be seriously underestimated.

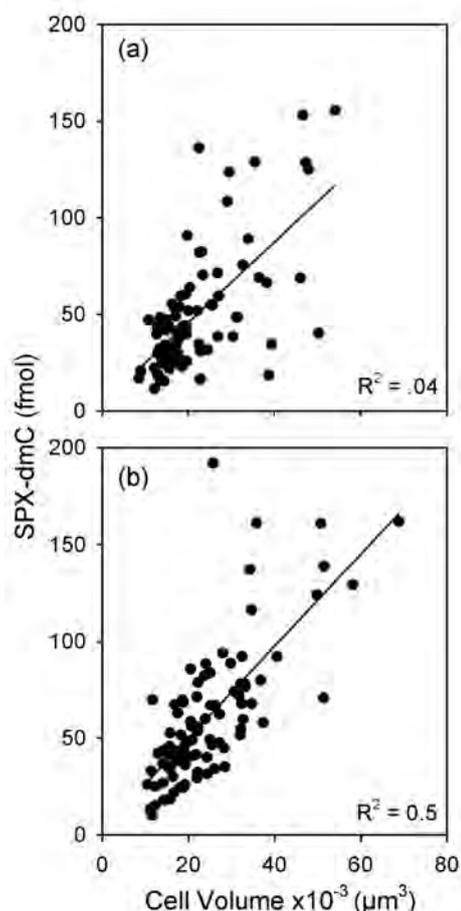
Cells of AOSH1 in clonal culture exhibited wide variation ( $9 - 69 \times 10^3 \mu\text{m}^3$ ) in volume. Minor spirolide analogues occurred in very low concentrations, potentially biasing the profile, and therefore SPX-dmC alone was correlated with cell volume. Cell quota of SPX-dmC varied from 10 – 192  $\text{fmol cell}^{-1}$  and was positively correlated to cell volume (Figs 3a, 3b). A slightly better correlation was observed in cells



**Figure 2.** (a) Toxin cell quota (fmol cell<sup>-1</sup>) from pooled cell samples over the sampling period and (b) the variability that occurred in single cells ( $n = 16$ , mean  $\pm$  SD) from that sample.

selected during the dark period ( $R^2 = 0.5$ ) than in the light ( $R^2 = 0.4$ ). Some of the observed variation may be attributed to differences in the physiological state of the cell over the diurnal cycle. Correlation between SPX-dmC cell quota and cell volume varied for each sampling period. Some of the observed variability may be due to differences in the time at which the culture was sampled. Although growth was not synchronised, cell division was still occurring as indicated by the increase in cell concentration and extracted chlorophyll. A better correlation was achieved when the size range of cells selected from the sample was maximised. Cells lost or broken during the isolation process produced low or undetectable levels of SPX-dmC and were not included in the analyses.

The processes by which secondary metabolites such as spirolides are produced can be complex and is likely controlled by a combination of many factors. Environmental conditions such as light, salinity, nutrients, and temperature may affect cell toxin quota directly or by influencing growth rate. Intrinsic factors such as age in culture or mitotic cell phase may



**Figure 3.** Spirolide SPX-dmC concentration in single cells correlated with cell volume during (a) light and (b) dark sampling periods.

also play a role as in the production of paralytic shellfish toxins (Taroncher-Oldenburg 1997). In this study, environmental conditions remained constant yet the spirolide cell quota varied significantly (Fig. 2b). A strong correlation between cell volume and cell toxin quota has been demonstrated (see Fig. 3), but clearly cell volume does not account for all of the variability. For instance, mean cell volume was also significantly greater ( $p = 0.026$ ) at night ( $24 \pm 10 \times 10^3 \mu\text{m}^3$ ) than during the day ( $22 \pm 10 \times 10^3 \mu\text{m}^3$ ). From this experiment we were unable to determine whether photoperiod affects toxin quota directly or indirectly via cell volume. Synchronization of cell division can be difficult to achieve with slow-growing species such as *A. ostensfeldii*, which responds adversely to dark adaptation. When dividing cells were observed in the sample they were selected for analysis, but too few of these cells were isolated to reliably interpret the effect of cell division on toxin concentration. This technique would be an ideal method of measuring the production of toxic metabolites because dividing cells can be analysed individually.

**References**

- Cembella, A.D., Lewis, N.I. & Quilliam, M.A. (1999). *Nat. Tox.* 7: 197-206.
- Guillard, R.R.L. & Hargraves, P.E. (1993). *Phycologia* 32: 234-236.
- John, U., Quilliam, M., Medlin, L. & Cembella, A. (2001). In: *Harmful Algal Blooms*, Hallegraeff G.M., Blackburn S.I., Bolch, C.J. & Lewis R.J. (eds), UNESCO, Paris, pp. 299-302.
- Macleay, C., Cembella, A.D. & Quilliam, M.A. (2003). *Bot. Mar.* 46: 466-476.
- Menden-Deuer, S., Lessard, E.J. & Satterberg, J. (2001). *Mar. Ecol. Prog. Ser.* 222: 41-50.
- Quilliam, M.A., Lewis, N.I., Aasen, J. & Hardstaff, W. (2007). This volume.
- Richard, D., Arsenault, E., Cembella, A. & Quilliam, M. (2001). In: *Harmful Algal Blooms*, Hallegraeff G.M., Blackburn S.I., Bolch, C.J. & Lewis, R.J. (eds), UNESCO, Paris, pp. 383-386.
- Taroncher-Oldenburg, G., Kulis, D.M. & Anderson, D.M. (1997). *Limnol. Oceanogr.* 42: 1178-1188.

## Nitric oxide (NO) generation by the harmful red tide phytoplankton *Chattonella marina*

T. Oda, D. Kim and K. Yamaguchi

Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Bunkyo-machi 1-14, Nagasaki 852-8521, Japan, t-oda@net.nagasaki-u.ac.jp

### Abstract

The harmful red tide phytoplankter *Chattonella marina* is known to exhibit potent fish-killing activity. Previous studies have demonstrated that *C. marina* produces reactive oxygen species (ROS), and a ROS-mediated ichthyotoxic mechanism has been postulated. In this study, we found that *C. marina* is producing relatively high level of nitric oxide (NO) under normal growth conditions. We measured NO by a fluorometric assay using a highly specific fluorescent indicator of NO. The response was diminished by the addition of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), a specific NO scavenger. The NO level in *C. marina* decreased significantly with the addition of NG-Nitro-L-arginine methyl ester (L-NAME), a specific NO synthase inhibitor. The addition of L-arginine resulted in an increase in NO levels, whereas an increased supply of NaNO<sub>2</sub> had no effect. NO generation by *C. marina* was also confirmed by a chemiluminescence (CL) reaction between NO and luminol-H<sub>2</sub>O<sub>2</sub>. These results suggest that a NO synthase-like enzyme is mainly responsible for NO generation in *C. marina*.

### Introduction

The red tide phytoplankter *Chattonella marina* is known to exhibit potent fish-killing activity. Although the exact ichthyotoxic mechanism of *C. marina* is still controversial, suffocation due to gill tissue dysfunction is generally believed to be the direct cause of fish death by this genus (Shimada *et al.* 1983; Endo *et al.* 1985). Several studies have demonstrated that *Chattonella* spp. generate reactive oxygen species (ROS) such as O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> (Kawano *et al.* 1996; Oda *et al.* 1997). Although a number of laboratory studies repeatedly demonstrated that these raphidophytes produce significant levels of ROS, controversy has continued over whether the levels of ROS detected in these genera are sufficient to kill fish or whether other toxic factors are involved (Twiner *et al.* 2001; Marshall *et al.* 2003; de Boer *et al.* 2004).

In considering toxic factors other than ROS, we suspected that *C. marina* produces nitric oxide (NO) under normal growth conditions without external stimuli. NO, originally described as the endothelium-derived relaxing factor (Palmer *et al.* 1987), has been shown to be involved in a number of physiological and pathological processes in mammals (Stamler 1994). In addition, evidence for the existence of an endogenous pathway for NO synthesis in the plant kingdom has increased in recent years (Durner *et al.* 1998). Since *C. marina* is a phototroph, these findings prompted us to measure the generation of NO in

*C. marina*. The results obtained by several NO detection systems such as a fluorometric assay using a NO-specific fluorescent probe supported the generation of NO by *C. marina*. Our results provide new insight into the physiology of *C. marina* as a NO-generating unicellular phytoplankter.

### Materials and Methods

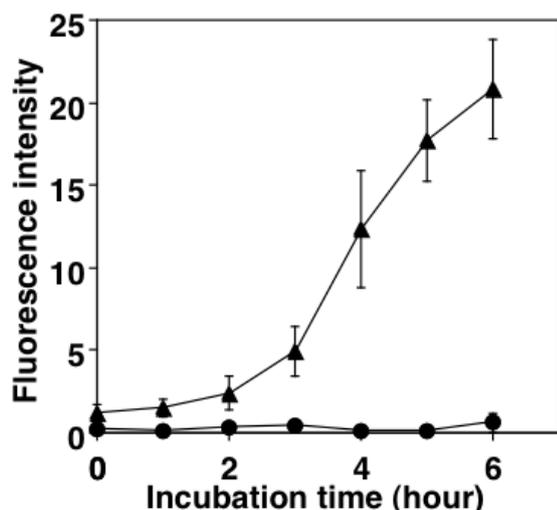
*Chattonella marina* isolated from Kagoshima Bay in 1985 was generously provided by the Kagoshima Prefectural Fisheries Experimental Station, Japan. It was cultured at 26 °C in Erd-Schreiber modified (ESM) medium, pH 8.2, under illumination from a fluorescent lamp (30 μmol photons m<sup>-2</sup> s<sup>-1</sup>) and a 12:12 L:D cycle. For the detection of NO, we used the membrane permeable ester derivative and NO-reactive fluorescent probe diaminofluorescein-FM diacetate (DAF-FM DA, Kojima *et al.* 1999). Once in living cells, DAF-FM DA is hydrolyzed by intracellular esterases to release DAF-FM, which is then converted to the fluorescent triazole derivative DAF-FM T after reaction with NO. The *C. marina* cell suspension was incubated with DAF-FM DA (final 10 μM) at 27 °C. The fluorescence reaction of NO was measured at 515 nm using a Hitachi High F-4500 fluorescence spectrophotometer with a 500 nm excitation wavelength. For microscopic observation, *C. marina* cells incubated with DAF-FM DA (final 10 μM) were observed with a fluorescence microscope (Carl Zeiss Axiovert

200). As an independent measurement technique for the detection of NO, we also tested chemoluminescence (CL) analysis using luminol- $H_2O_2$  (Kikuchi *et al.* 1993). After simultaneous addition of  $H_2O_2$  (final 2 mM) and luminol (final 50  $\mu$ M) or with carboxy-PTIO (c-PTIO, a NO-specific scavenger, Pfeiffer *et al.* 1997) to a *C. marina* cell suspension in ESM medium, the CL emission was recorded immediately with a luminometer (Bio Orbit 1254-001 Luminova). All CL experiments were done at 27 °C. Since the maximal CL response of *C. marina* was attained within the first 10 s, quantitative analysis for the activity of *C. marina* to induce CL was expressed in terms of relative intensity of integrated emission during the first 10 s. Each value represents an average of triplicate samples; bars represent standard deviations. Statistical analysis was performed using the student's t-test.

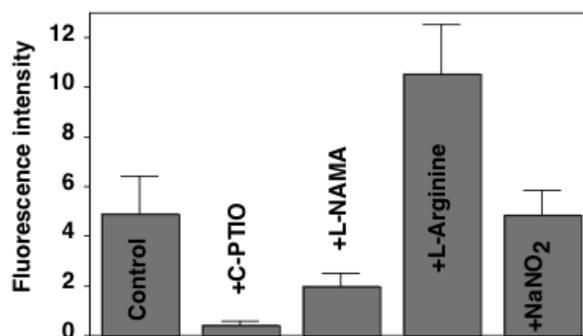
## Results

After addition of DAF-FM DA into *C. marina* cell suspensions, a gradual increase in fluorescence intensity was observed. The increase was completely inhibited by the addition of carboxy-PTIO, a specific NO scavenger (Fig. 1).

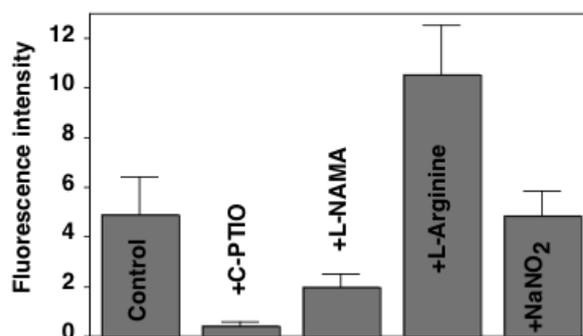
Fluorescence microscopy also suggested that NO was produced inside *C. marina* cells, and the bright fluorescence from *C. marina* was prevented by carboxy-PTIO addition (Fig. 2). L-NAME, a NO synthase specific inhibitor, inhibited NO production by *C. marina* as well (Fig. 3). Furthermore, the addition of L-arginine, a substrate for NO synthase (NOS),



**Figure 1.** Kinetics of NO production estimated by the fluorescence indicator DAF-FM DA. *C. marina* cell suspension incubated with (▲) or without (△) 2 mM of carboxy-PTIO for 5 min at 27 °C.



**Figure 2.** Fluorescence images of NO production in *C. marina*.



**Figure 3.** Effects of various agents on NO production in *C. marina*.

resulted in increased NO production. In the luminol- $H_2O_2$  assay, similar basic results were obtained (Fig. 4): when 2 mM of  $H_2O_2$  was added to *C. marina* simultaneously with luminol, increased CL was observed. The luminol- $H_2O_2$ -mediated CL was also suppressed by carboxy-PTIO. These results suggest that a NO synthase-like enzyme is involved in NO generation in *C. marina*. Nitrite-dependent NO production was not evident (Fig. 3).

## Discussion

Our DAF-FM DA results demonstrated that the unicellular marine phytoplankter, *C. marina*, produced a considerable amount of NO under normal growth conditions. Further, the chemoluminescence (CL) reaction of NO with luminol- $H_2O_2$ , reported as a sensitive and specific NO detection method (Kikuchi *et al.* 1993), indicated that *C. marina* induced potent CL emission that was abolished by carboxy-PTIO, a NO scavenger. The NO production in *C. marina* was inhibited by the NO synthase inhibitor, L-NAME, suggesting that the main source of NO production in this alga is through a NO synthase-like enzyme. In agreement with this concept, addition of the NO synthase

substrate L-arginine to the *C. marina* cell suspension resulted in a significant ( $p < 0.05$ ) increase of NO. The specific stimuli or culture conditions that influence the level of NO in *C. marina* have not been discerned to date.

Whether NO can cause harmful effects on fish is unclear, but several possibilities can be considered. Since NO is a diffusible and highly reactive radical, NO produced by *C. marina* may cause detrimental effects on fish through reaction with various biological molecules (Wink and Mitchell 1998). NO has been shown to be an important regulator of mucus secretion in the stomach (Brown *et al.* 1992, 1993; Ichikawa *et al.* 1999) and administration of NO donors stimulated mucus release from isolated gastric mucus-cell fraction (Brown *et al.* 1993). Thus, it is conceivable that NO alone or in combination with ROS may induce over-secretion of mucoid substances when *C. marina* cells pass between the gill lamellae, which in turn may result in the blockage of respiratory water flow. Further studies are required to clarify the involvement of NO in the ichthyotoxicity of this common raphidophyte.

#### Acknowledgements

This study was supported by the Nagasaki Prefecture Collaboration of Regional Entities for Advancement of Technological Excellence and the Japan Science and Technology Agency.

#### References

- Brown, J.F., Hanson, P.J. & Whittle, B.J.R. (1992). *Eur. J. Pharmacol.* 223: 103-104.
- Brown, J.F., Keates, A.C., Hanson, P.J. & Whittle, B.J.R. (1993). *Am. J. Physiol.* 265: G418-G422.
- de Boer, M.K., Tyl, M.R., Vrieling, E.G. & van Rijssel, M. (2004). *Aquat. Microb. Ecol.* 37: 171-181.
- Durner, J., Wendehenne, D. & Klessig, D.F. (1998). *Proc. Natl. Acad. Sci. USA* 95: 10328-10333.
- Endo, M., Sakai, T. & Kuroki, T. (1985). *Mar. Biol.* 87: 193-197.
- Ichikawa, T., Ishihara, K., Kusakabe, T., Kawakami, T. & Hotta, K. (1999). *Life Sci.* 65: 41-46.
- Kawano, I., Oda, T., Ishimatsu, A. & Muramatsu, T. (1996). *Mar. Biol.* 126: 765-771.
- Kikuchi, K., Nagano, T., Hayakawa, H., Hirata, Y. & Hirobe, M. (1993). *Anal. Chem.* 65: 1794-1799.
- Kojima, H., Urano, Y., Kikuchi, K., Higuchi, T., Hirata, Y. & Nagano, T. (1999). *Angew. Chem. Int. Ed. Engl.* 38: 3209-3212.
- Marshall, J.A., Nichols P.D., Hamilton, B., Lewis, R.J. & Hallegraeff, G.M. (2003). *Harmful Algae* 2: 273-281.
- Oda, T., Nakamura, A., Shikayama, M., Kawano, I., Ishimatsu, A. & Muramatsu, T. (1997). *Biosci. Biotechnol. Biochem.* 61: 1658-1662.
- Palmer, R.M.J., Ferrige, A.G. & Moncada, S. (1987). *Nature* 327: 524-526.
- Pfeiffer, S., Leopold, E., Hemmens, B., Schmidt, K., Werner, E.R. & Mayer, B. (1997). *Free Radical Biol. Med.* 22: 787-794.
- Shimada, M., Murakami, T.H., Imahayashi, T., Ozaki, H.S., Toyoshima T. & Okaichi, T. (1983). *Acta Histochem. Cytochem.* 16: 232-244.
- Stamler, J.S. (1994). *Cell* 78: 931-936.
- Twiner, M.J., Dixon, S.J. & Trick, C.G. (2001). *Limnol. Oceanogr.* 46: 1400-1405.
- Wink, D.A. & Mitchell, J.B. (1998). *Free Radical Biol. Med.* 25: 434-456.

## First evidence for implication of nitric oxide in a mouse model for ciguatera fish poisoning

S. Pauillac<sup>1</sup>, F. Vernel-Pauillac<sup>2</sup>, S. Kumar-Roine<sup>3</sup>, M-P. Sauviat<sup>4</sup>, E. Benoit<sup>5</sup>, M. Chinain<sup>6</sup> and D. Laurent<sup>3</sup>

<sup>1</sup>Laboratoire des Biotoxines, Institut Pasteur de Nouvelle-Calédonie, BP61, 98845 Nouméa, New Caledonia, spauillac@pasteur.nc, <sup>2</sup>Laboratoire de Recherche en Bactériologie, Institut Pasteur de Nouvelle-Calédonie, BP61, 98845 Nouméa, New Caledonia, fpauillac@pasteur.nc, <sup>3</sup>UMR 152, IRD-Université Paul Sabatier, Toulouse III, Centre IRD de Nouméa, BP A5, 98848 Nouméa, New Caledonia, Shilpa.Kumar-Roine@noumea.ird.nc and dominique.laurent@noumea.ird.nc, <sup>4</sup>INSERM U696, UMR CNRS 7645, X / ENSTA, Ecole Polytechnique, 91128 Palaiseau Cedex, France, martin-pierre.sauviat@polytechnique.fr, <sup>5</sup>UPR 9040, Institut de Neurobiologie Alfred Fessard, CNRS, 91198 Gif-sur-Yvette Cedex, France, benoit@nbcn.cnrs-gif.fr, <sup>6</sup>Laboratoire des Microalgues Toxiques, Institut Louis Malardé, BP 30, Papeete, Tahiti, French Polynesia, MChinain@ilm.pf

### Abstract

The involvement of the nitric oxide (NO) pathway in ciguatera fish poisoning (CFP) has been investigated, *in vitro* and *in vivo*, in a mouse-ciguatoxin (CTX) model. The induction of inducible nitric oxide synthase (iNOS) synthesis at the mRNA level was kinetically measured using a real-time PCR protocol based on the LightCycler® technology. CTX-pulsed cultured Neuro-2a cells (0.1 ng/ml) and peripheral blood mononuclear cells (PBMCs) from CTX-injected mice (0.1 ng/g) were demonstrated to express iNOS in a time-dependent manner with a peak expression around 4 h 30. This strongly suggests that NO radicals might be responsible for certain ciguatera symptoms (e.g. hypotension, allergenic effects and chronic fatigue syndrome) which can not be solely explained by CTX-induced activation of voltage-gated sodium channels. This hypothesis is further supported by the most currently used drugs for the treatment of CFP being free radical scavengers. In conclusion, the implication of NO in CFP paves the way for new therapies for both occidental and traditional medicines, together with new CTXs detection and clinical diagnostic tools.

### Introduction

Ciguatera fish poisoning (CFP) occurs from the consumption of certain species of tropical marine fish which have accumulated naturally occurring toxins through their diet. These toxins, referred to as ciguatoxins (CTXs), are potent lipid-soluble polyether compounds, originally produced by certain strains of benthic dinoflagellates of the genus *Gambierdiscus* (e.g. Lehane and Lewis 2000) that live in association with macroalgae attached to dead corals. CTXs enter the food chain starting with herbivorous and spreading up to carnivorous fish that prey on them, and ultimately to humans. More than 50,000 people are intoxicated annually with CTXs and suffer from multiple clinical symptoms including severe gastrointestinal, neurological and cardiovascular disorders (Marquais and Sauviat 1999; Nicholson and Lewis 2006). Until now, western therapy remains primarily symptomatic and supportive, hence, in the South Pacific area, traditional herbal medicine is still widely practiced (Bourdy *et al.* 1992). CTXs have been reported to exert their damaging effects (e.g. membrane hyperexcitability and cell swelling) primarily through activation of voltage-gated sodium channels of vari-

ous excitable cells, resulting in elevation of cytoplasmic sodium and calcium concentrations (Mattei *et al.* 1999; LePage *et al.* 2005; Nicholson and Lewis 2006). More recently, direct activation of L-type voltage-gated calcium channels has also been implicated in this influx (Sauviat *et al.* 2006). The latter mechanism involves participation of the cGMP pathway by increasing nitric oxide (NO) production via activation of inducible nitric oxide synthase (iNOS), an enzyme that catalyzes the NADPH-dependent oxidation of L-arginine into L-citrulline and free radical NO·. The fact that NO· is an important modulator of immune, endocrine and neuronal functions, and is deeply involved in the regulation of cardiovascular function, prompted us to investigate its possible implication in CFP. For this purpose, iNOS mRNA expression levels were monitored by a two-step, real-time, reverse transcriptase polymerase chain reaction (RT-PCR), comparatively to those of 3 housekeeping genes, both *in vivo* (CTX-injected mice) and *in vitro* (CTX-treated Neuro-2a, a mouse neuroblastoma-derived cell line). In the latter case, NO production was quantified by measuring nitrite levels in the cell culture medium using Griess reagents (Amano and Noda 1995).

## Materials and Methods

### Material

Pacific CTX-1B was purified within the LMT facilities (Tahiti, French Polynesia) from moray eel liver extracts, using organic solvent extraction and a combination of low and high pressure liquid chromatography as previously described (Legrand *et al.* 1989). Brevetoxin PbTx-3 was purchased from Latoxan (France). All molecular biology kits and reagents were from Roche Applied Science (New Zealand) employing protocols based on the LightCycler technology (Roche Diagnostics Inc.), unless otherwise stated.

### Cell culture

Unless otherwise stated, all reagents were from Sigma (France). Mouse neuroblastoma cells (Neuro-2a, ATCC, CCL131) were routinely cultured in a RPMI 1640 medium supplemented with 10 % foetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cultures were maintained at 37 °C under a humidified atmosphere of 5 % CO<sub>2</sub>.

### Determination of nitrite production

At the initial time point, Neuro-2a cells were seeded in the RPMI based-medium at a density of  $2.5 \times 10^5$  cells/ml in 96-well tissue culture plates. After 24 h of incubation, this complex medium was replaced by Earle's Balanced Salt Solution (EBSS). Brevetoxin PbTx-3 was added and the plates were further incubated for 12 h. Cell supernatants were harvested and tested for their nitrite content using Griess reagent and a Technicon Autoanalyzer III plus NaNO<sub>2</sub> to generate a standard curve.

### Mice exposure to CTX

Single sublethal P-CTX-1B doses (0.1 ng/g) were administered to 11 female OF1 mice averaging 20 g. Their peripheral blood mononuclear cells (PBMCs), were sequentially harvested over a 12-h period and analyzed as described below. Mice injected with 0.1 % Tween 60 were used as control (n = 6).

### Real-time PCR experiments

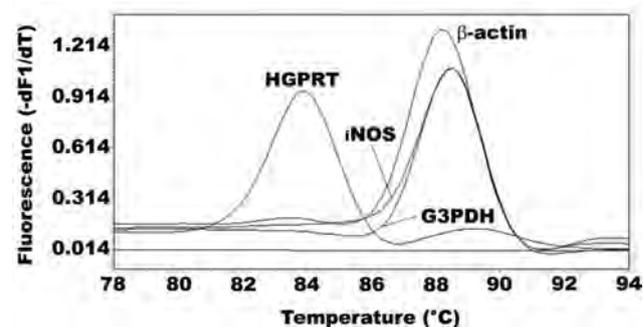
*In vitro* toxin-pulsed Neuro-2a cells or CTX-injected mice PBMCs together with their relevant controls were assayed for iNOS and housekeeping genes mRNA expression level. The following housekeeping genes were used throughout: G3PDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin and HGPRT (hypoxanthine guanine phosphoribosyl transferase). Sequences of all primers were designed with the LightCycler Primer Probe Design Software 2.0 (according to gene sequences retrieved from the

GenBank® database) and synthesized by Proligo Singapore Pte Ltd (Singapore). Neuro-2a cells ( $1 \times 10^6$  cells) or 1.5 ml of mouse blood were first treated with PaxGene Blood RNA system (Qiagen), then total RNA samples were obtained using the High Pure RNA isolation kit. cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit with a supplied random hexamers as priming strategy. RT-PCR experiments were conducted using the specific couple of primers and the LightCycler FastStart DNA Master SYBR Green I kit in a LightCycler® 2.0 Instrument. Finally, the specificity of the amplicons was assessed by melting curve analysis and agarose gel electrophoresis.

## Results and Discussion

In order to investigate the implication of NO radicals in CFP using a CTX/mouse model, we employed PbTx-3- and P-CTX-1B-pulsed Neuro-2a cells and OF1 mice primed with P-CTX-1B (n = 11), making use of molecular biology technique (RT-PCR) and chemical method (nitrite determination).

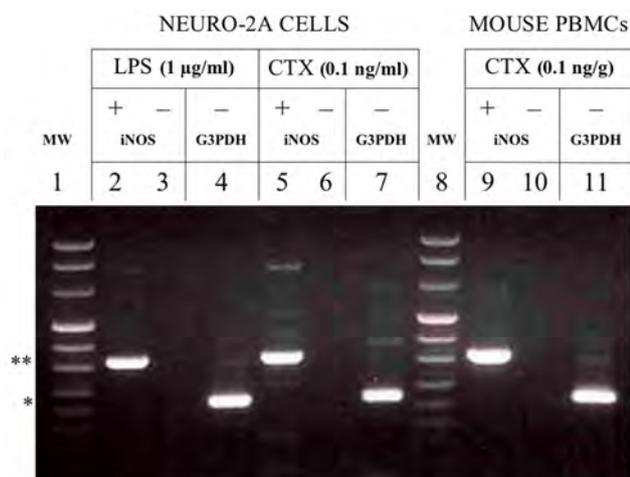
Typical results (Figs 1, 2) clearly show both *in vitro* and *in vivo* CTX- and time-dependent induction of iNOS with a peak expression around 4-6 h, leading to NO production as evidenced by the *in vitro* increase in nitrite levels (see below). It is noteworthy that in spite of the varying susceptibility of outbred mice population to CTXs, the observation of symptoms strongly correlated with iNOS expression. This can further explain the variation in peak expression of iNOS mRNA and the control-like behaviour of mice exhibiting no symptoms upon CTXs injection.



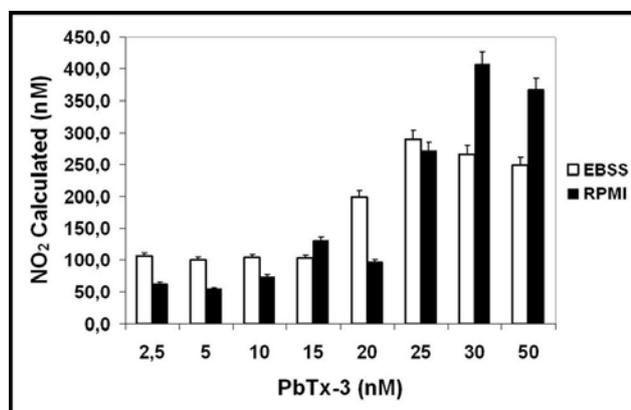
**Figure 1.** Typical melting curve analysis of RT-PCR-generated amplicons from CTX-pulsed Neuro-2a cells or CTX-injected mouse PBMCs.

The data of RT-PCR were fully correlated with those obtained *in vitro* with bacterial lipopolysaccharide (LPS), a well-known iNOS inducer.

As controls for gene expression, the 3 housekeeping mRNAs were consistently detected regardless of testing conditions.



**Figure 2.** Representative agarose gel electrophoresis of RT-PCR-generated amplicons from LPS or CTX-pulsed Neuro-2a cells and from CTX-injected mouse PBMCs compared with untreated controls. \*\*: iNOS (349 bp); \*: G3PDH (213 bp).



**Figure 3.** PbTx-3-induced nitrite production in Neuro-2a cells as determined by the Griess reagent (n=6).

As model for P-CTX-1B studies, PbTx-3 was found to induce a dose-dependent increase in  $\text{NO}_2^-$  level in both media (Fig. 3). At low PbTx-3 concentration, more  $\text{NO}_2^-$  can be detected in EBSS than in RPMI, whereas at higher PbTx-3 concentrations (ie. 30 and 50 nM), RPMI  $\text{NO}_2^-$  levels are far higher. This observation could be due to the presence of interfering substances in the complex RPMI-based medium, while it seems that the lack of L-arginine substrate and co-factors in EBSS become the limiting factors for  $\text{NO}_2^-$  production at high PbTx-3 concentrations.

Consequently, NO might be responsible for certain long-lasting symptoms of CFP such as allergenic effects and chronic fatigue syndrome (CFS). A new hypothesis for the cause of CFS has been proposed (Pall 2002), according to which potent oxidant peroxynitrites, generated by the reaction of  $\text{NO}\cdot$  with superoxide radicals ( $\text{O}_2^{\cdot-}$ ), increase the level of both

$\text{NO}\cdot$  and  $\text{O}_2^{\cdot-}$ , thus producing a self-sustaining vicious cycle. Supporting this theory, serum levels of L-citrulline were found to be significantly elevated in CFS patients and positively correlated with the intensity of symptoms (Pall 2002).

Regarding chemotherapy, most of the drugs commonly used for the treatment of CFP are free radical scavengers. Even mannitol, which is currently administered intravenously to treat patients with acute CFP, is a free hydroxyl radical scavenger (Desesso *et al.* 1994). Similarly, some of the traditional remedies showing the best efficacy (e.g. *Spondias cytherea* or *Davallia solida*) contain well-known  $\text{NO}\cdot$  scavengers such as mangiferin (Garrido *et al.* 2004; Boydron-Le Garrec *et al.* 2005).

In conclusion, the implication of NO in CFP paves the way for new therapies, together with new CTXs detection and specific clinical diagnostic tools.

## References

- Amano, F. & Noda, T. (1995). FEBS Letters 368: 425-428.
- Bourdy, G., Cabalion, P., Amade, P. & Laurent, D. (1992). J. Ethnopharmacol. 36 : 163-174.
- Boydron-Le Garrec, R., Benoit, E., Sauviat M-P, Lewis, R.J., Molgó, J. & Laurent, D. (2005). Toxicol 46 : 625-634.
- Desesso, J.M., Scialli, A.R. & Goeringer, G.C. (1994). Teratology 49: 248-259.
- Garrido, G., Delgado, R., Lemus, Y., Rodríguez, J., García, D. & Núñez-Sellés, A.J. (2004). Pharmacol. Res. 50: 165-172.
- Legrand, A-M., Litaudon, M., Genthon, J.N., Bagnis, R. & Yasumoto, T. (1989). J. Appl. Phycol. 1: 1183-1188.
- Lehane, L. & Lewis, R.J. (2000). Int. J. Food Microbiol. 61: 91-125.
- LePage, K.T., Dickey, R.W., Gerwick, W.H., Jester, E.L. & Murray, T.F. (2005). Crit. Rev. Neurobiol. 17: 27-50.
- Marquais, M. & Sauviat, M-P. (1999). J. Soc. Biol. 193: 495- 504.
- Mattei, C., Dechraoui, M-Y., Molgó, J., Meunier, F-A., Legrand, A-M. & Benoit, E. (1999). J. Neurosci. Res. 55 : 666-673.
- Nicholson, G.M. & Lewis, R.J. (2006). Mar. Drugs 4: 82-118.
- Pall, M.L. (2002). J. Chronic Fatigue Syndr. 10: 37-41.
- Sauviat, M-P., Boydron-Le Garrec, R., Masson, J-B., Lewis, R.J., Vernoux, J-P., Molgó, J., Laurent, D. & Benoit, E. (2006). Blood Cells Mol. Dis. 36: 1-9.

## First evidence of spirolide accumulation in northwestern Adriatic shellfish

S. Pigozzi<sup>1,\*</sup>, L. Bianchi<sup>2</sup>, L. Boschetti<sup>3</sup>, M. Cangini<sup>1</sup>, A. Ceredi<sup>1</sup>, F. Magnani<sup>1</sup>, A. Milandri<sup>1</sup>, S. Montanari<sup>2</sup>, M. Pompei<sup>1</sup>, E. Riccardi<sup>1</sup> and S. Rubini<sup>2</sup>

<sup>1</sup>Centro Ricerche Marine, V.le A. Vespucci 2, 47042 Cesenatico (FC) Italy,

<sup>2</sup>Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna Sez. di Ferrara, Via Modena 483, 44044 Cassana (FE) Italy, <sup>3</sup>Azienda Unità Sanitaria Locale di Ferrara, Via Borgoleoni 128, 44100 Ferrara Italy

\*Corresponding author, [silvia.pigozzi@centroricerchemarine.it](mailto:silvia.pigozzi@centroricerchemarine.it)

### Abstract

In autumn 2003, shellfish samples from the northwestern Adriatic Sea, collected during a routine monitoring programme, tested positive by mouse bioassay for lipophilic toxins (Gazzetta Ufficiale della Repubblica Italiana 2002). Unusual symptoms and the extremely short survival times (as low as 5 min) alerted the monitoring operators, suggesting the presence of compounds previously undetected in Italy. During the event, the dinoflagellate *Alexandrium ostenfeldii* reached a maximum concentration of 15612 cells/L. The microalga, isolated and grown in culture, was demonstrated to produce mainly spirolide 13-desmethyl C and no PSP toxins (Ciminiello *et al.* 2006). Here we report the first evidence of spirolide accumulation in Adriatic mussels. Analyses were carried out by liquid chromatography tandem mass spectrometry.

### Introduction

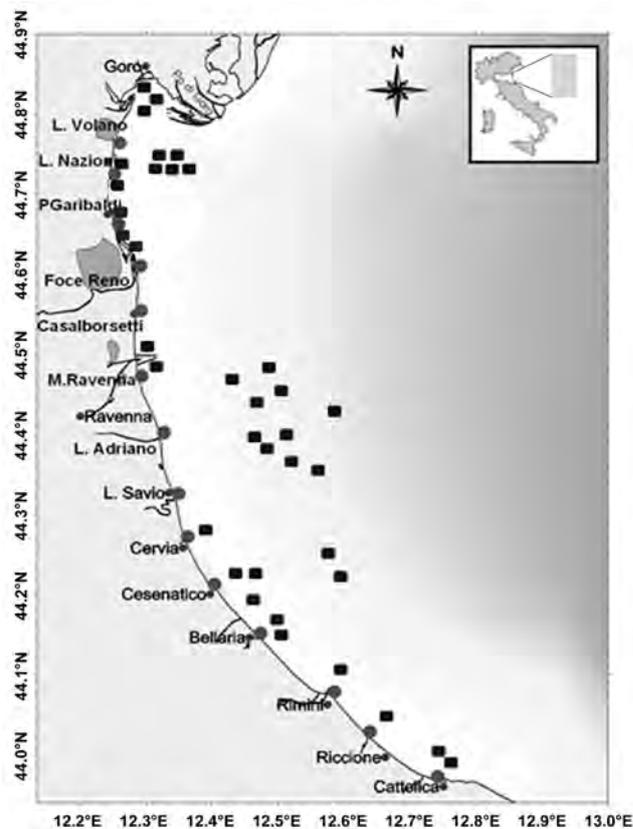
Since 1989, prolonged and regular toxicity episodes attributed to okadaic acids and yessotoxins have been reported in mussels from Italy's (Emilia Romagna – northwestern Adriatic Sea) breeding areas and natural banks. In autumn 2003 unusual symptoms and extremely short survival times (as low as 5 min) during mouse bioassays alerted the monitoring operators, suggesting the presence of compounds previously undetected in Italy and, as a consequence, shellfish production areas were closed. During this event, the dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech et Tangen 1985, potential producer of PSP toxins and spirolides, was detected. In this study we report the first evidence of spirolide accumulation in Adriatic shellfish (*Mytilus galloprovincialis*).

### Materials and Methods

Shellfish (*Mytilus galloprovincialis* and *Tapes philippinarum*) were collected weekly or bimonthly in natural banks and breeding areas off Italy's Emilia Romagna coast, from Goro to Cattolica, according to the national legislation concerning the monitoring plans for evaluation of hygienic and sanitary conditions of live bivalve molluscs.

Phytoplankton net samples were collected at the same sites and at the same time as bivalves. Additional water samples were collected inshore using Niskin bottles as part of the environmental monitoring programme (Fig. 1), which is usually carried out weekly and is not necessarily related to the sampling of bivalves. Water samples were preserved in dark glass

bottles with formalin and counted using an inverted microscope according to the Utermöhl method (Utermöhl 1958). Species identification was based on observation of relevant morphological features as described by Balech (1995). For this purpose the calcofluor white method was used (Fritz and Triemer 1985).



**Figure 1.** *Mytilus galloprovincialis* and *Tapes philippinarum* breeding areas/natural banks along the Emilia Romagna coast (■); inshore monitoring stations (●).

Shellfish samples were tested for the presence of both water-soluble and lipophilic biotoxins as prescribed by Italian Ministry Decree “Tenori massimi e metodiche di analisi delle biotossine algali nei molluschi bivalvi vivi, echinodermi, tunicati e gasteropodi marini” dated 16 May 2002. In particular, mouse bioassays (MBA) were carried out for PSP and lipophilic toxins according to the AOAC (1990) method and to the so-called “Yasumoto 2001” method, respectively. The latter method was a submission to the EU Reference Laboratory that was later reported in the *Gazzetta Ufficiale della Repubblica Italiana* (2002). It is composed of two protocols: the 2nd protocol involves a partition of the crude extract between dichloromethane and aqueous methanol and is particularly suitable for separation of yessotoxins from the other lipophilic toxins. Spirolides are thus concentrated in the DCM fraction.

Further investigations were carried out by liquid chromatography tandem mass spectrometry on the positive MBA residues that were not injected into mice (Quilliam *et al.* 2003). LC-MS-MS analyses were performed using a 1200-L triple quadrupole mass spectrometer (Varian Inc., Walnut Creek, CA, USA). LC separation of spirolides was performed using a 3- $\mu$ m Hypersil C8 BDS, 50  $\times$  2,1 mm column (Thermo/Keystone, Bellafonte, PA, USA) at 30 °C. The mobile phase consisted of two components: water (A) and acetonitrile/water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. The flow rate was 0,2 mL/min. A gradient elution was programmed. Several multiple reaction monitoring (MRM) experiments were carried out in the positive ion mode to identify the spirolide profile of our samples. For further confirmation, the material was cleaned-up on OASIS-HLB SPE cartridges (Aasen *et al.* 2006). Spirolide 13-desMeC was quantified against NRC (Halifax, Canada) certified reference standard dilutions.

## Results and Discussion

The presence in shellfish of spirolides, a group of marine biotoxins belonging to the cyclic imines, was first discovered in lipophilic extracts of scallop and mussel digestive glands harvested in Nova Scotia (Canada) because of their acute toxicity when injected intraperitoneally into mice (Hu *et al.* 1995).

The causative organism producing these toxins was identified as the dinoflagellate *Alexandrium ostenfeldii* by Cembella *et al.* (1998, 1999). The presence of a toxic strain of this dinoflagellate in the Adri-

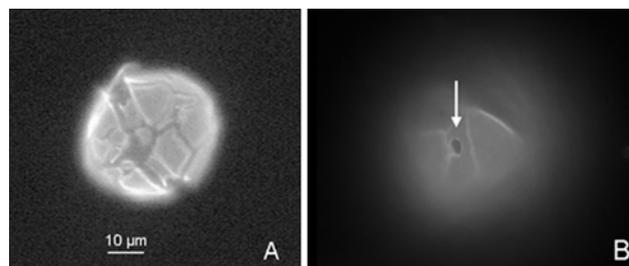
atic Sea has also been confirmed (Ciminiello *et al.* 2006).

In the past, specimens of *A. ostenfeldii* may have been misidentified in the study area. In fact, some cells appearing sporadically along the Emilia Romagna coastline could have been reported as *Alexandrium* spp. during routine work that generally involves only the use of light microscopy. It was only as a result of the anomalies during the MBA and the higher densities of these not fully identified *Alexandrium* cells at the end of November 2003 that a more comprehensive study was conducted. Net samples collected at the same sites as bivalve samples were used for qualitative phytoplankton analysis that also involved calcofluor white staining and epifluorescence. Morphological features of *Alexandrium* cells were fully consistent with those reported for *A. ostenfeldii* (Ciminiello *et al.* 2006) (Fig. 2 A-B). Abundance data were obtained from the analysis of bottle samples collected for the environmental monitoring programme. *Alexandrium ostenfeldii* was the only *Alexandrium* species in the seawater samples at the time, and it was present at all stations from mid November until February, with the highest densities in the northern area and, in particular, at stations Bagni di Volano and Porto Garibaldi on 24 November (Table 1).

No PSP toxicity was found in either *Mytilus galloprovincialis* or *Tapes philippinarum* throughout the study period.

Mouse bioassays for lipophilic toxins tested positive, with mice survival times as low as 5 min for samples of *M. galloprovincialis* collected on 25 November in the areas where *A. ostenfeldii* was highly concentrated on the previous day.

In the same samples, LC-MS-MS analyses confirmed the presence of spirolide 13-desMeC. Three characteristic MRM transitions were chosen for the unambiguous identification of 13-desMeC (m/z 692>674, 692>444 and 692>164) (Slencu *et al.* 2004) (Fig. 3) and the retention time was identical to the standard solution. We detected a maximum concen-



**Figure 2.** Vegetative cells of *Alexandrium ostenfeldii* stained with Calcofluor white (A); detail of the 1st apical plate showing the ventral pore (B, arrow).



This finding supports the need for alternative methods to the MBA for monitoring purposes, as the presence of spirolides in shellfish samples strongly interferes with the results of the official controls, resulting in closures of mussel farms at toxin concentrations in shellfish well below the hypothetical regulatory limit of 0,4 mg/kg.

### Acknowledgements

We wish to thank the staff of ARPA Emilia Romagna (Regional Agency for Environmental Prevention) – Daphne Oceanographic Structure for their constant help in water samples collection.

### References

- Aasen, J.A.B., Hardstaff, W., Aune, T. & Quilliam, M.A. (2006). *Rapid Commun. Mass. Spectrom.* 20: 1531-1537.
- AOAC (Association of Official Analytical Chemists) (1990). In: *Official Methods of Analysis*. 15th ed. Section 959.08, Hellrich, K. (ed.), Association of Analytical Chemists, Arlington, VA, pp. 881-882.
- Balech, E. (1995). *Sherkin Island Marine Station*, Sherkin Island, Co. Cork, Ireland, 151 p.
- Cembella, A.D., Quilliam, M.A., Lewis, N.I., Bauder, A.G. & Wright, J.L.C. (1998). *Harmful Algae* 1: 481-484.
- Cembella, A.D., Lewis, N.I. & Quilliam, M.A. (1999). *Nat. Toxins* 7: 197-206.
- Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Magno, S., Tartaglione, L., Cangini, M., Pompei, M., Guerrini, F., Boni, L. & Pistocchi, R. (2006). *Toxicon* 47: 597-604.
- Fritz, L. & Triemer, R.E. (1985). *J. Phycol.* 21: 662-664.
- Gazzetta Ufficiale della Repubblica Italiana n°165, 16 Luglio 2002. Decreto Ministeriale del 16 Maggio 2002 "Tenori massimi e metodiche di analisi delle biotossine algali nei molluschi bivalvi vivi, echinodermi, tunicati e gasteropodi marini".
- Hu, T., Curtis, J.M., Oshima, Y., Quilliam, M.A., Walter, J.A., Watson-Wright, W.M. & Wright, J.L.C. (1995). *J. Chem. Soc., Chem. Commun.* 1995: 2159-2161.
- Quilliam, M.A., Hardstaff, W.R. & Cembella, A.D. (2003). In: *Proc. HABTech2003 Workshop* Holland, P., Rhodes, L. & Brown L. (eds), Cawthron Report No. 906, Nelson, New Zealand, pp. 115-118.
- Richard, D., Arsenault, E., Cembella, A.D. & Quilliam M.A. (2001). In: *Harmful Algal Blooms 2000*, Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. & Lewis, R.J. (eds), UNESCO, Paris, pp. 383-386.
- Sleno, L., Windust, A.J. & Volmer, D.A. (2004). *Anal. Bioanal. Chem.* 378: 969-976.
- Utermöhl, H. (1958). *Mitt. Int. Ver. theor. angew. Limnol.* 9: 1-38.

## Ecotoxicology of different strains of *Lingulodinium polyedrum* from the Portuguese coast

M. Reis<sup>1</sup>, A. C. Kraberg<sup>2</sup>, K. Erler<sup>3</sup>, B. Luckas<sup>4</sup>, A. Amorim<sup>5</sup> and K. H. Wiltshire<sup>6</sup>

<sup>1</sup>Faculdade Ciências da Universidade de Lisboa, Instituto de Oceanografia, 1749-016 Lisboa, Portugal, mariana.a.reis@gmail.com, <sup>2</sup>Alfred Wegener Institute for Polar and Marine Research, Biologische Anstalt Helgoland, 27498 Helgoland, Germany, akraberg@awi-bremerhaven.de, <sup>3</sup>Department of Food Chemistry, Friedrich-Schiller University, Jena, Germany, katrin.erler@uni-jena.de, <sup>4</sup>Department of Food Chemistry, Friedrich-Schiller University, Jena, Germany, bernd.luckas@uni-jena.de, <sup>5</sup>Faculdade Ciências da Universidade de Lisboa, Instituto de Oceanografia, 1749-016 Lisboa, Portugal, ajamorim@fc.ul.pt, <sup>6</sup>Alfred Wegener Institute for Polar and Marine Research, Biologische Anstalt Helgoland, 27498 Helgoland, Germany, kwiltshire@awi-bremerhaven.de

### Abstract

In 2004 and 2005, blooms of *Lingulodinium polyedrum* were recorded along the south and west coasts of Portugal, leading to extensive precautionary beach closures. However, no information was available on the toxicity of this species in the area. The production of yessotoxins (YTX) and YTX-analogues, by four different strains of *L. polyedrum*, isolated at the time of the blooms and from cysts in sediments, was investigated using HPLC-MS. Although physiological differences were observed among the studied strains, YTX production could not be confirmed under the conditions tested.

### Introduction

*Lingulodinium polyedrum* (Stein) Dodge is a widely distributed species found from temperate to subtropical coastal waters. Blooms along European coasts have been reported since early 1900, regularly affecting the NW coast of the Iberian Peninsula (Lewis and Hallett 1997). In Portugal, the first record that relates this species with red-tide events dates back to 1944 (Santos-Pinto 1944). In 2004 and 2005, blooms of *L. polyedrum* were recorded along the south and west coasts of Portugal, leading to extensive precautionary beach closures. The blooms were associated with stratified water adjoining upwelled nutrient-enriched plumes (Amorim *et al.* 2003). No information regarding the toxicity of the episodes is available.

Since 1961, *L. polyedrum* has been associated with toxin production (Shimizu 1987). However, the reports are often contradictory, suggesting the existence of toxic and non-toxic strains. Recently, reports from the Adriatic Sea associated natural shellfish contamination with YTX and homo-YTX with blooms dominated by *L. polyedrum* (Draisci *et al.* 1999; Pavela-Vrancic *et al.* 2002). YTX production by *L. polyedrum* was also shown in culture in strains isolated from the N and S coasts of Spain (Paz *et al.* 2004).

Here, we investigate the potential production of YTX by strains of *L. polyedrum* isolated from the W and S coasts of Portugal (Fig. 1). Toxin production was investigated with HPLC-MS at different stages

of culture growth. Physiological differences among strains were also investigated by comparing growth characteristics of batch cultures under controlled environmental conditions.

### Materials and Methods

Cultures of *L. polyedrum* from the algal collection of the University of Lisbon (ALISU) (Fig. 1; Table 1) were grown in polycarbonate tissue culture flasks (270 ml) in GSe medium (Blackburn *et al.* 2001) (salinity 35 psu) at 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 14:10 h L:D cycle, and 19 $\pm$ 1 °C. Clonal cultures of all iso-



**Figure 1.** Map of Portugal showing the origin of the different strains of *L. polyedrum* (•)

**Table 1.** Sample location and culture origin of *L. polyedrum* isolates.

Isolates	Location	Isolation date	Culture Origin	Clonal
IO11-01	Ria Alvor	Jan. 2003	Cyst	No
IO11-02	37°08'N 08°36'W	2003		
IO11-03	Albufeira 37°05'N 08°15'W	Aug. 2004	Motile stage (bloom sample)	Yes
IO11-04	Sines 38°57'N 08°52'W	Aug. 2005		

lates were used in growth experiments. The volume of inocula varied between 10 ml (IO11-02) and 37 ml (IO11-04). For toxin determination, non-clonal cultures of IO11-01 and IO11-02 had to be used, as the high cellular densities needed were never achieved with clonal ones.

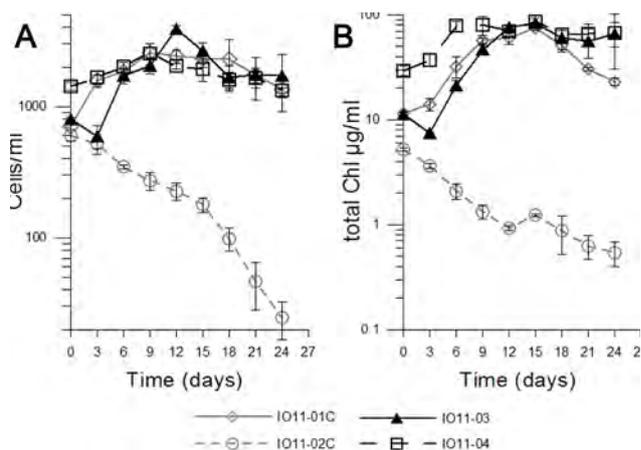
For growth measurements, 3 replicates of each strain were sampled every 2-3 days for cell enumeration. Growth was also followed using *in vivo* fluorescence (BBE Cuvette Fluorometer, BBE Moldaenke GmbH, Kiel, Germany). Cells were counted with LM (Olympus CX41) in a Sedgwick-Rafter chamber. A minimum of 400 cells was counted. The growth rates were calculated by least-squares regression.

For toxin analysis, 3 replicates of each isolate and growth stage were established in 1-L polycarbonate bottles. 500 ml of culture at the different growth stages were filtered using Whatman 1.2- $\mu$ m GF/C filters, 25 mm  $\varnothing$ . Each filter was extracted in 1.0 ml methanol/0.1 M acetic acid, and YTX toxins were analysed by LC-MS according to Luckas and Erler (Department of Food Chemistry, Friedrich-Schiller University, Jena, Germany). YTX-toxins were separated on a C18 HPLC column (5  $\mu$ m, 150 mm x 2.0 mm i. d.) (Phenomenex, Germany), with gradient elution. The eluent contained 53 mM formic acid and 5 mM ammonium formate as buffer, in a water/acetonitrile solution. The quota of acetonitrile in eluent A was 10 % and in eluent B 90 %. The HPLC system was equipped with a PE series 200 quaternary pump and a PE series 200 autosampler (Perkin Elmer, Germany). All measurements were performed on an API 165 SCIEX mass spectrometer with pneumatic-assisted atmospheric pressure ion (API) source operating in turbo ion-spray mode (PE Sciex, Canada). Selected ion monitoring (SIM) experiments were carried out in negative ion mode by selecting the following [M-H]-

ions at m/z 1141,5 (YTX), m/z 1157,5 (45-OH-YTX), m/z 1155,5 (Homo-YTX), and m/z 1171,5 (45-OH-Homo-YTX).

## Results and Discussion

Fig. 2 shows the growth curves obtained for the different clones during the 24 days of the experiment. No viable cultures could be established for clone IO11-02 (Fig. 2). Statistically significant differences between the growth curves of IO11-01, IO11-03 and IO11-04 were found (repeated-measures ANOVA, F<sub>2, 8</sub>=6,542; p<0.001). Clones IO11-01 and IO11-04 showed no lag-phase and reached lower cell densities when compared to IO11-03. The latter was the only one that had an evident lag-phase, typical of batch cultures. IO11-03 reached the highest density with 3903 cells/ml (day 12) and the highest growth rate (0.19 d<sup>-1</sup>) (Fig. 2; Table 2). For all clones, the exponential phase lasted for 9 days, irrespective of culture age (Fig. 2). Total chlorophyll (Chl) content and cell density correlated positively during the exponential phase. However, again for all clones, the maximum

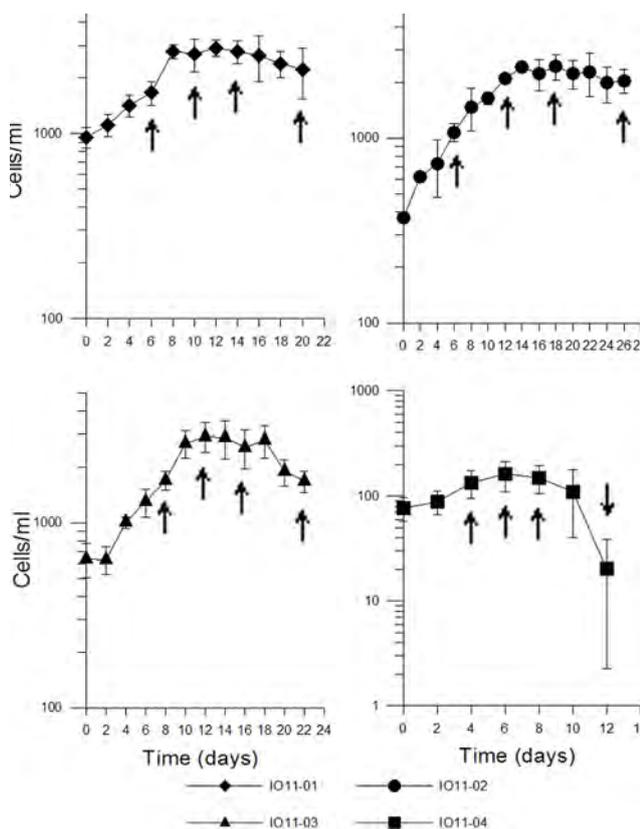
**Figure 2.** Growth curves obtained for the different clones of *L. polyedrum*: (A) cell counts and, (B) *in vivo* fluorescence**Table 2.** Cell concentration at day 0, maximum cell concentration and growth rates of the different clones of *L. polyedrum*

Isolates	[Day0]* (cells/ml)	Max. [cell] (cells/ml)	$\mu$ max (d <sup>-1</sup> )
IO11-01C	703	2586	0,14
IO11-02C	605	-	-
IO11-03	806	3903	0,19
IO11-04	1429	2553	0,06

\*inoculum from mid-exponential phase

cell concentration was reached before the total Chl maximum. When cell numbers started to decay, Chl content continued to increase for 3-6 days (Figs 2 A, B). This may indicate an increase in Chl per cell of *L. polyedrum* as a photoadaptation response due to self-shading at high cell densities (Prézlin and Alberte 1978; Prézlin and Sweeny 1978).

Fig. 3 shows the growth curves for the different cultures used in the toxin experiments and harvesting days. The production of YTX or YTX- analogues could not be detected for any of the isolates at any growth stage. This contrasts with previous reports of YTX production in cultures of *L. polyedrum* from the same geographical region (Huelva and Coruña, Spain) (Paz *et al.* 2004). However, the latter results were recorded using culturing conditions and analytical procedures different from the ones used herein (namely L1 medium without silicates, salinity 34 psu, 17±1 °C, 12:12 h L:D cycle; LC-MS coupled to fluorometric detection using the dienophile reagent DMEQ-TAD) (Paz *et al.* 2004).



**Figure 3.** Growth curves of the different *L. polyedrum* isolates used for the toxin experiments. Arrow (→) indicates day of harvest.

Our results suggest that further work should be carried out in order to clearly establish if the observed differences in the production of YTX toxins by *L. polyedrum* are strain specific or may reflect environmental triggering. Understanding the mechanisms of toxin production in this species is a vital prerequisite for monitoring activities and to enable environmental managers to develop appropriate remedial strategies.

### Acknowledgements

We thank the EU for financial support to M. Reis through the Leonardo da Vinci programme and Plankton\*Net "Structuring the European Research Area" RIAM no 026071. Thanks are also due to the Alfred Wegener Institute, Biological Station Helgoland for the opportunity to carry out this work and for the unconditional support given to M. Reis.

### References

- Amorim, A., Moita, M.T. & Oliveira, P. (2003) In: Harmful Algae 2002, Steidinger, K.A., Landsberg, J.H., Tomas, C.R. & Vargo, G.A. (eds), Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, & Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, Florida, USA, pp. 89-91
- Blackburn, S.I., Bolch, C.J.S., Haskard, K.A. & Hallegraeff, G.M. (2001). *Phycologia* 40: 78-87.
- Draisci, R., Ferretti, E., Palleschi, L., Marchiafava, C., Poletti, R., Milandri, A., Ceredi, A. & Pompei, M. (1999). *Toxicon* 37: 1187-1193.
- Lewis, J. & Hallet, R. (1997). *Oceanogr. Mar. Biol., Ann. Rev.* 35: 97-161.
- Pavela-Vrancic, M., Mestrovic, V., Marasovic, I., Gillman, M., Furey, A. & James, K. (2002). *Toxicon* 40: 1601-1607.
- Paz, B., Riobó, P., Fernández, M.L., Fraga, S. & Franco, J. M. (2004). *Toxicon* 44: 251-258.
- Prézlin, B.B. & Alberte, R. (1978). *Botany* 75: 1801-1804.
- Prézlin, B.B. & Sweeny, B.M. (1978). *Mar. Biol.* 48: 27-35.
- Santos-Pinto, J. (1949). Separatas dos Arquivos do Instituto Nacional de Saúde (Lisboa), IV: 252-262.
- Shimizu, Y. (1987). In: *The Biology of Dinoflagellates*, Taylor, F.J.R. (ed.), Blackwell Scientific Publications, Oxford, pp. 282-315.

## ***Ostreopsis siamensis* and palytoxin-related compounds in New Zealand: a risk to human health?**

Lesley Rhodes<sup>1</sup>, Rex Munday<sup>2</sup> and Lyn Briggs<sup>2</sup>

<sup>1</sup>Cawthron Institute, Private Bag 2, Nelson, New Zealand, lesley.rhodes@cawthron.org.nz;

<sup>2</sup>AgResearch, Private Bag 3123, Hamilton, New Zealand, rex.munday@agresearch.co.nz

### **Abstract**

*Ostreopsis siamensis* occurs in New Zealand's subtropical waters and produces potent novel toxins related to palytoxin. Summer blooms of *O. siamensis* have been linked to sea urchin (*Evechinus chloroticus*) mortalities in the north of New Zealand over the last few years. Techniques for mass production of the microalga have been developed, and allelopathic effects of bacteria and fungi on growth and toxin production have been identified. Larval bioassays using sea urchins, Greenshell mussels™ (*Perna canaliculus*) and paua (abalone; *Haliotis iris*) indicated differential toxicity to these animals (tD50 of 6 h, 12 h, and no effect, respectively). Extracts of the alga were highly toxic to mice by intraperitoneal injection. Analysis of the extract by the haemolysis-neutralisation assay indicated that the toxin(s) was of similar toxicity to palytoxin itself. *O. siamensis* cells were fed incrementally to shellfish and sea urchins (a total of  $46 \times 10^6$  cells over three days) in order to establish the degree of uptake of the toxin(s) into edible tissues. After feeding, Greenshell mussels (whole shellfish) contained 13.7 µg of palytoxin equivalents/kg; scallops (minus hepatopancreas; *Pecten novaezealandiae*) 7.7 µg/kg, Pacific oysters (*Crassostrea gigas*) 16.1 µg/kg, and sea urchins 1.2 µg/kg. These results indicate a low risk to humans, at least for the shellfish and sea urchins investigated in this study.

### **Introduction**

The production of analogues of palytoxin by dinoflagellates in the genus *Ostreopsis* Schmidt (Ostreopsidaceae) has been known for some time. *O. mascarensis* Quod produces mascarenotoxins (Lenoir *et al.* 2004), and strains of *O. ovata* Fukuyo (Penna *et al.* 2005) and *O. lenticularis* Fukuyo (Mercado *et al.* 1994; Meunier *et al.* 1997) also produce palytoxin-like compounds. An *O. ovata* bloom in the Italian Riviera (2005) resulted in beachgoers requiring treatment for fever, headaches and breathing problems (source Associated Press, 20 July 2005) and a risk warning for palytoxin in fish and shellfish was posted.

Strains of *Ostreopsis siamensis* Schmidt produce the palytoxin derivative ostreocin D (Usami *et al.* 1995; Ukena *et al.* 2001), although a New Zealand strain produces a different, as yet uncharacterized, derivative (Rhodes *et al.* 2002). It has been suggested that *O. siamensis* is involved in seafood poisoning, including human deaths, after eating contaminated bottom-feeding fish or crabs (Lenoir *et al.* 2004).

*Ostreopsis siamensis* occurs epiphytically on seaweeds throughout subtropical New Zealand, and a strain from Rangiputa, Northland, contained  $\leq 0.4$  pg cell<sup>-1</sup> palytoxin equivalents, as measured using a haemolysis-neutralisation assay (HNA; Bignami 1993). The extract exhibited slow haemolytic activity, characteristic of palytoxin, and haemolysis was suppressed by an anti-palytoxin antibody. This extract

was also toxic to mice by intraperitoneal injection (i.p.; Briggs *et al.* 1998) and caused hyperpolarisation of hippocampal rat brain slices (Rhodes *et al.* 2000), as described by Meunier *et al.* (1997) for palytoxin.

*Ostreopsis siamensis* has been found in the gut contents of wild mussels (*Mytilus edulis planulatus*) from Tasmania, Australia (Pearce *et al.* 2001), and a palytoxin-like material has been detected in extracts of Pacific oysters (*Crassostrea gigas*) collected from Northland, New Zealand (Briggs *et al.* 1998). During an epiphytic bloom of *O. siamensis* on brown seaweeds in 2004 ( $1.0 \times 10^6$  per g wet seaweed), mass mortalities of sea urchins were reported (Shears 2004).

In a previous study, in which low numbers of *O. siamensis* were fed to Greenshell mussels, scallops and Pacific oysters, no palytoxin-like material was detected in whole mussels. In contrast, whole oysters and the hepatopancreas (hp) of scallops contained detectable amounts of toxin (Rhodes *et al.* 2002). In the present study, higher cell concentrations of the microalga were fed to Greenshell mussels, scallops, oysters and sea urchins and the uptake determined by HNA. The effect of *O. siamensis* on shellfish larvae was also investigated to ascertain the potential for the larvae to be used as bioassay organisms and to assess whether blooms may impact on shellfish recruitment.

The potential involvement of bacteria in the production of palytoxin-like compounds has been reported (Ashton *et al.* 2003) and preliminary screenings

of allelopathic interactions between *O. siamensis* and marine bacteria and yeast strains, isolated from New Zealand waters, was carried out to determine whether allelopathy should be considered in future studies or shellfish biotoxin risk assessments.

## Methods

*Ostreopsis siamensis* (Cawthron Culture Collection, CAWD75) was cultured in F/2 medium (Guillard 1975) with added Cu (0.16  $\mu\text{M}$ ; Rhodes *et al.* 2006) under standard growth conditions (Rhodes *et al.* 2002). Mass cultures were grown in plastic bags (1.4 m by 0.2 m) under controlled temperature and light (Laczka 2002). Filtered air (0.45  $\mu\text{m}$ ) was pumped through the bags, and stationary-phase cultures were harvested by centrifugation and the pellet retained.

### Larval bioassays

Ten larvae (2 d old) were placed in 24-well tissue culture plates (Becton Dickinson, USA) in the presence of *O. siamensis* (1000, 2000, 8000 or 20,000 cells per test well). Assays were carried out in triplicate and the time until death of 50 % of the test organisms (tD50) was recorded.

### Allelopathy

*Halomonas* sp., *Cellulophaga lytica*, *Flavobacterium* sp., *Bacillus pumilis*, *Photobacterium* sp. and a yeast species were maintained on marine agar, and a loopful transferred to marine broth (10 ml in 50-ml Erlenmeyer flasks) with added sodium phosphate (2 mM Mountfort and Rhodes 1991). When cultures were at late exponential phase they were added to an early exponential phase culture of *O. siamensis* (5 % inoculum in 45 ml medium) in 150-ml Erlenmeyer flasks. Algal cell counts were carried out over 3 weeks and the cells were then harvested by centrifugation. Pellets and supernatant were analysed for toxin by the HNA (Bignami 1993).

### Feeding trials (Rhodes *et al.* 2002)

For each animal species tested, one batch was retained unfed and frozen as control (6 scallops, 7 mussels or Pacific oysters, or 3 sea urchins) and a second batch was fed a culture of *O. siamensis* over a 3-d period ( $45.5 \times 10^6$  cells total). Test animals were then frozen before toxin analysis.

### Toxin analysis

Microalgae and control and test animals, were assayed by the HNA as described previously (Rhodes *et al.* 2002).

## Results

*Ostreopsis siamensis* was toxic to larvae of sea urchins and Greenshell mussels, with tD50 of 6 h and 12 h, respectively, when  $20 \times 10^6$  algal cells were added. Paua larvae were not affected by the microalga.

Feeding of *O. siamensis* to shellfish and sea urchins resulted in uptake of palytoxin-like materials into edible tissue. Whole Greenshell mussels (fed a total of  $45.5 \times 10^6$  *O. siamensis* cells) contained 13.7  $\mu\text{g}/\text{kg}$  palytoxin equivalents and Pacific oysters 16.1  $\mu\text{g}/\text{kg}$ , whereas 7.7  $\mu\text{g}/\text{kg}$  was detected in scallops with the hp removed. Shellfish continued feeding throughout the experiment. Lower levels (1.2  $\mu\text{g}/\text{kg}$ ) were found in sea urchins, but the animals continuously tried to escape from the feeding environment and uptake of cells may have been low (Table 1).

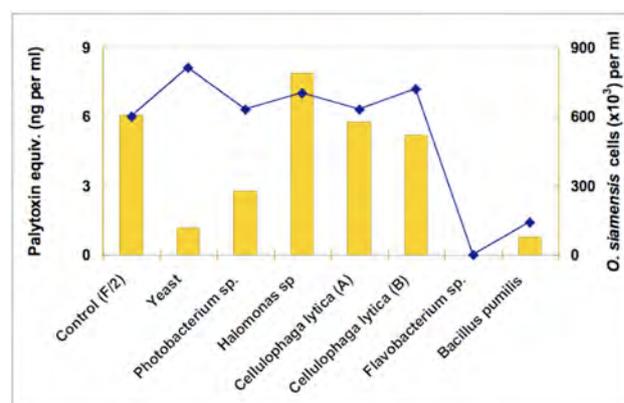
Marine bacteria and a yeast had major impacts on the growth of, and toxin production by, *O. siamensis* (Fig. 1). Growth was enhanced in cultures to which the yeast or *Cellulophaga lytica* (B) and *Halomonas* sp. were added, whereas *Bacillus pumilis* depressed growth and *Flavobacterium* sp. killed the microalga. Toxin production was depressed by the yeast and by all the bacterial isolates except *Halomonas* sp.

## Discussion

**Table 1.** Uptake of palytoxin equivalents ( $\mu\text{g}/\text{kg}$ ) by Greenshell mussel, scallop, Pacific oyster and sea urchins during *Ostreopsis siamensis* 3-d feeding trials.

Test animal → Total microalgal cell count ( $\times 10^6$ ) ↓	Whole mussel	Scallop minus hp	Whole oyster	Whole sea urchin
45.5	13.7	7.7	16.1	1.2
8.6	nd	nd	Trace	NT

(hp: hepatopancreas; nd: not detected; NT: not tested)



**Figure 1.** Effect of addition of marine bacteria and yeast isolates (10 ml late exponential phase cultures) to growth of (-♦-), and toxin production by (■), *Ostreopsis siamensis*.

*Ostreopsis siamensis* has been recorded around New Zealand's North Island (routine phytoplankton monitoring data) and *O. ovata* and *O. lenticularis* have also been recorded in Northland waters (Chang 1999). Therefore, if *Ostreopsis*-derived toxins are taken up by marine animals, human exposure to these toxins will occur. As with previous experiments, mussels, scallops and oysters contained palytoxin-like material after feeding on *O. siamensis* cells, as did sea urchins. The levels of toxin present in edible tissues, however, suggest that human intoxication is unlikely to occur via this route.

The LD50 of palytoxin by i.p. injection in mice is 0.72 µg/kg; in a previous study mice became lethargic soon after administration of palytoxin, with hunching and piloerection. At lethal doses, respiration became slow and laboured before death. Extracts of *O. siamensis* (NZ) are of similar toxicity to palytoxin. However, the LD50 of palytoxin by gavage is 510 µg/kg, >700 times less toxic than by injection. Similarly, an extract of *O. siamensis* (NZ) was >1000 times less toxic by gavage than by i.p. (Munday and Rhodes 2004).

The acute oral "no observable adverse effects level" (NOAEL) of palytoxin is 320 µg/kg. If a safety factor of 300 is applied, the acute reference dose for humans would be 1.07 µg/kg body weight. Based on a serving size of 250 g, the guidance level would then be calculated as 256 µg/kg shellfish. If a standard serving is approximately 20 Greenshell mussels, then toxin accumulated during the feeding experiments (≈7 µg in a simulated bloom contaminated serving) may be estimated as ≈ 10 % of the calculated guidance level.

Scallops with the hp removed contained even less toxin than mussels, whereas the level in oysters was slightly higher.

Uptake of palytoxin-like toxin(s) by sea urchins was extremely low, possibly due to avoidance of the alga.

The potential effects of environmental conditions, including microflora and differences in geographic strains of microalga, need to be considered in risk assessments. Addition of *Halomonas* sp. to *O. siamensis* cultures enhanced both biomass and toxin production, whereas addition of a yeast isolate resulted in an increase in biomass, but a decrease in palytoxin equivalents per cell of *O. siamensis*. The addition of a selected microflora to maximise mass cultures warrants further investigation.

The susceptibility of sea urchin and mussel larvae to *Ostreopsis* could provide a sensitive bioassay

for these algae. Both are more susceptible than *Artemia salina* (Rhodes *et al.* 2000). The high toxicity of the algae to larvae could have a significant impact on the population density of these animals after an *Ostreopsis* bloom.

Overall, the low uptake by shellfish and sea urchins consuming *Ostreopsis*, combined with the relatively low toxicity in mice by gavage suggests that shellfish may be of low risk to consumers. However, the very high concentrations of palytoxin recorded in crabs (Alcala *et al.* 1970; Yasumoto *et al.* 1986) and in some fish species (Taniyama *et al.* 2003) suggests that these groups require a cautionary approach until more data become available.

### Acknowledgements

Technical support: J. Adamson, T. Dodgshun, K. Ayers (Caw), J. Fitzgerald (AgR); bacterial and yeast isolates: D. Mountfort, M. Packer (Caw). The study was funded by NZ FRS&T, Contract CAWX0301.

### References

- Alcala, A. & Halstead, B. (1970). Clin. Toxicol. 3: 609.
- Ashton, M., Rodao, W., Govind, N. Tosteson, T. (2003). Toxicon 42: 419-424.
- Bignami, G. (1993). Toxicon 31: 817-820.
- Briggs, L., Rhodes, L., Munday, R. & Towers, N. (1998). Proc. 10th NZ Marine Biotoxin Science Workshop, Wellington, NZ, pp. 91-97.
- Chang, F. (1999). Water and Atmosphere 7: 15-16.
- Guillard, R. (1975). In: Culture of Marine Invertebrate Animals, Smith, W. and Chanley, M. (eds), Plenum Press, NY, pp. 29-60.
- Laczka, O. (2003). Report for Ecole Supérieure d'Ingénieur de Luminy (ESIL-Marseille), France.
- Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.-P., Bernard, C. & Hennion, M.-C. (2004). J. Phycol. 40: 1042-1051.
- Mercado, J., Viera, M., Gonzales, I., Escalona de Motta, G., Tosteson, T. Gonzalez, I. & Silva, W. (1994). Toxicon 32: 256.
- Meunier, F., Mercado, J., Molgo, J., Tosteson, T. & Gotta, G. (1997). Brit. J. Pharmacol. 121: 1224-1230.
- Mountfort, D. Rhodes, L. (1991). Appl. Env. Microbiol. 57: 1963-1968.
- Munday, R. & Rhodes, L. (2004). Proc. 20th NZ Marine Biotoxin Science Workshop, Wellington, NZ, pp. 23-36.
- Pearce, I., Marshal, J.-A. & Hallegraef, G. (2001).

- In: Harmful Algal Blooms, Hallegraeff, G., Blackburn, S., Bolch, C. & Lewis, R. (eds), IOC of UNESCO, pp. 54-57.
- Penna, A., Vila, M., Fraga, S., Giacobbe, M., Andreoni, F., Reobo, P. & Vernesi, C. (2005). *J. Phycol.* 41: 212-225.
- Rhodes, L., Adamson, J., Suzuki, T., Briggs, L. & Garthwaite, I. (2000). *N.Z. J. Mar. Freshw. Res.* 34: 371-384.
- Rhodes, L., Selwood, A., McNabb, P., Briggs, L., van Ginkel, R., Adamson, J. & Laczka, O. (2006). *Afr. J. Mar. Sci.* 28(2), in press.
- Rhodes, L., Towers, N., Briggs, L., Munday, R. & Adamson, J. (2002). *N.Z. J. Mar. Freshw. Res.* 36: 631-636.
- Shears, N. (2004). New Zealand Marine Science Society Conference (abstract).
- Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takani, T., Mahmud, Y. & Noguchi, T. (2003). *Toxicon* 42: 29-33.
- Ukena, T., Satake, M., Usami, M., Oshima, Y., Naoki, H., Fujita, T., Kan, Y. & Yasumoto, T. (2001). *Bio-sci. Biotechnol. Biochem.* 65: 2585-2588.
- Usami, M., Satake, M., Ishida, S., Inoue, A., Kan, Y. & Yasumoto, T. (1995). *J. Am. Chem. Soc.* 117: 5389-5390.
- Yasumoto, T. (1986). *Agricult. Biol. Chem.* 50: 163.

## The effects of chloramphenicol, arginine and temperature on PST-production by *Cylindrospermopsis raciborskii* strain D9

Katia Soto<sup>1,2</sup>, Karina Stucken<sup>1,2</sup>, Marco A. Méndez<sup>3</sup>, Néstor Lagos<sup>4</sup>, Allan D. Cembella<sup>5</sup>,  
Bernd Krock<sup>5</sup> and Mónica Vásquez<sup>1,2</sup>

<sup>1</sup> Laboratorio de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile, mvasquez@bio.puc.cl; <sup>2</sup> Millennium Nucleus on Microbial Ecology and Environmental Microbiology and Biotechnology, Pontificia Universidad Católica de Chile, Alameda 340, 651349; <sup>3</sup> Laboratorio de Bioinformática y Expresión Génica, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Avda. El Líbano 5524, Santiago, Chile, mmendez@.inta.cl; <sup>4</sup> Laboratorio de Bioquímica de Membrana, ICBM, Universidad de Chile, Avda. Independencia 1027, Santiago, Chile, nlagos@med.uchile.cl; <sup>5</sup> Alfred Wegener Institute, Am Handelshafen 12, 27570 Bremerhaven, Germany, allan.cembella@awi.de, bernd.krock@awi.de

### Abstract

The filamentous cyanobacterium *Cylindrospermopsis raciborskii* strain D9, a non-axenic culture isolated from a freshwater reservoir in Brazil, produces PSTs - mainly saxitoxin (STX) and gonyautoxins (GTX2/3), and low amounts of dcSTX and dcGTX2/3. In order to establish conditions to stimulate STX production, we analyzed the effect on growth and toxin production caused by the antibiotic chloramphenicol, arginine and temperature. Quantification was performed by HPLC with post-column derivatization. The toxin profile was confirmed by LC-MS/MS. Chloramphenicol (1 µg ml<sup>-1</sup>) inhibits growth and STX and GTX2/3 synthesis after 24 h exposure. Supplementation with arginine (10 mM) diminished cellular STX levels by 78 % after 48 h, while GTX levels were not affected. Growth of this strain was faster at 25 °C than at 19 °C but intracellular PST concentration remained stable until 100 h. The results were substantially different from those previously obtained with *C. raciborskii* strain T3, another non-axenic culture producing STX and the N-sulfocarbamoyl toxins C1-C2. Although the presence of heterotrophic bacteria in both cultures could have an influence on the results obtained, our data suggest that physiological differences between strains are probably related to differential gene expression in the toxin biosynthetic pathways.

### Introduction

The paralytic shellfish toxins (PSTs) are a group of tetrahydropurine neurotoxins that selectively block Na<sup>+</sup> channels in excitable cells. Among PSTs, saxitoxin (STX) is the most potent analogue. The toxins are produced by representatives of widely divergent phyla, primarily by marine dinoflagellates and cyanobacteria from marine and freshwater ecosystems throughout the world (Llewellyn 2006). *Cylindrospermopsis raciborskii* is a freshwater planktonic cyanobacterium, comprising strains that can produce cylindrospermopsin (CYL), a potent hepatotoxic alkaloid, or PSTs, but many do not produce either toxin. These filamentous strains are always maintained as non-axenic cultures, i.e. accompanied by heterotrophic bacteria. The biosynthetic pathways involved in CYL production are well defined, but the genetics of PST synthesis in cyanobacteria and dinoflagellates are not yet elucidated and regulatory factors remain unknown.

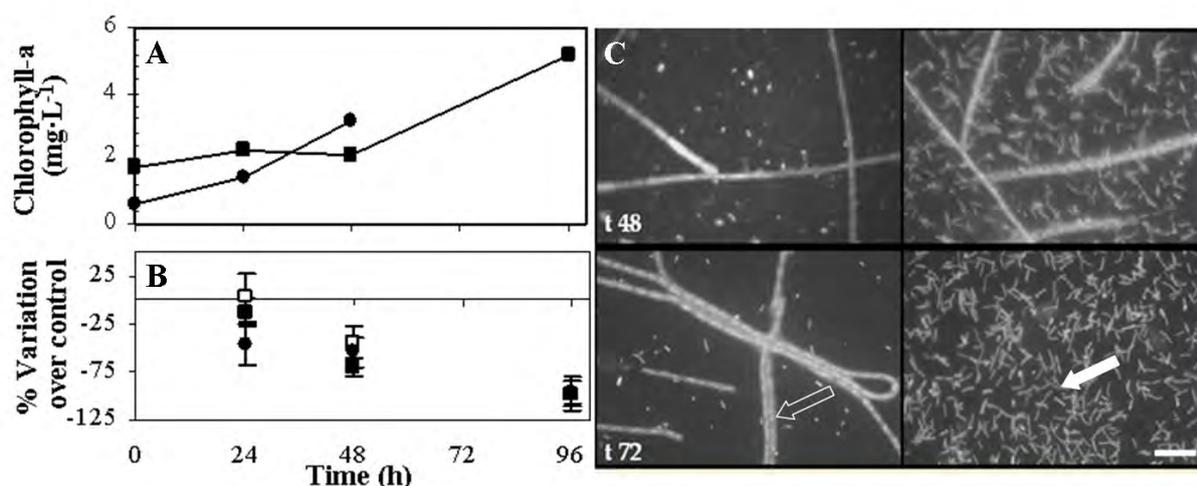
Our aim was to characterize the PST production of *C. raciborskii* strain D9 under different culture

conditions that might induce or suppress toxin biosynthesis. We supplemented cultures with arginine, one of the substrates putatively involved in STX synthesis. Toxin biosynthesis and cell toxin content was also evaluated in the presence of the antibiotic chloramphenicol (CAM). This reversible inhibitor of ribosomal protein synthesis was previously shown to affect PST production in *C. raciborskii* strain T3, also isolated from a freshwater habitat in Brazil (Pomati *et al.* 2004). Finally, we determined the effect of temperature on growth and toxin production in strain D9, as an external factor potentially affecting toxin biosynthesis.

### Methods

#### *Cyanobacterial cultures and treatments*

*Cylindrospermopsis raciborskii* strain D9 was clonally isolated from a mixed culture SPC338 kindly provided by María Teresa de Paiva from the Billings water reservoir near Sao Paulo, Brazil. It was maintained in 250-ml glass flasks with MLA medium (Castro 2004) at 18-23 °C under a 12:12 h L:D photoperiod with



**Figure 1.** Effect of arginine on growth (A) in cultures with culture medium MLA (■) and arginine 10mM added (●) and in intracellular toxin levels (B) of GTX 3 (■) GTX 2 (□) and STX (●) in cultures of *C. raciborskii* D9. Data points represent the percentage mean variations over control ( $\pm$  standard error). In (C) the left panel shows cyanobacteria (open arrow) and heterotrophic bacteria (closed arrow) cultured in MLA; the right panel corresponds to cultures with arginine added. Scale bar=10 $\mu$ m.

33  $\mu$ mol photons  $m^{-2} s^{-1}$ . To evaluate effects of CAM and arginine on toxin production, cultures of *C. raciborskii* strain D9 were exposed to CAM (1  $\mu$ g  $ml^{-1}$ ) and sampled for toxin analysis at 0, 8 and 24 h after exposure. We first determined the CAM concentration and the range for which no measurable effects on chlorophyll *a* synthesis were observed. These conditions were: 1  $\mu$ g  $ml^{-1}$  CAM and between 0 and 8 h culture (initial chlorophyll *a* concentration 0.3 mg  $L^{-1}$ ). Cultures exposed to arginine (10 mM final concentration) were harvested at 24, 48 and 96 h for toxin quantification. Temperature effects on toxin production were evaluated in cultures grown at 19 and 25 °C under continuous white light at 114  $\mu$ mol photons  $m^{-2} s^{-1}$ . Growth of cultures was estimated by chlorophyll quantification. Cell integrity was evaluated by epifluorescence microscopy in samples stained with acridine orange (Hobbie 1977).

#### Toxin preparation and analysis

One ml of cyanobacterial culture was harvested by centrifugation at 16000  $\times$  g for 15 min and the cell pellet was lyophilized. PSTs were extracted in 300  $\mu$ l of 0.05M acetic acid by bath-sonicating twice for 30 min, with 1 h of soaking between sonications; samples were then centrifuged at 5220  $\times$  g for 10 min. The supernatant was filtered through a 0.45- $\mu$ m pore size membrane filter and stored at -20 °C until analysis. In addition, samples of the culture medium after growth (but minus cells) were harvested for analysis of extracellular toxins.

Initial quantitative analysis by high performance liquid chromatography with fluorescence detection (HPLC-FLD) was carried out according to the method described in Lagos (1999), using a Jasco HPLC chromatograph PU-2089 coupled with Jasco FP2020Plus fluorescence detector, with some minor modifications. Toxin analysis was performed in reverse-phase on a Kromasil C-8 column (100  $\times$  4.6 mm i.d.), with oxidant and acidifying reagent flow rates at 0.7  $ml min^{-1}$ .

Mass spectral measurements were carried out on a triple quadrupole mass spectrometer (API 4000 QTrap, ABI-Sciex) with turbo-spray ionization in negative ion mode. The analytical column was a Hypersil BDS C8 (50  $\times$  2 mm, 3  $\mu$ m, 120 Å), with a flow rate of 0.3  $ml min^{-1}$  using a binary elution gradient (A: water and B: 95 % acetonitrile/methanol (1:2 v/v) and 5 % water. Both eluants contained 2.0 mM ammonium formate and 50 mM formic acid. Initial composition was 40 % Eluant B with a linear gradient to 100 % B at 6 min, isocratic 100 % B until 15 min, then returning to initial conditions.

Toxin identity was confirmed by comparison of mass spectral data from sample peaks with external standards of certified reference PST obtained from the CRMP programme from NRC, Halifax, Canada.

#### Results

The LC-MS/MS analysis showed that *C. raciborskii* strain D9 produces the following PST profile: STX, GTX2/3, dcSTX, and dcGTX2/3 (Table 1). The main toxins detected in both intracellular and extracellular fractions were STX and GTX2/3. Neosaxitoxin

(NEO) was only detected in the extracellular fraction.

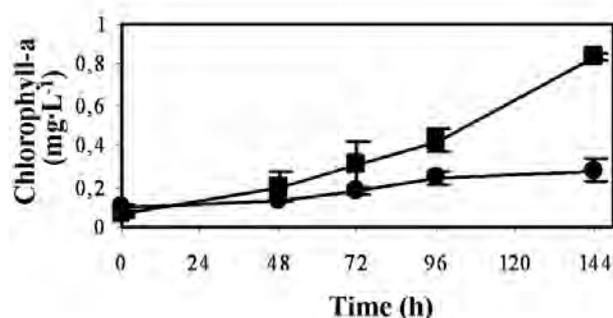
The experiments performed under the specified antibiotic conditions showed that CAM increased STX cell content (90 % of variation over control), but not the intracellular concentration of GTX2/3 (data not shown). After 24 h in culture, total cell lysis was observed.

**Table 1.** Determination of PSTs by LC-MS/MS in *C. raciborskii* D9.

	Extracellular (ng·µl <sup>-1</sup> )	Intracellular (ng·mg <sup>-1</sup> )
STX	0.131	59.2
NEO	0.021	nd*
dcSTX	0.004	2.9
GTX2/3	1.369	1,242
dcGTX2/3	0.074	8.9

\* Not detected

Arginine at 10 mM concentration did not affect growth until 48 h of exposure in culture (Fig. 1A), but mean intracellular STX levels nevertheless decreased 48 % relative to controls after 24 h. GTX2/3 levels were not affected during this exposure (Fig 1B). The amount of heterotrophic bacteria in the medium increased in cultures supplemented with arginine, with concomitant cyanobacterial cell lysis (Fig. 1C).



**Figure 2.** Effect of the temperature on growth at 19 °C (●) and 25 °C (■) of *C. raciborskii* D9.

Growth of *C. raciborskii* D9 was four-fold higher at 25 °C than at 19 °C by 144 h as determined by chlorophyll-specific biomass measurements (Fig. 2). However, intracellular toxin content, standardized by dry weight, remained stable in that period. No changes were observed in the morphology of filaments under growth at the different temperatures.

## Discussion

The toxin profile of *C. raciborskii* strain D9 (STX, dcSTX, GTX2/3 and dcGTX2/3) is radically different from that of the previously described strain T3 (STX, C1 and C2) (Pomati *et al.* 2004). The PST profile differences indicate functional modifications of the basic PST biosynthetic pathway, related to the expression and activity of carbamoylase in strain D9, versus putative N-aminosulfotransferase activity in strain T3. These differences are genetically determined and intracellular processes, unrelated to the presence of heterotrophic bacteria. Nevertheless, detection and confirmation by mass spectral analysis of NEO in the extracellular medium indicates that biotransformation (possibly mediated with heterotrophic bacteria) also occurs in cultures of strain D9.

Among other differences between *C. raciborskii* strain D9 and the homologous strain T3, CAM treatment differentially affected STX production. We observed an increase in STX cell content in strain D9 at CAM concentration of 1 µg ml<sup>-1</sup>. This may be an indication of greater antibiotic sensitivity of strain D9, with the rate of STX biosynthesis uncoupled from the declining growth rate of the cells and eventual lysis. Nevertheless, it is also possible that the negative effect on growth rate of heterotrophic bacteria could indirectly influence the cyanobacterial response. The presence of high arginine in the medium also had a different effect on strain D9 than on T3. In D9, the 48 % decrease in cell STX quota after 24 h exposure to arginine can be compared to the reported 476 % increase in strain T3 (Pomati 2004). Arginine is a putative precursor of STX, and therefore it is reasonable to expect an induction of STX production, and an increase in cell quota unless the growth rate is proportionally increased (apparently not the case). In strain D9, when arginine was supplemented, growth of heterotrophic bacteria in the cultures was greatly induced, presumably as a response to provision of a heterotrophic growth substrate. Although at 24 h in culture this effect was not dramatic, we cannot discard the possibility that negative allelochemical interactions between cyanobacteria and heterotrophic bacteria may have been stimulated.

We previously described that over a long period in culture (50 days), temperature had an effect on PST production in strain C10 (Castro 2004). However, in D9 cultures, we did not detect temperature-dependent inhibition or stimulation of PST production over 144 h. For cylindrospermopsin-producing strains, an in-

crease in toxin cell quota was observed when the cultures were grown below optimal temperatures (Saker and Griffiths 2000). However, that was not the case for PST in the D9 strain. High genotypic variation among strains, in growth, metabolism and biosynthetic rates argues strongly for caution in forming general conclusions on regulation of toxin synthesis by environmental factors.

#### **Acknowledgements**

Grants Fondecyt 1050433 and Millennium Nucleus EMBA P04/007

#### **References**

- Lewellyn, L. (2006). *Nat. Proc. Rep.* 23: 200-222.
- Pomati, F., Moffitt, M.C., Cavaliere, R. & Neilan, B.A. (2004). *Biochim. Biophys. Acta* 1674: 60-67.
- Castro, D., Vera, D., Lagos, N., García, C. & Vásquez, M. (2004). *Toxicon* 44: 483-489.
- Hobbie, J.E., Daley, R.J. & Jasper, S. (1977). *Appl. Environ. Microbiol.* 33: 1225-1228.
- Lagos, N., Onodera, H., Zagatto, P., Andrinolo, D., Azevedo, S. & Oshima, Y. (1999). *Toxicon* 37: 1359-1373.
- Saker, M.L. & Griffiths, D.J. (2000). *Mar. Freshwater Res.* 52: 907-915.

## The genus *Ostreopsis* in recreational waters of the Catalan Coast and Balearic Islands (NW Mediterranean Sea): is this the origin of human respiratory difficulties?

M. Vila<sup>1</sup>, M. Masó<sup>1</sup>, N. Sampedro<sup>1</sup>, H. Illoul<sup>1</sup>, L. Arin<sup>1</sup>, E. Garcés<sup>1</sup>, M.G. Giacobbe<sup>2</sup>,  
J. Alvarez<sup>3</sup> and J. Camp<sup>1</sup>

<sup>1</sup> Institut de Ciències del Mar, CSIC, Pg. Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain, magda@icm.csic.es, <sup>2</sup> Istituto per l'Ambiente Marino Costiero, CNR, Messina, Italy,

<sup>3</sup> Departament de Salut de la Generalitat de Catalunya Barcelona, Spain

### Abstract

*Ostreopsis* spp. blooms have been associated with human respiratory irritation in Mediterranean coastal areas. Here, we present their distribution along beaches of the NW Mediterranean (Catalan Coast and Balearic Islands) during August 2004. The build-up of *Ostreopsis* spp. to relatively high concentrations coincided only once with a harmful episode in Llanereres beach. We summarize and discuss situations characterized by relatively high *Ostreopsis* spp. concentrations with or without records of harmful effects.

### Introduction

One of the main emergent problems related with harmful algae is the exposure to marine aerosols containing e.g. brevetoxins, which causes respiratory symptoms in humans (Pierce *et al.* 1990). Occurrences of *Ostreopsis* spp. during summer periods have been associated with similar human health problems in some Mediterranean coastal localities since 1998. Respiratory irritation, fever, and skin irritations affected people exposed to contact with seawater or marine aerosols by recreational activities on the beach (Sansoni *et al.* 2003; Gallitelli *et al.* 2005). Benthic fauna mortality - possibly due to anoxia - and even fish kills have also been related with the occurrence of *Ostreopsis* species (Sansoni *et al.* 2003). Concern over this topic was voiced in the International Workshop 'Ostreopsis: is it a problem for the Mediterranean Sea?' held in Genova (Italy) in November 2005 ([www.apat.gov.it/site/\\_files/ConvegnoOstreopsis.pdf](http://www.apat.gov.it/site/_files/ConvegnoOstreopsis.pdf)).

Two *Ostreopsis* species known to produce palytoxin-like toxins have recently been identified in the Mediterranean Sea: *Ostreopsis* cf. *siamensis* and *O. ovata* (Penna *et al.* 2005). These algae are epiphytic, loosely attached to macroalgae, and have the capability to detach and swim in the water column (Vila *et al.* 2001). Studies on their distribution are few but urgent due to the potentially harmful effects. Extensive sampling in recreational waters has been conducted along the Catalan Coast (CC) and Balearic Islands (BI) in the summer 2004. Here, we discuss the distribution of the two species and their relationship with an epidemic respiratory irritation outbreak that occurred in Llanereres (CC; Alvarez *et al.* 2005).

### Materials and Methods

During the summer 2004, an extensive monitoring along beaches used for swimming was conducted in two Mediterranean areas. Between 3 and 9 August, seawater at 234 beaches, and at the end of August 65 beaches, were sampled at the surface along the CC and BI, respectively. On June 2001, *Ostreopsis* spp. in the surface water were followed during 4 nycthemeral cycles in Paguera (Majorca), with sampling every 2 h for the first day, and every 4 h the following days. Water samples (250 ml) were fixed with Lugol's solution, the phytoplankton settled in 50-ml counting chambers for one day and cells counted using a Leica inverted microscope.

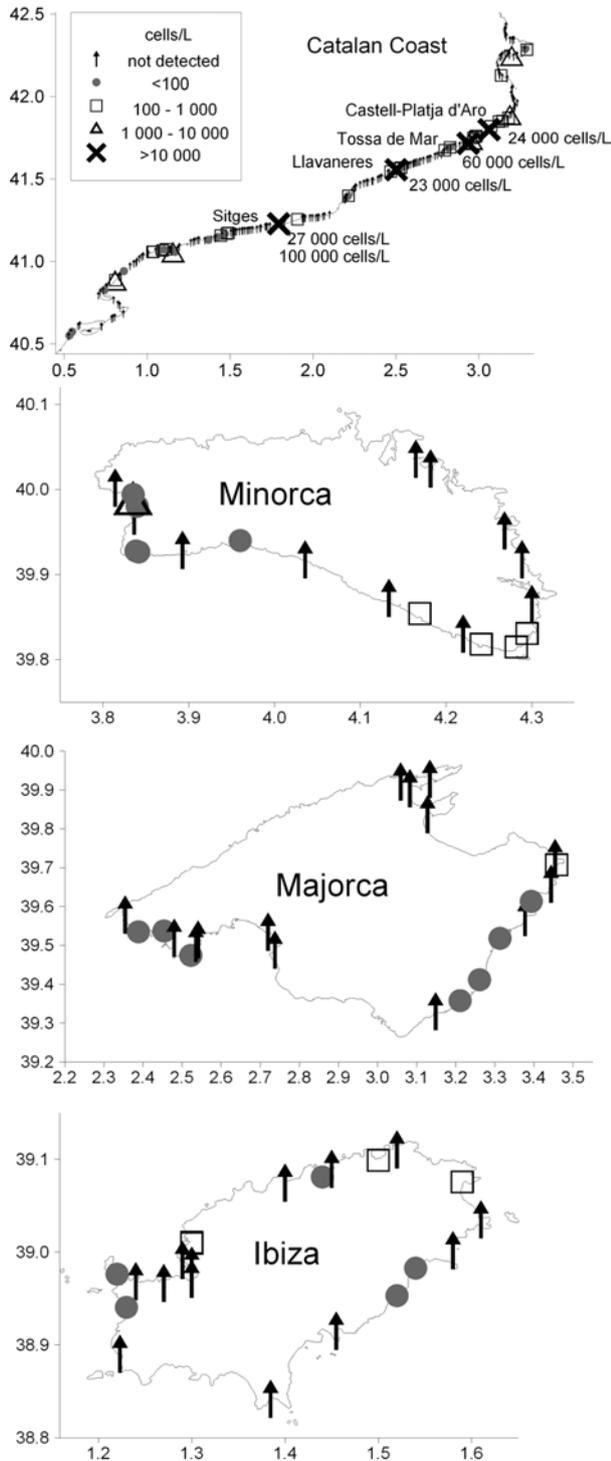
Sixty-two among the known 74 affected people in Llanereres were interviewed for symptoms and duration of the epidemic outbreak.

Meteorological data were provided by the Malgrat de Mar station, located 25 km north of Llanereres.

### Results

The two *Ostreopsis* spp. identified in this study, *O. cf. siamensis* and *O. ovata*, were detected in 22 % of the samples from CC and 42 % from BI (Table 1). At BI, the concentrations were below 100 cells·l<sup>-1</sup> in 26 % of the samples, with a maximum of 1280 cells·l<sup>-1</sup> in Sa Caleta (Minorca). Along the CC, 8 % of the samples had *Ostreopsis* concentrations below 100 cells·l<sup>-1</sup>, 10 % between 100 and 1000, 2 % between 1000 and 10,000, and in only five samples (2 %) did the cell concentrations exceed 104 cells·l<sup>-1</sup> (Fig. 1). From 4 to 10 August 2004, an epidemic respiratory irritation outbreak affected 74 people in Llanereres beach. The main symptoms were rhinorrhoea (74.2 %), nose ir-

ritation (66.1 %), throat irritation (62.9 %), coughing (59.7 %), expectoration (51.6 %), eye irritation (41.4 %) and migraine (40.3 %). The estimated incubation period was around 3 h and the symptoms lasted about 45 h. Sick people were beachgoers of Lllavaneres (basically a rocky area), or residents of flats located very near a rocky artificial area constructed to protect the

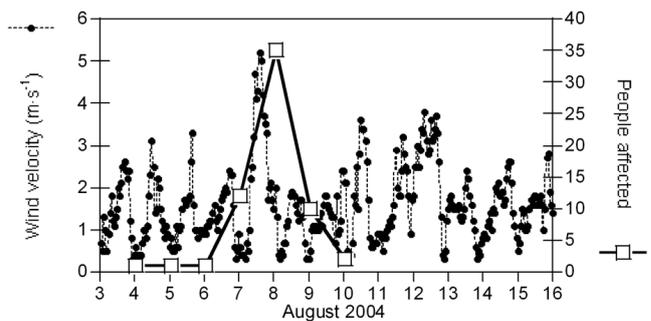


**Figure 1.** *Ostreopsis* distribution along the Catalan Coast and Balearic Islands (latitude: N; longitude: E) in August 2004. Arrows indicate no detection of cells.

shore line. Most cases of sick people (91 %) occurred between 7 and 9 August, with a maximum reported on 8 August (56 %) (Fig. 2). Unusually high concentrations of *Ostreopsis* were detected in the surface water ( $2.3 \cdot 10^4\text{ cells}\cdot\text{l}^{-1}$ ) on 6 August, decreasing to a maximum of  $4 \cdot 10^3\text{ cells}\cdot\text{l}^{-1}$  on 13 and below  $2 \cdot 10^3\text{ cells}\cdot\text{l}^{-1}$  on 17 August.

**Table 1.** Number of beaches affected (%) and maximum of *Ostreopsis* cell concentrations recorded in August 2004.

	Catalan (CC)	Balear (BI)
Stations sampled	234	65
Max. Conc. ( $\text{cells}\cdot\text{l}^{-1}$ )	106 655	1 280
% Stations affected	22	41.5
<i>Ostreopsis</i> $\text{cells}\cdot\text{l}^{-1}$	Affected beaches: num. (%)	
Not detected ( $<40$ )	182 (78)	38 (58.5)
$<100$	18 (8)	17 (26)
100-1 000	24 (10)	9 (14)
1 000-10 000	5 (2)	1 (1.5)
$>10\ 000$	5 (2)	-



**Figure 2.** Wind velocity and number of persons affected by respiratory symptoms in Lllavaneres.

Meteorological conditions during the event and the preceding week were as follows: air temperature  $27.4\text{--}29.5\text{ }^\circ\text{C}$ , maximum solar irradiation  $752\text{--}832\text{ W}\cdot\text{m}^{-2}$ , and maximum wind velocity reached  $5.2\text{ m}\cdot\text{s}^{-1}$  the day before the maximum of affected people was recorded (Fig. 2). Interestingly, on this day (8 August) wind intensity was low (maximum  $1.9\text{ m}\cdot\text{s}^{-1}$ ); however, an on-shore wind was prevalent. Symptoms increased during the evening (40 %), probably as a consequence of increased *Ostreopsis* cell concentrations in the water during light-time and breeze direction, and diminished when people left the affected area. A high daily variability was detected in surface waters of Majorca, with *Ostreopsis* concentrations increasing up to two orders of magnitude during the light hours vs. night (Fig. 3).

## Discussion

The genus *Ostreopsis* was widespread in the NW Mediterranean recreational waters sampled. However, blooms ( $>10^4\text{ cells}\cdot\text{l}^{-1}$ ) have been detected only at a

few beaches, and have been associated with harmful effects in only two cases (Table 2).

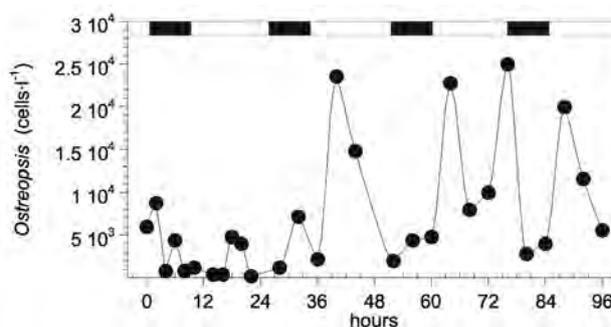
**Table 2.** Summary relating *Ostreopsis* events and harmful effects in the study area.

EVENT	MAX. CELL. CONC. (cells·l <sup>-1</sup> )	HARMFUL EFFECTS
Garraf and Blanes harbours, autumn 1997, CC	7.8·10 <sup>4</sup> and 9.8·10 <sup>4</sup> (after storms)	Not known
Fosca beach (annual cycle: 1997-1998), CC	>1·10 <sup>4</sup> 5.9·10 <sup>5</sup> cells·g <sup>-1</sup> Fresh Weigh*	Not known
Llavaneres (near Riera Argentona) August 1998, CC	2·10 <sup>5</sup> (after heavy rain)	Benthic fauna mortality. Fish mortality in an aquarium
Paguera beach Majorca, June 2001, BI	2.5·10 <sup>4</sup>	Not known
Llavaneres, August 2004, CC	2.3·10 <sup>4</sup>	Respiratory irritation in beach users
Several beaches, 2004, CC (Fig. 1.)	From 2.4·10 <sup>4</sup> to 1·10 <sup>5</sup>	Not known

\**O. cf. siamensis* cells·g<sup>-1</sup> Fresh Weight macroalgae, in Vila *et al.* 2001; CC: Catalan Coast; BI: Balearic Islands

As shown in Table 2, until now, only one case has been documented at the CC of unusually high *Ostreopsis* spp. concentrations in surface waters to coincide with human respiratory difficulties. In a previous study, the concentrations of epiphytic *Ostreopsis* on macroalgae recorded in Fosca beach (CC) were among the highest recorded anywhere in the world. They were not reported to be related with human health problems. No respiratory outbreak was reported at any of the other beaches with *Ostreopsis* concentrations similar or higher than those of Llavaneres, nor in harbours.

Apparently a combination of several factors must occur to cause an outbreak of human respiratory irritation. The area affected at Llavaneres is a shallow rocky habitat colonised by *Corallina elongata*, which can support high *Ostreopsis* concentrations (Vila *et al.* 2001). The microalgal growth is probably stimulated by high temperatures, reflected in the high concentrations of cells detected in surface water (10<sup>4</sup> cells·l<sup>-1</sup>). During the day, *Ostreopsis* cell concentrations increase in the water as observed at Paguera beach (Fig. 3). Detachment from macroalgae must be studied further to give an exact explanation for the diurnal increases in cell concentration. When the *Ostreopsis* population is



**Figure 3.** *Ostreopsis* daily cycles in Paguera beach (Majorca) in June 2001.

well developed, toxin content is probably released to the water and air as documented for *Karenia brevis* in the Gulf of Mexico. Unarmoured *K. brevis* cells under physical stress have been suggested to lyse, thereby releasing the endotoxins to the water (Pierce *et al.* 1990). Finally, the prevalent winds blowing onshore increase the risk of people being exposed to marine biotoxin aerosols. The detection of biotoxins in water and marine aerosols will be addressed in future studies.

#### Acknowledgements

This work was supported by “Pla de vigilància de fitoplàncton nociu i tòxic a la Costa Catalana” (CSIC-Agència Catalana de l’Aigua, Generalitat de Catalunya) and “Calidad de las aguas costeras de las Islas Baleares” (CSIC- Conselleria de medi ambient, Govern de les Illes Balears). We thank the Servei Meteorològic de Catalunya for meteorological data.

#### References

- Alvarez, J. and 13 co-authors (2005). In: XXIII Reunión de la Sociedad Española de Epidemiología, Las Palmas de Gran Canaria (Spain).
- Gallitelli, M., Ungaro, N., Addante, L.M., Procacci, V., Silver, N.G. & Sabbà, C. (2005). *J. Am. Med. Ass.* 293: 2599-2600.
- Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobó, P. & Vernesi, C. (2005). *J. Phycol.* 41: 212-245.
- Pierce, R.H., Henry, M.S., Proffitt, S. & Hasbrouck, P.A. (1990). In: *Toxic Marine Phytoplankton*, Granéli, E., Sundström, B., Edler, L. & Anderson, D. M. (eds), Elsevier, Amsterdam, pp. 397-402.
- Sansoni, G., Borghini, B., Camici, G., Casotti, M., Righini, P. & Rustighi, C. (2003). *Biologia Ambientale* 17: 17-23.
- Vila, M., Garcés, E. & Masó, M. (2001). *Aquat. Microb. Ecol.* 26: 51-60.

## 10. TOXIN ANALYSIS AND SYNTHESIS



12TH INTERNATIONAL  
CONFERENCE ON  
HARMFUL ALGAE



COPENHAGEN, 2006

## Studies on cryptic PSP toxicity depends on the extraction procedure

M.J. Botelho, S.S. Gomes, S.M. Rodrigues and P. Vale

Instituto Nacional de Investigação Agrária e das Pescas –IPIMAR, Av. Brasília, 1449-006 Lisboa, Portugal

### Abstract

The recent modifications of the extraction solvent for the pre-chromatographic oxidation LC method of Lawrence *et al.* (2005) were tested with shellfish harvested during blooms of *Gymnodinium catenatum* on the NW coast of Portugal in 2005/2006. The toxin profile of several shellfish species was obtained using acetic acid extraction and compared with hydrochloric acid extraction. Since acetic acid, unlike hydrochloric acid, does not promote conversion of *N*-sulfocarbamoyl analogues to carbamoyl analogues, an underestimation of toxicity was observed in most of the samples tested. This did not compromise the protection of public health, as samples with levels close to the regulatory limit are easily detected by HPLC methodology and trigger intensive sampling.

### Introduction

During the autumn of 2005, bivalve molluscs contaminated with PSP (paralytic shellfish poisoning) toxins due to blooms of *Gymnodinium catenatum* were recorded on the NW coast of Portugal, the first case since 1995. The toxin profiles observed in most of the bivalves were typically representative of contamination by the European strain of *G. catenatum*, with high proportion of *N*-sulfocarbamoyl toxins (Oshima *et al.* 1993). *Spisula solida* and *Scrobicularia plana* showed profiles dominated by decarbamoyl analogues resulting from carbamoylase activity (Artigas *et al.* 2007). In the remaining bivalve species the presence of large amounts of *N*-sulfocarbamoyl toxins represented a cryptic toxic potential upon gastric digestion at low pH in the stomach of consumers.

The official methods for PSP surveillance worldwide include mild acid extraction (hydrochloric acid 0.1M) (AOAC 1990; Irwin 1970). High-performance liquid chromatography (HPLC) methods developed to replace mouse bioassay have relied so far on the same extraction procedure, which promotes partial conversion of *N*-sulfocarbamoyl toxins into their respective carbamoyl analogues (Lawrence *et al.* 1991; Thielert *et al.* 1991; Sullivan *et al.* 1993; Oshima 1995).

The last modification of the Lawrence method, using acetic acid (HAc) as extractant, was recently subjected to an interlaboratory collaborative study to support its acceptance as an official AOAC method (Lawrence *et al.* 2005). However the use of a weak acid may lead to an under-estimation of toxicity. The complete release of cryptic toxicity of *N*-sulfocarbamoyl analogues is only achieved after hydrolysis in strong acidic media (HCl 1M) (Band-Schmidt *et al.* 2005).

We aimed at verifying whether the adoption of the Lawrence *et al.* (2005) methodology in a monitoring programme would provide the same level of public health protection as traditionally conveyed by AOAC extraction (1990), using the profiles found in Portuguese bivalves.

### Materials and Methods

Contaminated bivalves samples were collected from Aveiro lagoon in autumn 2005 and from Óbidos lagoon and Lisboa/Peniche offshore in summer 2006 (NW coast of Portugal), as part of the ongoing monitoring programme. 23 samples were selected from commercial species (*Mytilus galloprovincialis*, *Cerastoderma edule*, *Tapes decussatus*, *Venerupis pullastra*, *Solen* spp., *Scrobicularia plana*, *Solen marginatus*, *Crassostrea* spp. and *Donax* spp.).

The extraction procedure was performed in triplicate using a 5-g portion of whole flesh sample vortex mixed with 5 ml of HCl 0.1M or HAc 1 % for 2 min and boiled for 5 min at temperature > 95 °C. The mixture was centrifuged for 10 min at 5000 rpm (2800 x g), and the extracts were filtered using a 0.22-µm nylon disposable syringe filter. For complete hydrolysis, 400 µl HCl extracts were heated with 100 µl 1M HCl for 30 min at 80 °C and neutralised with 100 µl 2M ammonium formate.

Toxins were analysed by HPLC with automated peroxide pre-column oxidation and fluorescence detection (Vale and Taleb 2005). The equivalent of 1.25 mg extract (2.5 µl) was oxidized and injected on the column without SPE clean-up. Only peroxide oxidation was used to detect non-hydroxylated analogues. Periodate oxidation results in a large number of hydroxylated analogues, for which a limited number of

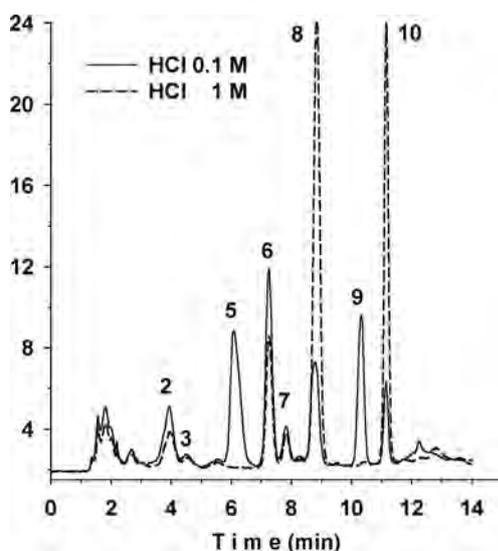
standards are available. For calculation of sample toxicity, NRC standards were used (dcGTX2+3, dcSTX, B1, GTX2+3, STX), with the exception of C1+2, where the fluorescent response was assumed to be identical to B1. Toxicity was determined by multiplying the concentration of each toxin with the respective specific toxicity (Oshima 1995). For toxins determined together, the highest toxicity factor of the two isomers was used.

## Results and Discussion

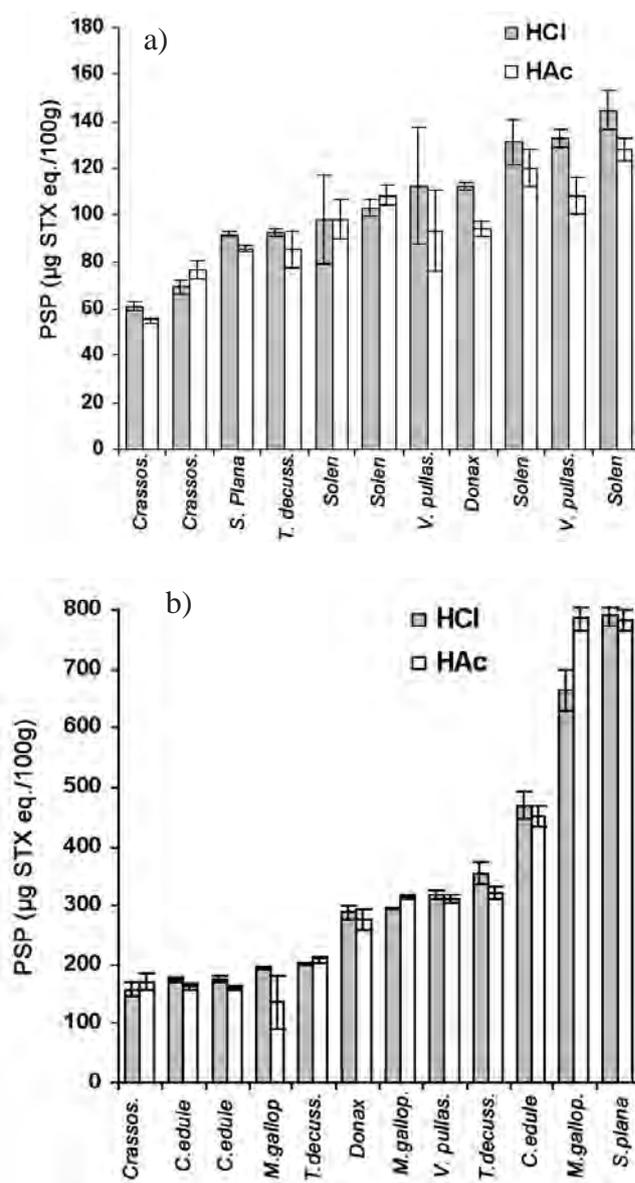
Bivalves contaminated with *G. catenatum* contained a large amount of cryptic toxins, as exemplified with a mussel sample analysed with conventional AOAC extraction, and the same extract after complete hydrolysis (Fig. 1). *N*-sulfocarbamoyl analogues (peaks 5 and 9, corresponding to C1+2 and B1, respectively) disappeared completely after hydrolysis, resulting in an increment of carbamoyl analogues (peaks 8 and 10, corresponding to GTX2+3 and STX, respectively).

Each bivalve species was tested with 0.1M HCl and 1% HAc extraction in triplicate, sorted after contamination levels, below 160  $\mu\text{g STX eq. /100 g}$  and above 160  $\mu\text{g STX eq. /100 g}$ . In the overall samples analysed toxicity obtained by acetic acid extraction was on average 5 % lower than with hydrochloric acid, independently of the contamination levels and the matrices studied (Fig. 2). Occasionally, HAc extraction led to higher toxicity values.

A good correlation was obtained between the two extractions procedures (Fig. 3).

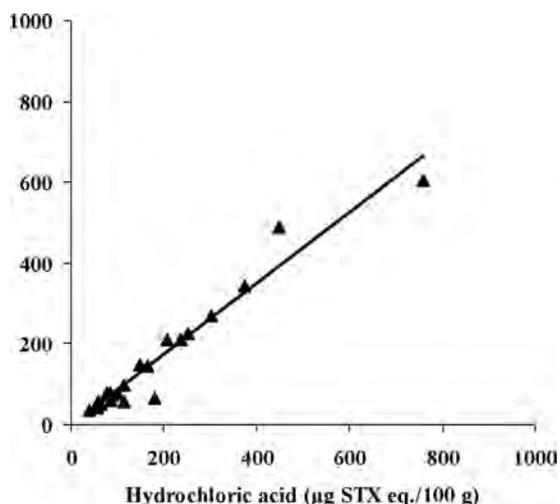


**Figure 1.** Chromatogram of pre-column peroxide oxidation of PSP toxins extracted from mussel with HCl 0.1M, and overlaid with same extract after complete hydrolysis with HCl 1M (2 and 3 = dcGTX2+3, 5 = C1+2, 6 and 7 = dcSTX, 8 = GTX2+3, 9 = B1, 10 = STX).



**Figure 2.** Non-hydroxylated analogues contribution to sample toxicity in different bivalve species collected in 2005, with increment levels after HCl and HAc extraction: a) levels below 160  $\mu\text{g STX eq. /100g}$ ; b) above 160  $\mu\text{g STX eq. /100g}$  (solid line = regulatory limit of 80  $\mu\text{g STX eq. /100g}$ ); (each bar represents mean  $\pm$  SD, n = 3).

*N*-sulfocarbamoyl analogues were dominant in the toxin profiles. On a molar basis they reached around 76 % of the total toxin content, as exemplified by a cockle sample (Table 1). Mild hydrochloric acid extraction promoted only a 0.6 % increase of carbamoyl toxins, contributing only 3.1 % of the total toxicity, due to the already large contributions from decarbamoyl and *N*-sulfocarbamoyl toxins. In turn, this translated to toxicity increasing from 161 to 175  $\mu\text{g STX eq. /100g}$ .



**Figure 3.** Relationship between PSP toxicity obtained by HCl and HAc extraction with peroxide oxidation for all the samples analysed. Toxin levels ranged from 61 to 789 µg STX eq./100g ( $y=1.05x-17.43$ ,  $r=0.98$ ,  $n=23$ ).

**Table 1.** Toxin profiles of a *Cerastoderma edule* sample collected in December 2005 at Aveiro lagoon and obtained by 0.1M HCl or 1 % HAc extraction with per-oxide oxidation (molar percent). Percent contribution of each toxin component to sample toxicity by HCl or HAc extraction.

	Molar percent (%)		Toxicity percent (%)	
	HCl	HAc	HCl	HAc
B1	43.8	41.6	16.3	14.9
C1+2	33.7	33.6	18.7	18.0
STX	0.4	0.0	2.5	0.0
GTX2+3	0.2	0.0	0.6	0.0
dcGTX2+3	3.4	4.8	7.4	10.1
dcSTX	18.4	19.9	54.5	57.0
Sum N-sulfo	77.5	75.2	35.0	32.9
Sum Carbamoyl	0.6	0.0	3.1	0.0
Sum Decarbamoyl	21.9	24.8	61.9	67.1
PSP (µg STX eq. /100 g)			175.3	161.4

## Conclusions

Although acetic acid extraction could result in lower toxin levels than HCl extraction, underestimation of toxicity does not jeopardise the protection of public health; samples with levels close to the regulatory limit are easily detectable by this highly sensitive HPLC methodology and trigger intensive sampling.

## Acknowledgements

“Safety, Surveillance and Quality of Bivalve Molluscs” (QCAIII /med.4/ MARE Programme) supported this work. The authors would like to thank Jorge Lameiras for technical assistance in sample preparation.

## References

- AOAC (1990). In: Hellrich, K. (ed.) Official Method of Analysis. 15th Edition, Sec 959.08. AOAC, Arlington, Virginia, USA, pp. 881-882.
- Artigas, M.L., Vale, P., Gomes, S.S., Botelho, M.J., Rodrigues, S.M. & Amorim, A. (2007). J. Chromatogr. A. 1160: 99-105.
- Band-Schmidt, C.J., Bustillos-Guzmán, J., Gárate-Lizárraga, I., Lechuga-Devéze, C.H., Reinhardt, K. & Luckas, B. (2005). Harmful Algae 4: 21-31.
- Irwin, N. (1970). Americ. Public Health Ass., Inc., Washington DC, 4th ed., pp. 61-65.
- Lawrence, J. F., Ménard, C, Charbonneau, C.F. & Hall, S. (1991). J. AOAC 74 : 404-409.
- Lawrence, J.F., Niedzwiadek, B. & Ménard, C. (2005), J. AOAC International 88: 1714-1732.
- Oshima, Y. (1995). J. AOAC International 78: 528-532.
- Oshima, Y., Blackburn, S.I. & Hallegraeff, G.M. (1993). Mar. Biol. 116: 471-476.
- Sullivan, J.J. & Iwaoka, W.T. (1993) J. Assoc. Offic. Anal. Chem. 66 : 297-303.
- Thielert, G., Kaiser, I. & Luckas, B. (1991). In: J.M. Freymy (eds.) Proc. Symposium Mar. Biotoxins, Editions CNEVA, Maisons-Alfort, pp. 121-125.
- Vale, P. & Taleb, H. (2005) Food additives and Contaminants 22: 838-846.

## Rapid field-based monitoring systems for the detection of toxic cyanobacteria blooms: microcystin ImmunoStrips and fluorescence-based monitoring systems

G.L. Boyer<sup>1</sup>, E. Konopko<sup>1</sup> and H. Gilbert<sup>2</sup>

<sup>1</sup>Department of Chemistry, State University of New York, College of Environmental Science and Forestry, Syracuse, USA, glboyer@esf.edu and <sup>2</sup>Agdia Inc., Elkhart, USA, hgilbert@agdia.com

### Abstract

The occurrence of toxic cyanobacteria is an increasing problem as the world places more demand on its water supplies. Current monitoring strategies are labour intensive, when many discrete samples are collected and returned to the laboratory for taxonomic, chemical or biochemical analysis. This approach is slow and difficult to implement in less-developed regions. Here we describe two alternative approaches for monitoring cyanobacterial blooms and their toxins. The first uses fluorescence of the cyanobacterial pigment phycocyanin. Dual fluorometers were installed in a research vessel and used to monitor for chlorophyll and phycocyanin-containing blooms while the ship was underway. Autonomous ship-board monitoring on Lake Ontario clearly detected a cyanobacterial bloom, and the toxicity of this bloom was determined using a lateral flow immunoassay (ImmunoStrip) for the hepatotoxin, microcystin-LR. With a concentration step, this ImmunoStrip successfully detected soluble microcystins at concentrations below 0.5 µg L<sup>-1</sup> and when used in parallel with the fluorescent detection, could quickly determine if a toxic bloom was present without returning the samples to the laboratory.

### Introduction

The occurrence of toxic cyanobacteria is an increasing problem as the world places more demand on its water supplies. Traditional monitoring strategies usually require the collection of discrete samples, their return to the laboratory and subsequent analysis using enzyme assays such as the protein phosphatase inhibition assay, enzyme linked immunoassays (ELISA) or HPLC with photodiode array (PDA) or mass selective (MS) detection. This approach is both slow and man-power intensive, especially when the sample locations are remote and there is no easy access to the analytical lab. Field-based methods that could provide a rapid assessment of the presence or absence of a bloom and its potential toxicity would be of benefit for monitoring programs in both undeveloped and developed countries.

The Laurentian Great Lakes located along the eastern United States and Canadian border has seen an increase in harmful algal blooms over the past several years. Toxic blooms of *Microcystis aeruginosa* initially reported in the western basin of Lake Erie in the mid 1990s (Brittain *et al.* 2000) have now been observed across Lake Erie, in Lake Ontario and Lake Huron (Budd *et al.* 2001; Boyer 2007). Monitoring for these blooms outside of confined embayments is difficult due to the large size of the lakes and the patchy nature of the bloom events. For example, in a recent lower food web study in Lake Ontario (LOLA), sampling stations were often 30-40 km apart whereas

the bloom event could be sub-kilometer in size. Time and distance constraints made it impractical to stop the ship and collect samples at a greater frequency.

The European Ferry-Box project addressed this issue by installing a suite of autonomous sampling instruments on the ferry ships that routinely cross the Baltic and North Sea. Cyanobacteria blooms can easily be detected without stopping the ship by measuring in situ fluorescence of chlorophyll and the accessory pigment phycocyanin (Rabtaharvu *et al.* 1998). Cyanobacteria are common members of the phytoplankton community in the Great Lakes and detection of a bloom event does not necessarily indicate that it is toxic. To determine toxicity, samples are usually collected and returned to a laboratory for analysis. Lateral flow immunoassays (dipstick assays) provide a rapid approach for estimating the toxicity on board ship. Recently an ImmunoStrip has been developed for the hepatotoxic microcystins. Anti-microcystin antibodies were conjugated to particulate gold, and interactions between the immunogold conjugate and microcystin toxin bound to a detection strip cause the gold to precipitate and form a visible detection band. A second control strip binds directly to the immunogold particle and ensures that the test is working. The presence of free toxin in the water competes with the binding of the immunogold particle to the detection strip, causing the detection band to disappear. Sensitivity of the ImmunoStrip is between 5-10 µg microcystin-LR equivalents L<sup>-1</sup>.

Here we report on the combined application of continuous fluorescence detection of algal pigments and use of an ImmunoStrip for microcystins to monitor for toxic cyanobacterial blooms in the Laurentian Great Lakes.

## Methods

Turner Designs 10-AU fluorometers equipped with phycocyanin and in vivo chlorophyll filter sets were installed in series on the bow water intake system of the CCGS Limnos during the 2004, 2005 and 2006 Taste and Odor cruises on Lake Ontario. The fluorometers were preceded by a de-bubbling unit to remove entrapped air bubbles, and a YSI 6600 series sonde to measure basic water quality parameters. Data were collected at 1-min intervals and linked to a global positioning system to record the location of each sample. The 1-min intervals corresponded to ~300 m when the ship was in motion. At predetermined stations, discrete water samples (20 L for microcystin analysis, 1 L each for chlorophyll and phycocyanin) were collected at 1 m depth (same as the bow intake) and filtered onto 934-AH glass fiber filters. Toxin filters were extracted in 50 % methanol using ultrasound, and the microcystin concentrations were determined by the protein phosphatase inhibition assay (PPIA) and confirmed by HPLC with PDA or MS detection (Hotto *et al.* 2005). Chlorophyll was determined after extraction with 90 % acetone, using the method of Welschmeyer (1994). Phycocyanin was determined fluorometrically using a modification of the method of Siegelman and Kytica (1978; E. Konopko, submitted). Chlorophyll and phycocyanin concentrations from the discrete water samples were plotted against the average fluorescence obtained while stopped on station and the regressions were used to calibrate the continuously acquired data. Distribution maps of the pigment concentrations were generated using ArcView 9.0.

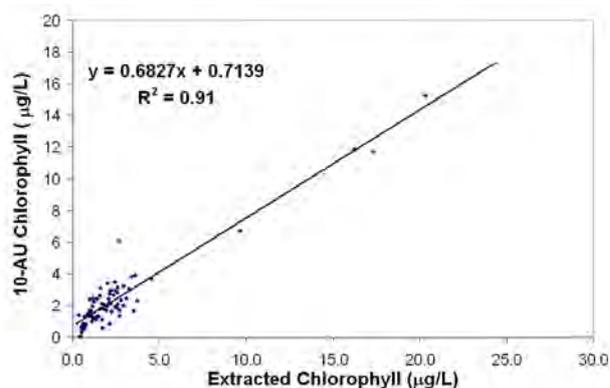
Microcystin ImmunoStrips® with a nominal detection limit of 5-10  $\mu\text{g L}^{-1}$  were obtained from Agdia Inc. (Elkhart, Indiana) and run in two different formats. For samples run on board ship, a small amount of lake water was filtered through 25-mm polycarbonate filter with a nominal pore size of 2  $\mu\text{m}$ . The filters were placed in a screw cap microfuge tube and boiled for 5 min to release the microcystins (Metcalf and Codd 2000). Microcystin ImmunoStrips were also used to confirm toxicity of the 50 % methanolic extracts prepared in the lab. These extracts were highly concentrated and were therefore diluted with distilled water to achieve an apparent concentration factor of 50-fold over raw lake water (detection limit 0.2  $\mu\text{g L}^{-1}$ ). This

also served to dilute the methanol concentration in the extracts to below 5 %.

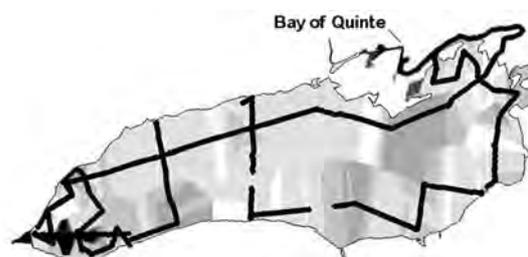
## Results and Discussion

The flow-through system for monitoring cyanobacterial pigments was deployed on three separate cruises on Lake Ontario. The cruises took place in late August when cyanobacterial blooms are at their maximum. More than 80 discrete stations were sampled on each 5-6 day cruise and approximately 8,000 readings were collected per cruise from the fluorometers. The calibration curves for chlorophyll (Fig. 1) and phycocyanin (data not shown) were heavily weighted towards the lower values due to the oligotrophic nature of the Laurentian Great Lakes, but showed excellent linearity ( $R^2 > 0.90$ ). Drift from the 10-AU fluorometers was typically less than 3 % over the course of the 6-day cruise.

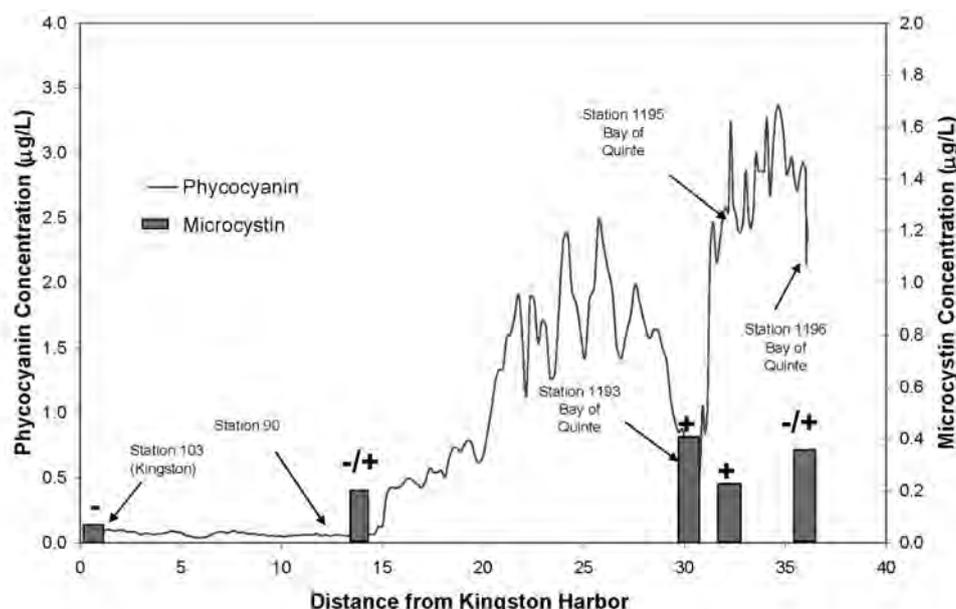
Distribution maps generated from this pigment information (Fig. 2) illustrated the highly patchy nature of algal blooms in Lake Ontario. In 2004, high levels of chlorophyll and phycocyanin were observed in both the western end of Lake Ontario and in the Bay of Quinte.



**Figure 1.** Relationship between chlorophyll as measured using the 10-AU flow-through system and extracted chlorophyll at the discrete stations.



**Figure 2.** Distribution of algal chlorophyll in Lake Ontario obtained from the flow-through system in August 2004. The image is plotted in grey scale from 0 (white) to  $>10 \mu\text{g L}^{-1}$  chlorophyll (black). The cruise track is shown as a dark black line.



**Figure 3.** The phycocyanin fluorescence on a transect running from Kingston Harbor into the Bay of Quinte in August 2004. Microcystin concentrations are indicated by the vertical bars. Results from the microcystin ImmunoStrip at the individual stations are indicated as plus (positive) or minus (negative).

To further illustrate the usefulness of the flow-through system, the fluorescence output from the phycocyanin sensor was plotted versus distance from Kingston Harbor for a single transect into the Bay of Quinte (Fig. 3). Two distinct bloom events were observed as the ship entered the more eutrophic Bay from the open waters of Lake Ontario. Samples for microcystin analysis were taken at five predetermined stations. These stations did not necessarily correspond to the locations of highest bloom density. Microcystin concentrations ranged from 0.05 to 0.4  $\mu\text{g L}^{-1}$  and gave a different impression of the bloom, namely that it was uniformly toxic across the Bay of Quinte. Results of the ImmunoStrip at a detection threshold of 0.2  $\mu\text{g L}^{-1}$  were inconsistent. They accurately predicted the toxicity at the high (#1193, #1195) and low (#103) stations, but did not correct predict toxicity at Station #1196 and #90. Samples run at a lower detection threshold (0.1  $\mu\text{g L}^{-1}$ ) did correctly indicate the toxicity of these intermediate stations.

In general, the correlation between the immuno-assay and laboratory-based PPIA results was good, given that they measure toxicity versus structure and that the PPIA is a more sensitive technique than the ImmunoStrip. Rapid detection techniques such as in situ fluorescence and ImmunoStrips can provide a valuable tool for monitoring of harmful algal blooms, if for no other reason than to indicate that additional sampling and analysis are needed. As such, they can be important components of a tier-based monitoring strategy for harmful algal blooms.

### Acknowledgements

This work was sponsored by NOAA's Coastal Oceans Program for Monitoring and Event Response in the Lower Great Lakes (MERHAB-LGL) under grant #NA160P2788. We thank the captain and crew of the CCGS Limnos for assistance and Michael Satchwell for the PPIA results.

### References

- Boyer, G.L. (2007). *Adv. Exp. Med. Biol.* In press.
- Brittain, S.M., Wang, J., Babcock-Jackson, L., Carmichael, W.W., Rinehart, K.L. & Culver, D.A. (2000). *J. Great Lakes Res.* 26: 241-249.
- Budd, J.W., Beeton, A.M., Stumpf, R.P., Culver, D.A. & Kerfoot, W.C. (2001). *Verh. Int. Ver. Limnol.* 27: 3787-3793.
- Hotto, A., Satchwell, M. & Boyer, G. (2005). *Environ. Toxicol.* 20: 243-248.
- Rabtharvu, E., Olsonen, R., Hällfors, S., Leppainen, J.M., Raateoja, M. (1998). *UCES J. Mar. Sci.* 55: 697-704.
- Siegelman, H.W. & Kycia, J.H. (1978). In: *Handbook of Phycological Methods*, Volume 2, Hellebust, J. & Craigie, J.S. (eds), Cambridge University Press, Cambridge, pp. 71-80.
- Welschmeyer, N.A. (1994). *Limnol. Oceanogr.* 39: 1985-1992.

## The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method

P. Ciminiello<sup>1</sup>, C. Dell'Aversano<sup>1</sup>, E. Fattorusso<sup>1</sup>, M. Forino<sup>1</sup>, G. S. Magno<sup>1</sup>, L. Tartaglione<sup>1</sup>, C. Grillo<sup>2</sup>, and N. Melchiorre<sup>2</sup>

<sup>1</sup>Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, via D. Montesano 49, 80131, Napoli, Italy, ciminiel@unina.it, <sup>2</sup>Agenzia Regionale per la Protezione dell'Ambiente Ligure (ARPAL), Dipartimento La Spezia, via Fontevivo 21, 19125, La Spezia, Italy

### Abstract

A new method based on the combination of reversed phase liquid chromatography with mass spectrometry (LC-MS) is proposed for sensitive, specific and direct determination of palytoxin. The technique was used for analysis of a plankton sample collected along the Genoa coasts in July 2005, when respiratory illness in people exposed to marine aerosols occurred. The study indicated putative palytoxin as the causative agent responsible for patients' symptoms and demonstrated for the first time the presence of such a toxin in Italian waters.

### Introduction

During summer 2005, symptoms of rhinorrhea, cough, fever, bronchoconstriction with mild dyspnea and wheezes were observed in about 200 people exposed to marine aerosols by recreational or working activities on the beach and promenade of Genoa (Italy). Conjunctivitis was also observed in some cases, and twenty people required extended hospitalization.

A careful look at the marine plankton brought to light that the tropical microalga *Ostreopsis ovata* was blooming along the investigated coastal areas during the toxic outbreak. Some *Ostreopsis* strains are regarded as producing palytoxin (Fig. 1) and its analogues, a complex polyether macromolecule and one of the most potent marine toxins so far known<sup>1</sup>. Thus, the need arose to set up a method for detection of palytoxin to investigate whether the *O. ovata* bloom was producing this toxin.

A new approach to the analysis of palytoxin based on reversed phase LC-ESI-MS/MS is proposed. The systematic investigation of MS and chromatographic parameters led to an optimized analytical technique and was applied to analysis of the Mediterranean *O. ovata*. The results obtained point to putative palytoxin as the causative agent of the Genoa outbreak.

### Material and Methods

#### Collection and identification of the plankton

A concentrated plankton sample was collected in July 2005 by operators of the Regional Environmental Protection Agency (ARPAL) by several horizontal net hauls, using an Apstein plankton net (20- $\mu$ m mesh). Inverted microscope analyses showed *Ostreopsis ovata* to be the major species ( $1.8 \times 10^6$  cells  $L^{-1}$ ) present in the sample, together with a few cells

of diatoms (*Coscinodiscus* spp.) and other potentially toxic dinoflagellates, namely *Coolia monotis*, *Prorocentrum lima* and *Amphidinium* sp. The sample was shipped to the Department of Natural Product Chemistry, University of Naples "Federico II", for chemical analysis.

#### Extraction

The concentrated plankton sample was centrifuged at 2000 x g for 20 min to separate particles from the seawater. The pellet was sonicated with 4 mL of methanol/water (1:1, v/v) and centrifuged at 2000 x g. The supernatant was decanted and the pellet washed twice with 3 mL of extraction solvent. The extracts were combined (V = 10 mL) and analyzed by LC-MS. Sea water (500 mL) was added to an equal volume of methanol and extracted twice with 1 L of dichloromethane. The dichloromethane layers were evaporated to dryness, dissolved in methanol (2 mL) and analyzed by LC-MS. The aqueous layer was extracted twice with 500 mL of butanol, which was evaporated, dissolved in 4 mL of methanol/water (1:1, v/v) with 0.5 % formic acid and analyzed by LC-MS.

#### Mouse bioassay

Mouse lethality of both pellet and butanol extracts was tested on female mice CD1 Swiss (14-17 g). One percent of each sample was dried and dissolved in 1 mL of Tween 60 for intraperitoneal injection into mice.

#### Liquid chromatography-mass spectrometry

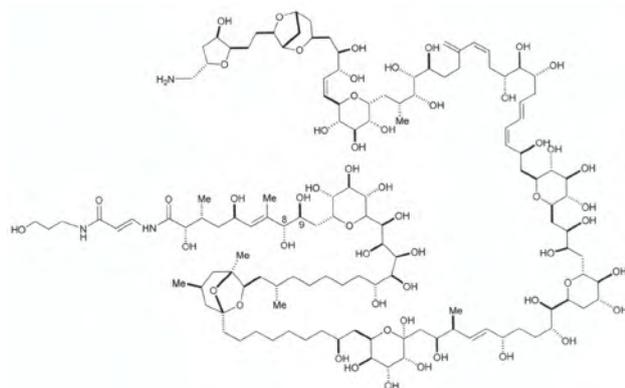
MS experiments were performed using a PE-SCIEX (Concorde, ON, Canada) API-2000 triple-quadrupole mass spectrometer equipped with a Turbospray® source, coupled to an Agilent (Palo Alto, CA, USA) model 1100 LC. A 3- $\mu$ m hypersil C8 BDS (50  $\times$  2.1

mm) column and a 5- $\mu\text{m}$  gemini C18 (150  $\times$  2.0 mm) column (Phenomenex, Torrance, CA, USA) were employed. The following key parameters were investigated: type of organic modifier, buffer and acid character, and acid concentration. The final conditions recommended for routine operation are the following: the 5- $\mu\text{m}$  gemini C18 (150  $\times$  2.00 mm) column eluted at 0.2 mL min<sup>-1</sup> with water (A) and 95% acetonitrile/water (B), both eluents containing 30 mM acetic acid (gradient: 20-100 % B over 10 min and hold 4 min). MS detection was carried out in positive ion mode. Selected ion monitoring (SIM) experiments were carried out using a turbogas temperature of 300 °C, an ionspray voltage of 5500 V, a declustering potential (DP) of 8 V, a focusing potential of 350 V, and an entrance potential of 11 V. A collision energy of 50 eV and a cell exit potential of 10 V were used in multiple reaction monitoring (MRM) experiments. The bi-charged ion at m/z 1340 and the tri-charged ion at m/z 912 were monitored in SIM experiments (500 ms, dwell time) and the transitions 1340  $\rightarrow$  327 (DP = 8 V) and 912  $\rightarrow$  327 (DP = 50 V) were monitored in MRM experiments (dwell time = 500 ms). The presence of okadaic acid (OA), dinophysistoxin-1 (DTX-1), 13-desmethyl-C spirolide, azaspiracids (AZA-1, AZA-2, and AZA-3), yessotoxin (YTX), brevetoxins (PbTx-2 and PbTx-9), paralytic shellfish poisoning (PSP) toxins and domoic acid (DA) was also investigated.<sup>2-4</sup>

## Results and Discussion

### Optimization of mass spectrometry

The full scan mass spectrum (FS-MS) of palytoxin dissolved in acetonitrile-water (1:1, v/v) with 2 mM ammonium formate and 50 mM formic acid was dominated by the presence of: i) a major cluster of ions in the mass range m/z 1240-1370; ii) a cluster of ions in the range m/z 900-920; and iii) a dominant ion at m/z 327. Palytoxin has a molecular weight of 2680 Da. Thus, the ions at m/z 1368, 1360, 1352, 1340 could be assigned to the bi-charged ions  $[\text{M}+\text{K}+\text{NH}_4]^{2+}$ ,  $[\text{M}+\text{K}+\text{H}]^{2+}$ ,  $[\text{M}+\text{Na}+\text{H}]^{2+}$ , and  $[\text{M}+2\text{H}]^{2+}$ , respec-



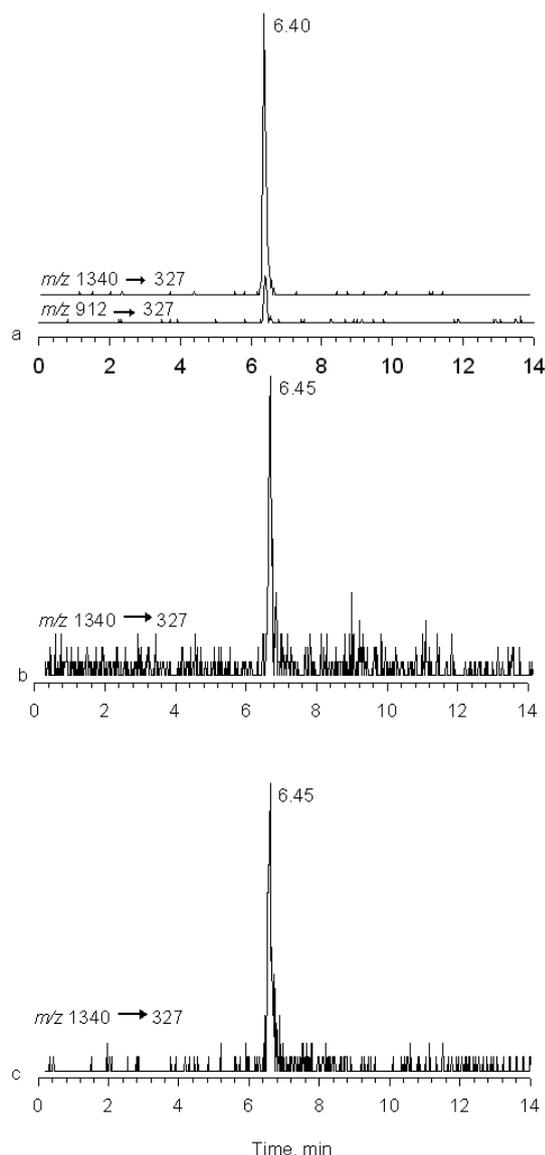
**Figure 1.** Structure of palytoxin.

tively. Multiple losses of water molecules (1-10 H<sub>2</sub>O) from the  $[\text{M}+2\text{H}]^{2+}$  ion produced the ions at m/z 1332, 1323, 1314, 1305, 1296, 1287, 1278, 1269, 1260, and 1251. The ions at m/z 912, 906 and 900 could be assigned to the tri-charged  $[\text{M}+3\text{H}+3\text{H}_2\text{O}]^{3+}$ ,  $[\text{M}+3\text{H}+2\text{H}_2\text{O}]^{3+}$ , and  $[\text{M}+3\text{H}+\text{H}_2\text{O}]^{3+}$  ions, respectively. The base peak at m/z 327 could arise from the cleavage between C8 and C9 and the additional loss of a molecule of water (Tan and Lau 2000), which occurred in the ion source under the conditions used. The ions at m/z 1340,  $[\text{M}+2\text{H}]^{2+}$  and 912,  $[\text{M}+3\text{H}+3\text{H}_2\text{O}]^{3+}$  were chosen for SIM experiments. All source parameters were further optimized by repeated flow injections of palytoxin standard. The above ions were also selected as precursors for MS/MS product ion scan experiments. The fragmentation pattern contained an intense ion at m/z 327, whatever precursor ion was used. Thus, the following transitions at m/z 1340  $\rightarrow$  327 and 912  $\rightarrow$  327 were selected for MRM experiments. The collision energy, the cell exit potential and the collision gas settings were optimized to achieve the highest sensitivity.

### Optimization of chromatography

To set up chromatographic conditions for determination of palytoxin, we first considered the LC-MS method by Lenoir *et al.* (2004) for determination of palytoxin as well as the system proposed by Quilliam *et al.* (2001) for the comprehensive analysis of a wide range of phycotoxins. In both cases a hypersil C8 BDS column was used. Unfortunately, under the proposed LC conditions, palytoxin response was very poor: the chromatographic peak was broad and showed significant tailing.

A number of eluting systems were then tested on the hypersil column. Particular attention was paid to character and percentage of organic modifier and buffer. Both acetonitrile and methanol were tested as possible organic modifiers and the former was preferred for providing a sharper peak and shorter retention time. The use of an acidic mobile phase (pH 2.5-3.2) without addition of ammonium salts was preferred since it allowed improvement of signal intensity in positive ion mode. Both formic acid and acetic acid were tested at different concentrations in the range 10-50 mM. The best results in the signal to noise ratio for all the transitions monitored were achieved by using 20 mM formic acid. A different stationary phase, namely a gemini C18 column, was also considered and its performance compared to that of the hypersil column. Optimization experiments were performed as outlined above. The gemini C18 column provided better results than the hypersil C8 BDS in terms of both sensitivity and peak shape when using water as



**Figure 2.** LC-MS analyses in positive MRM mode of (a) a 1- $\mu\text{g}/\text{mL}$  standard solution of palytoxin, (b) pellet, and (c) butanol extracts from a plankton sample dominated by *Ostreopsis ovata*.

eluent A and acetonitrile/water (95:5, v/v) as eluent B, both containing 30 mM acetic acid. A gradient elution (20-100 % B over 10 min and hold 4 min) allowed determination of palytoxin in a 15-min analysis, as shown for a 1- $\mu\text{g}/\text{mL}$  standard solution of palytoxin in Fig. 2a.

#### *Application to plankton analysis: the Genoa 2005 outbreak*

In late July 2005, the respiratory distress observed in humans was concurrent with a massive bloom of *O. ovata* along the Genoa coasts and disappeared when the algal population declined.

The plankton sample ( $9 \times 10^5$  cells) was centrifuged to obtain a pellet and a supernatant that were extracted separately. Both extracts caused mice to die

within 30 min. Thus, they were directly analyzed by the newly developed method. The presence of peaks at 6.45 min for the transitions  $m/z$  1340  $\rightarrow$  327 and 912  $\rightarrow$  327, which matched perfectly in retention time, fragmentation and ion ratios those of a reference sample injected under the same experimental conditions, indicated that putative palytoxin was contained in both pellet (Fig. 2b) and butanol (Fig. 2c) extracts at levels of 1.35  $\mu\text{g}$  and 1.95  $\mu\text{g}$ , respectively. This indicated a palytoxin content of 3.6 pg/cell, which matched well with the mouse bioassay results.

To assess whether putative palytoxin was the only toxin responsible for human complaints, the samples were also investigated for the presence of OA, DTX-1, 13-desmethyl-C spirolide, AZA-1, AZA-2, and AZA-3, YTX, PbTx-2 and PbTx-9 by using the method developed by Quilliam *et al.* (2001), as well as for the presence of PSP toxins and DA (Ciminiello *et al.* 2005; Dell'Aversano *et al.* 2005). None of the above toxins was detected in the samples. The results obtained suggested that putative palytoxin could be inferred as the cause of the Genoa 2005 outbreak. Since palytoxin has been recognized as the causative agent of clupectoxism (Tan and Lau 2000), a lethal human seafood poisoning following consumption of contaminated sardines and herrings, our findings represent a warning for palytoxin and its analogues as toxins that need to be carefully monitored in Italian seas and seafood to prevent clupectoxism outbreaks.

#### Acknowledgement

This work is a result of a research supported by EU project BIOTOXmarin 2005.

#### References

- Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Quilliam, M.A., Tubaro, A. & Poletti, R. (2005). Rapid Comm. Mass Spectrom. 19: 2030-2038.
- Dell'Aversano, C., Hess, P. & Quilliam, M.A. (2005). J. Chromat. A 1081: 190-201.
- Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.P., Bernard, C. & Hennion, M. C. (2004). J. Phycol. 40: 1042-1051.
- Quilliam, M. A., Hess, P. & Dell'Aversano, C. (2001). In: Mycotoxins and Phycotoxins in Perspective at the Turn of the Millennium, deKoe, W.J., Samson, R.A., Van Egmond, H.P., Gilbert, J. & Sabino, M. (eds), deKoe, W. J., Wageningen, The Netherlands, pp. 383-391.
- Tan, C.H. & Lau, C.O. (2000). In: Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection, Botana, L.M. (ed.), Marcel Dekker Inc., New York, pp. 533-547.

## Application of capillary electrophoresis-mass spectrometry to the determination of lipophilic marine toxins

P. de la Iglesia<sup>1</sup>, A. Gago-Martínez<sup>1</sup> and T. Yasumoto<sup>2</sup>

<sup>1</sup>Departamento de Química Analítica y Alimentaria, Facultad de Química, Campus Universitario, Universidad de Vigo, 36310 Vigo, Spain, anagago@uvigo.es, <sup>2</sup>Japan Food Research Laboratory, Tama Laboratory, 6-11-10 Nagayama, Tama-shi, Tokyo 206-0025, Japan, yasumotot@jfri.or.jp

### Abstract

Capillary electrophoresis (CE) was applied in this work for analysis of yessotoxins as an alternative to the conventional HPLC method. Capillary electrophoresis-mass spectrometry (CE-MS) has the advantage of combining an efficient separation technique with highly selective detection. CE analysis was performed using 10 mM ammonium acetate at pH 8.5 as running buffer, and mass spectrometric detection was carried out using an electrospray ionisation source. The limit of detection achieved was 0.02 µg/mL, which corresponded to 1.25 pg of yessotoxin loaded onto the capillary. Under these conditions, yessotoxin and its analogue 45-hydroxy-yessotoxin were clearly determined by CE-MS in several shellfish samples as well as in marine phytoplankton cultures of *Protoceratium reticulatum*. Additionally, the method was successful for determination of one other lipophilic toxin present in mussel samples such as pectenotoxin-6. The results clearly demonstrate the potential of CE, especially when coupled with MS, as an alternative method for determination of these particular compounds.

### Introduction

Capillary Electrophoresis (CE) is considered one of the most efficient separation techniques and offers several advantages over other separation techniques such as minimal sample and solvents consumption. CE coupled with mass spectrometry (CE-MS) has gained attention in the last years for both food and environmental analysis (Simó *et al.* 2005; Klampfl 2006). In this work, CE-MS is being applied for the analysis of yessotoxins (YTXs) as an alternative to the conventional LC-MS which is the most common approach used for this purpose. Moreover, the methodology presented has been also applied for the determination of other lipophilic toxins such as pectenotoxins (PTXs), and the ability of CE-MS to be applied for the analysis of YTXs and PTXs present in different matrices is presented in this work.

### Materials and Methods

#### *Chemicals and materials*

Research grade analytical standard of YTX was provided by Dr. Paul McNabb from Cawthron Institute (Nelson, New Zealand). Research-grade analytical standard of 45-hydroxy-yessotoxin (45-OHYTX) and pectenotoxin-6 (PTX-6) were provided by Prof. Takeshi Yasumoto as well as crude extracts of mussels naturally contaminated with YTXs and PTXs from Japan. Phytoplankton cultures of *Protoceratium reticulatum* (CAWD40) were kindly provided by Dr. Kevin James from Cork Institute of Technology (Cork, Ire-

land) and Dr. A. Lincoln MacKenzie from Cawthron Institute (Nelson, New Zealand). Stock standard solutions of YTXs and PTX-6 were prepared in methanol and stored at -18 °C for further analysis. HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). HPLC-grade 2-propanol and hexane were obtained from Scharlau (Sentmenat, Spain) and Panreac (Barcelona, Spain), respectively. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). NH<sub>3</sub> 25 % and ammonium acetate for analysis ACS-ISO were from Panreac (Barcelona, Spain).

#### *Sample preparation*

Extraction and clean-up conditions previously described by Yasumoto *et al.* (1997) were slightly modified. One gram of shellfish hepatopancreas homogenate was extracted with 9 mL of MeOH/H<sub>2</sub>O (8:2, v/v) solvent. The supernatant was obtained after 3 min homogenization using Ultra-Turrax T25 (IKA-Labortechnik, Germany) and 10 min centrifugation at 825 × g. After extraction, the crude extract was washed twice with 5 mL of hexane. For phytoplankton cultures, aqueous aliquots (12-14 mL) were sonicated with a VibraCell™ ultrasonic homogenizer (Sonics & Materials, Danbury, CT, USA) for 3 min in a cold bath to release YTXs from cells to the culture media. The extracts were filtered through Millex®-HV 0.45-µm syringe filters (Millipore Corporation, Bedford, MA, USA) and subsequently submitted to clean-up procedure. SPE clean-up followed for shellfish sam-

ples was described by Gago-Martínez *et al.* (in press). The clean-up procedure for algal cultures was similar to that described for shellfish except that 10 mL crude extract was mixed with 10 mL phosphate buffer for SPE loading.

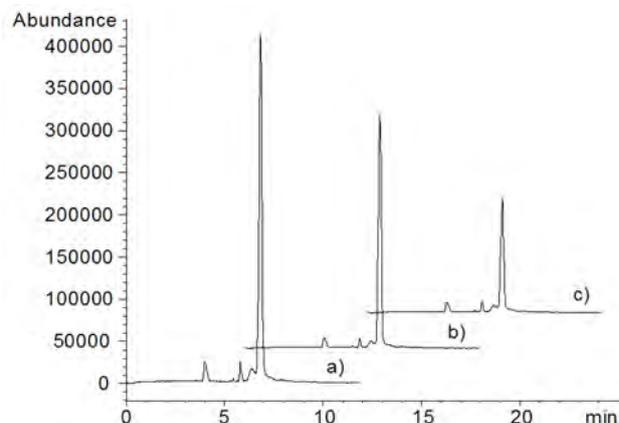
#### Capillary electrophoresis-mass spectrometry

CE-MS analyses were performed on an Agilent<sup>3D</sup>CE system (Waldbronn, Germany). An Agilent coaxial sheath-flow CE-ESI-MS interface was used to connect the CE system to the Agilent 1100 series single quadrupole mass spectrometer. A binary pump Agilent 1100 series equipped with a 1:100 splitter delivered the sheath flow. Agilent<sup>3D</sup>Chemstation was used for the entire instrument control, data acquisition and data analysis. All separations were performed using uncoated fused-silica capillaries with a total length of 80.0 cm and 50  $\mu$ m ID from Composite Metal Services (The Chase, Hallow, UK). The capillary temperature was set at 25 °C. The background electrolyte (BGE) consisted of 10 mM ammonium acetate adjusted at pH 8.5. It was freshly prepared daily, filtered through Millex®-HV 0.45- $\mu$ m membranes (Millipore, Bedford, MA, USA) and degassed before use. An on-line sample pre-concentration approach based on field-amplified sample stacking (Osborn *et al.* 2000) was applied to increase the sensitivity. Samples prepared in methanol/background buffer (7:3) solution were loaded hydrodynamically by application of 50 mbar pressure for 20 s, then stacked and separated by application a running voltage of 20 kV.

#### Results and Discussion

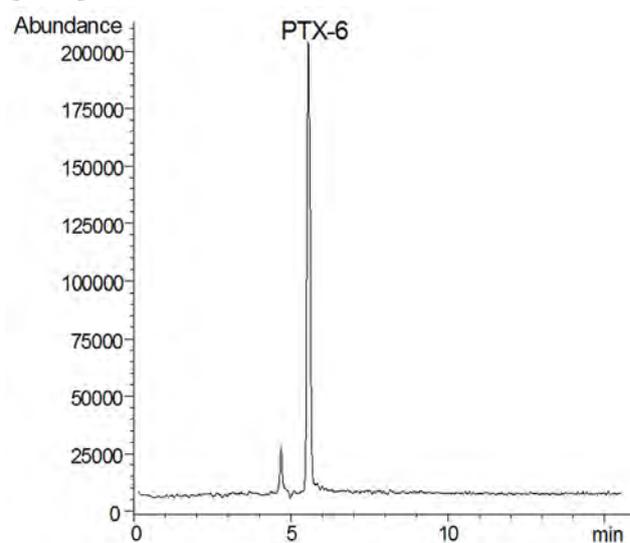
Previously to CE-MS coupling, flow injection analysis (FIA)-mass spectrometric experiments were carried out on the yessotoxin standard solution (12  $\mu$ g/mL) at 400  $\mu$ L/min. Full-scan mass spectra acquired in negative mode from 50 to 1200 m/z for YTX standard gave majority signals at m/z 1163, 1141 and 1061, which corresponded to  $[M-Na]^-$ ,  $[M-2Na+H]^-$  and  $[M-2Na-SO_3+H]^-$  respectively (Murata *et al.* 1987; Draisci *et al.* 1998). However, the ion  $[M-2Na]^{-2}$  at m/z 570 was not observed for the experimental conditions. MS parameters were adjusted and the optimised instrument settings were as follows: capillary voltage 4500 V, fragmentor 275 V, gain 3.0, bandwidth 0.30, high-resolution mode. Sheath-liquid consisted of a 2-propanol/water (80:20) solution with 0.1 mM ammonium acetate which was pumped toward the ESI interface at 7  $\mu$ L flow rate. The sheath flow performs two functions: the first is to supplement the CE flow to a level adequate for electrospray operation and the second is to close the electrical circuit. ESI source

conditions were also examined and optimized for drying gas flow and temperature (nitrogen, 4L/min, 350 °C) and nebulizer pressure (17 psi). Molecular ions at 1141 and 1157 m/z were chosen for monitoring YTX and its major analog 45-OHYTX, respectively, in selected ion monitoring (SIM) mode.



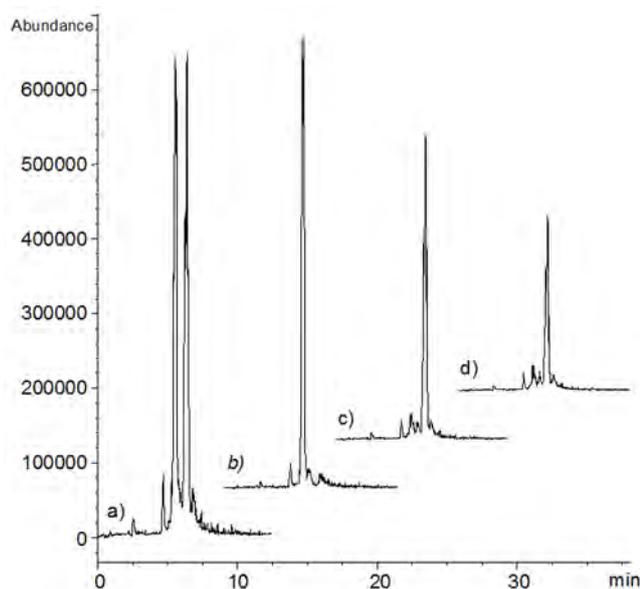
**Figure 1.** CE-MS analysis of a YTX standard mixture containing 6  $\mu$ g/mL of YTX and 3  $\mu$ g/mL of 45-OHYTX. a) total ion electropherogram (TIE), b) extracted ion electropherogram (EIE) at 1141 m/z and c) EIE at 1157 m/z. Conditions described in text.

Fig. 1 shows an electropherogram obtained for a CE-MS analysis of a YTX standard mixture where the high performance and fast analysis provided by this technique can be observed. Despite complete resolution was not achieved on capillary between YTX and 45-OHYTX, it could be reached by means of a mass filter. CE-MS conditions initially developed for YTX analysis were also applied for PTX determination. Fig. 2 shows a CE-MS analysis of a PTX-6 standard solution of 20  $\mu$ g/mL in which the molecular ion  $[M-H]^-$  at 887 m/z was added for SIM mode.



**Figure 2.** CE-MS analysis of a PTX-6 standard solution of 20  $\mu$ g/mL. Conditions described in text.

CE-MS was evaluated for YTX analysis. It showed good linearity ( $r = 0.9985$ ) over the calibration range (0.25-6.0  $\mu\text{g/mL}$ ). LOD estimated on the basis of a  $S/N = 3$  criterion was 0.02  $\mu\text{g/mL}$  for YTX, which corresponded to 1.25 pg loaded onto capillary.



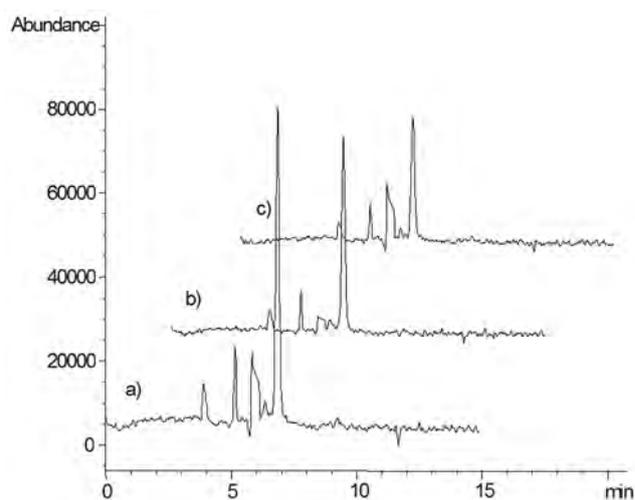
**Figure 3.** CE-MS analysis of a digestive gland blue mussel sample from Japan naturally contaminated with lipophilic toxins. a) total ion electropherogram, b) extracted ion electropherogram (EIE) at 887  $m/z$ , c) EIE at 1141  $m/z$  and d) EIE at 1157  $m/z$ . Conditions described in text.

The high efficiency provided by CE as well as the on-line pre-concentration approach applied allowed to reach a LOD similar to that achieved in LC-MS with much lower mass consumption. Run-to-run and day-to-day precision for migration times were 3.9 and 4.1 % RSD, respectively. Higher values were found for peak areas (4.7 and 7.5 % RSD, respectively) although they were lower than 10 % RSD in all cases. Therefore CE-MS can be considered for quantitative purposes.

Fig. 3 and 4 show the application of the optimized conditions to determination of lipophilic toxins present in different samples naturally contaminated. The efficiency of CE as an alternative for this particular application, independent of the matrix, as well as the ability of MS for confirmation purposes, are clearly demonstrated in this work.

### Conclusions

Capillary electrophoresis resulted in a fast, sensitive and efficient alternative for the analysis of lipophilic toxins present in shellfish and phytoplankton samples. The potential of CE was significantly enhanced with the confirmation power provided by mass spectrom-



**Figure 4.** CE-MS analysis of phytoplankton culture sample of *Protoceratium reticulatum* from Ireland containing YTXs. a) total ion electropherogram, b) extracted ion electropherogram (EIE) at 1141  $m/z$ , c) EIE at 1157  $m/z$ . Conditions described in text.

etry detection, although further work should be taken into account to improve toxin resolution on capillary.

### Acknowledgements

Dr. Paul McNabb for providing YTX standard for this study as well as Dr Kevin J. James and Dr. Lincoln MacKenzie for providing phytoplankton samples. A doctoral fellowship of P. de la Iglesia was provided from EU project EU-011-01.

### References

- Chien, R.-L. & Burgi, D. S. (1992). *Anal. Chem.* 64: 1046-1050.
- Draisci, R., Giannetti, L., Lucentini, L., Ferretti, E., Palleschi, L. & Marchiafava, C. (1998). *Rapid Commun. Mass Spectrom.* 12: 1291-1296.
- Gago-Martínez, A., de la Iglesia, P., Vaquero, E., Leão, J.M. & Rodríguez-Vázquez, J.A. (2006). In: *Mycotoxins and Phycotoxins: Advances in Determination, Toxicology and Exposure Management*, H. Njapau, S. Trujillo, H.P. van Egmond & D.L. Park (eds), Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 309-320.
- Klampfl, C. W. (2006). *Electrophoresis* 27: 3-34.
- Murata, M., Kumagai, M., Lee, J. S. & Yasumoto, T. (1987). *Tetrahedron Lett.* 28: 5869-5872.
- Osborn, D.M., Weiss, D.J. & Lunte, C.E. (2000). *Electrophoresis* 21: 2768-2779.
- Simó, C., Barbas, C. & Cifuentes, A. (2005). *Electrophoresis* 26: 1306-1318.
- Yasumoto, T. & Takizawa, A. (1997). *Biosci. Biotechnol. Biochem.* 61: 1775-1777.

# LC-MS/MS determination of Paralytic Shellfish Poisoning (PSP) in seafood by application of a new hydrophilic interaction liquid chromatographic (HILIC) column

M. Diener and B. Luckas

Friedrich-Schiller-University of Jena, Department of Food Chemistry, Dornburger Str. 25, D-07743 Jena (Germany), marc.diener@uni-jena.de

## Abstract

Paralytic shellfish poisoning (PSP) is caused by approximately two dozen naturally occurring potent neurotoxins. The quality control of potentially contaminated seafood requires exact quantification of the PSP toxins with regard to international regulations for public health protection and international commerce. The broad toxicity range of different PSP toxins necessitates accurate and reliable analytical methods. The newly developed LC-MS/MS method is based on chromatographic separation of the PSP toxins using a zwitterionic (ZIC)-HILIC column. The stationary phase allows complete separation of all relevant PSP toxins in seafood. Low concentration of non-volatile buffer substances and absence of ion-pair reagents increase the sensitivity of the MS detection.

## Introduction

Paralytic shellfish poisoning (PSP) toxins (Fig. 1) are biosynthesized by marine dinoflagellates of the genus *Alexandrium*, *Gymnodinium* and *Pyrodinium*. In addition, some freshwater cyanobacteria such as *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii* and *Anabaena circinalis* can also produce PSP toxins. The toxins induce dangerous intoxications by acting as potent sodium channel blockers. The PSP toxins cause persistent problems due to their accumulation in shellfish. Therefore, methods for quantitative PSP analysis must be available to control seafood (Luckas 2000).

Present chemical methods are mostly based on ion-pair LC and complicate the on-line coupling to a mass spectrometer. The mobile phases contain alkyl-sulfonic acids such as ion-pair reagents, which cause suppression effects of the ionization of the PSP toxins (Diener *et al.* 2006). To solve this problem Lagos *et al.* (1999) used heptafluorobutyric acid, a volatile ion-pair reagent, for the separation of the PSP toxins. The method of Lawrence *et al.* (2005) is based on pre-chromatographic oxidations (hydrogen peroxide and periodate) of the PSP toxins, but it is not sensitive enough for controlling seafood by LC-MS/MS (Quilliam *et al.* 1993). Furthermore, hydrophilic interaction liquid chromatographic (HILIC) methods using TSK-gel Amide-80 as the stationary phase separate the PSP toxins only in groups such as gonyautoxin (GTX) 1/2 and GTX 3/4. In addition, inconstant retention times for PSP toxins in different seafood matrices were obtained (Aversano *et al.* 2005). As a consequence, the

development of a new method is urgently needed that will allow complete separation of all PSP toxins of relevance to seafood regulation using one chromatographic run followed by selective and sensitive quantification by mass spectrometric detection.

## Materials and Methods

PSP standards of GTX 1-4, decarbamoyl-gonyautoxin (dcGTX) 2/3, N-sulfocarbamoyl-gonyautoxin 2 and 3 (C1 and C2), neosaxitoxin (NEO), saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX) and N-sulfocarbamoyl-saxitoxin (B1) were purchased from the National Research Council, Marine Analytical Chemistry Standards Program (NRC-CRM), Institute for Marine Biosciences, Halifax, Nova Scotia, Canada. LC-MS/MS was performed using the following equipment: SIL-10ADvp intelligent autosampler, LC-10AT pumps, SCL-10Avp system controller, CTO 10ASvp column oven (Shimadzu, Duisburg, Germany), triple quadrupole API 365 PE Sciex mass spectrometer equipped with turbo ion spray (PE Sciex, Halifax, Canada). HILIC was performed with two eluents. Eluent A consisted of 10 mM ammonium formate and 10 mM formic acid in 100 % aqueous solution. Eluent B contained 80 % acetonitrile and 20 % water with 5 mM ammonium formate and 2 mM formic acid. The separation was accomplished by application of a 5- $\mu$ m zwitterionic HILIC column (250 x 4.6 mm, SeQuant, Haltern, Germany). A gradient formation system was used starting with 25 % A and 75 % B adjusting to 65 % B within 5 min; from 65 % B to 60 % B in 5 min; followed by isocratic elution from 10 to 20 min with

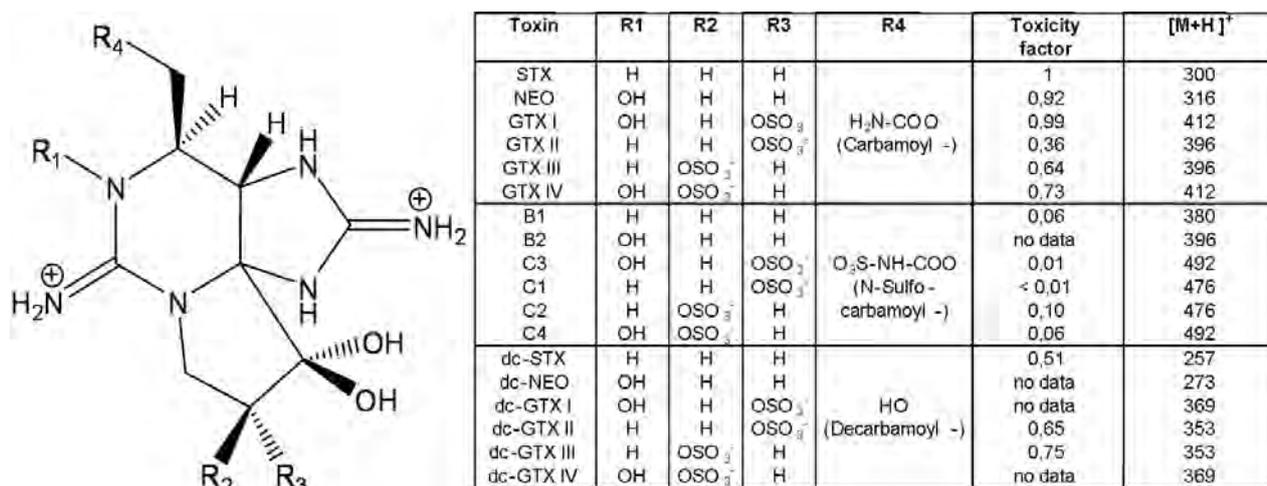


Figure 1. Chemical structure of PSP toxins.

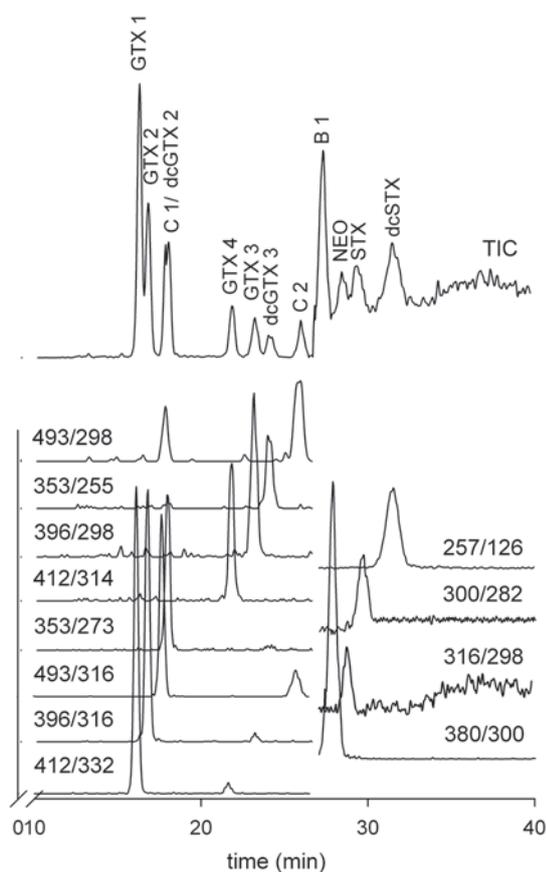


Figure 2. HILIC-MS/MS analysis of a PSP standard mixture containing GTX1 (6.6 ng), GTX2 (3.45 ng), C1 (4.2 ng), dcGTX2 (3.0 ng), GTX4 (2.1 ng), GTX3 (1.16 ng), dcGTX3 (0.84 ng), C2 (6.0 ng), B1 (4.9 ng), NEO (7.65 ng), STX (4.2 ng), and (dcSTX 4.8 ng). Experiments for time period 1 and 2 were carried out in SRM mode with an API 365 mass spectrometer.

PSP toxins	Retention time (min)
GTX 1	16.15 ± 0.05
GTX 2	16.65 ± 0.05
C 1	17.7 ± 0.75
dcGTX 2	17.9 ± 0.1
GTX 4	21.70 ± 0.1
GTX 3	23.03 ± 0.05
dcGTX 3	24.0 ± 0.1
C 2	25.7 ± 0.75
B 1	27.1 ± 0.1
NEO	28.25 ± 0.05
STX	29.2 ± 0.08
dcSTX	31.3 ± 0.08

Figure 3. Retention times for different PSP toxins analyzed by HILC-MS/MS.

60 % B. After 20 min re-equilibration with 80 % B (5 min), the run stopped at 40 min. The main flow rate was 0.7 mL/min and the mobile phase was transferred into MS using a split ratio of 1:1 (volume wasted/volume transferred). MS detection was carried out by selected reaction monitoring (SRM) and positive ion mode.

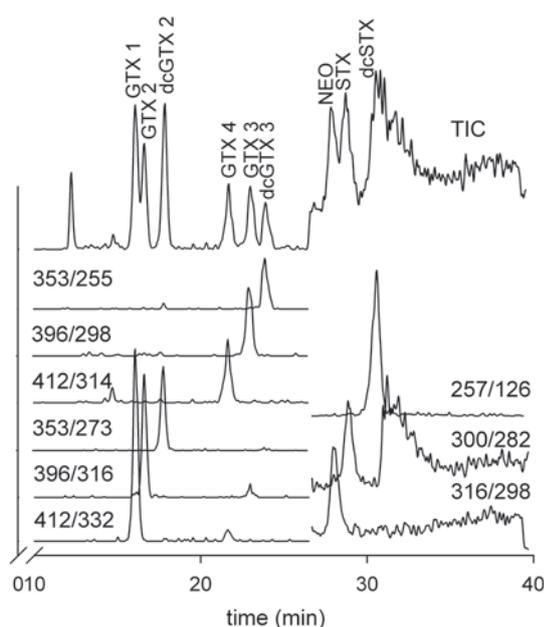
## Results

The method described above was developed for exact quantification of individual PSP toxins present in complex matrices such as seafood with regard to international regulations. A ZIC-HILIC column was applied to separate all regulation relevant PSP toxins in one chromatographic run (Fig. 2).

The PSP toxins were eluted in the order GTX1, GTX2, dcGTX 2, C1, GTX4, GTX3, dcGTX3, C2, B1, NEO, STX and dcSTX. (Fig. 3). Five-point calibration curves and statistical information for the mass

Toxin	$m/z > m/z$	Amount on column (ng)	Calibration equation (5 data points)	Correlation	LOD (ng)
GTX 1	412 > 332	4.4 – 13.2	$y = 2930x + 2230$	0.995	0.1
GTX 2	396 > 316	2.3 – 6.9	$y = 3610x + 985$	0.995	0.1
GTX 3	396 > 298	0.77 – 2.31	$y = 3640x - 92$	0.994	0.08
GTX 4	412 > 314	1.4 – 4.2	$y = 2160x - 600$	0.994	0.1
dcGTX 2	353 > 273	2.0 – 6.0	$y = 4070x - 1100$	0.999	0.03
dcGTX 3	353 > 255	0.56 – 3.2	$y = 2330x + 358$	0.990	0.07
NEO	316 > 298	5.13 – 15.4	$y = 682x + 1820$	0.995	0.6
dcSTX	257 > 126	3.2 – 9.6	$y = 2930x - 811$	0.997	0.3
STX	300 > 282	2.87 – 8.61	$y = 1570x + 545$	0.999	0.5

**Figure 4.** LOD (signal/noise (S/N) = 3) and equation of calibration curve for different amounts of PSP toxins analyzed by HILIC-MS/MS (SRM mode).



**Figure 5.** HILIC-MS/MS analysis of a PSP-free mussel extract (0.2 M HCl) spiked with a PSP standard mixture.

spectrometric detection are presented in Fig. 4. The retention times of each PSP toxin were stable for different sample matrices, e.g. mussel (Fig. 5). The selective MS detection of all relevant PSP toxins by application of SRM mode reduced the time of one analytical run to 40 min.

### Conclusions

The present HILIC method enables the separation of all relevant PSP toxins in one LC run. In addition, the

ZIC-HILIC column produces stable retention times for each PSP toxin in different sample matrices. The mobile phase contains a high amount of organic solvent and a low volatile buffer concentration without ion-pair reagents resulting in an improved sensitivity of the MS detection.

By application of an optimized gradient elution the new developed HILIC-MS/MS method allows complete separation of the carbamate and decarbamoyl toxins. Therefore, the same ZIC-HILIC column can also be used for control of seafood regarding PSP contamination by application of an HPLC device with post column oxidation unit and fluorescence detector.

### References

- Aversano, C.D., Hess, P. & Quilliam, M.A. (2005). *J. Chromatogr A*. 1081: 190-201.
- Diener, M., Erler, K., Hiller, S., Christian, B. & Luckas, B. (2006). *Eur. Food Res. Technol.* 224: 147-151.
- Lagos, N., Ondera, H., Zagatto, P.A., Andrinolo, D., Azevedo, S. & Oshima, Y. (1999). *Toxicon* 37: 1359-1373.
- Lawrence, J.F., Niedzwiadek, B. & Menard, C.J. (2005). *AOAC Int.* 88: 1714-1727.
- Luckas, B. (2000). In: *Seafood and Freshwater Toxins, Pharmacology, Physiology and Detection*, Botana, L.M. (ed.), Marcel Dekker Inc., New York, Basel, pp. 173-186.
- Quilliam, M.A., Janecek, M. & Lawrence, J.F. (1993). *Rapid Commun. Mass Spectrom.* 6: 14-24.

## Development of Real-Time PCR assays for the detection of *Cylindrospermopsis raciborskii*

S. Fuentes, H.J. Rick, P. Scherp, A. Chistoserdov and J. Noel

University of Louisiana at Lafayette, P.O. Box 42451, Lafayette, LA 70504-2451 and fsm2335@louisiana.edu, hansrick@louisiana.edu, peter@scherp.info, ayc6160@louisiana.edu, jodiecasey@yahoo.com

### Abstract

Blooms caused by toxic blue-green algae have repeatedly produced episodes of wild and domestic animal illness and death. The responsible cyanobacteria belong to about 40 genera including the most recently detected toxin-producing cyanobacterium, *Cylindrospermopsis*. Since 2002, *Cylindrospermopsis raciborskii* has been abundant in water samples of the Caernarvon Breton Sound estuary (Louisiana, USA), posing a potential risk of harmful blooms. In October 2004, the species was abundant in the entire Atchafalaya Basin. Monitoring these areas for the presence of *Cylindrospermopsis* is essential in assessing the potential for bloom formation. Microscopic detection and quantification of *Cylindrospermopsis* is problematic, as it is not easily distinguished from suspended sediment or other types of cyanobacteria. Therefore, we developed a real-time PCR assay for the quantification of *Cylindrospermopsis raciborskii*. In this assay, detection of amplified target DNA requires annealing of fluorescent-labelled probes, resulting in higher specificity compared to assays using traditional PCR methodology. Using this sensitive technique, it is possible to detect even low densities of this species. These data allow for the creation of real-time species abundance distribution maps, enabling an immediate response to increases in biomass of this harmful species and for timely notification of possible health risks to the public.

### Introduction

Due to its potential toxicity (Humpage *et al.* 2000), its ability to form dense blooms interfering with several kinds of water use and its invasive behaviour at midlatitudes, *Cylindrospermopsis raciborskii* is rated one of the most notorious cyanobacterial species (Briand *et al.* 2004). Its toxin, cylindrospermopsin, is a potent inhibitor of protein synthesis (Ohtani *et al.* 1992). It damages a range of organs, especially liver and kidney. Additionally there is growing apprehension about its role in gastrointestinal and liver cancer with long-time low-level exposure (Falconer 2005). Florida's eutrophic lakes and rivers, which are used frequently as drinking water supplies, often support very large *Cylindrospermopsis* populations (Chapman and Schelske 1997). Adjacent states have not been screened for this species but it might exist throughout the higher rainfall areas of the southern U.S. (Falconer 2004). Here we document the recent records of *C. raciborskii* from coastal Louisiana and introduce a newly developed real-time PCR assay for rapid detection of the species.

### Material and Methods

#### *Collection of Cylindrospermopsis samples*

Based on a 20-station grid, the Caernarvon Breton Sound estuary was surveyed monthly 2002-2004. Additionally, 39 stations were sampled in fall 2004 in the

Atchafalaya Basin. Plankton samples were stored in acid-cleaned PE bottles and fixed with formaldehyde (Thronsen 1978) for subsequent quantification of *C. raciborskii* using the inverted microscope method (Lund *et al.* 1958; Utermöhl 1958).

#### *Real-Time PCR assay*

A toxic strain of *C. raciborskii* (CCMP 1973) was obtained from Provasoli-Guillard culture center. It was grown in DY-V medium at 22 °C under a 16:8 L:D cycle. Cells collected from early stationary phase were stained with DAPI fluorescent dye and observed by UV fluorescence microscopy to determine the length to width ratio. Filaments were counted and measured in Sedgewick rafter chambers. Filament numbers were converted to cell numbers using the previously determined factor.

DNA extraction was performed using a modified protocol from Xu and Tabita (1996). In short, cells were ruptured by enzyme digestion coupled with a freeze thaw cycle. The DNA was purified using a modified phenol chloroform extraction followed by RNase A treatment. The purified DNA was then quantified using a Hoechst fluorometry protocol. DNA quantity was divided by the corresponding cell number to give an estimate of DNA content per cell.

Because there is only one copy per cell, the *nifH* gene, which is involved in nitrogen fixation, was used as a target for detection of *C. raciborskii*. We designed a

set of primers [forward primer (5'-attgcacttgctgctgagaaagg-3'), reverse primer (5'-ataccacgaccag-cacaacctaca-3')] to amplify this gene (amplicon length 135 bp). The specificity of the primers was preliminarily assessed by performing a BLAST database search ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) for DNA sequences. The correct primer concentration was defined using a combination of three different concentrations (250, 500 and 1000 nM) of each primer with a fixed amount of target template (232 ng) and using SYBR green I as the reporter dye. (SYBR-green master mix, Eurogentec, USA). The probe (5'-FAM-aagaagtaatgctggccggattccgt-TAMRA-3') concentration was optimized for the real-time PCR assay using a matrix of three different concentrations (50, 250 and 500 nM) with the previously optimized primer concentrations. All probe-based assays were carried out using PCR Mastermix for Probe Assays – No ROX (Eurogentec). The efficiency of the reaction was obtained from the slope of the standard curve of a ten-fold dilution series (185-0.185 ng of DNA per reaction) of template. All real-time PCR reactions were carried out on an ICycler IQ (Biorad, USA). The thermal cycling conditions were 10 min denaturation at 95 °C, 50 cycles of 15 s, denaturation at 95 °C, 20 s of annealing at 58 °C and 60 s of extension at 72 °C, followed by 30 s elongation at 55 °C.

## Results and Discussion

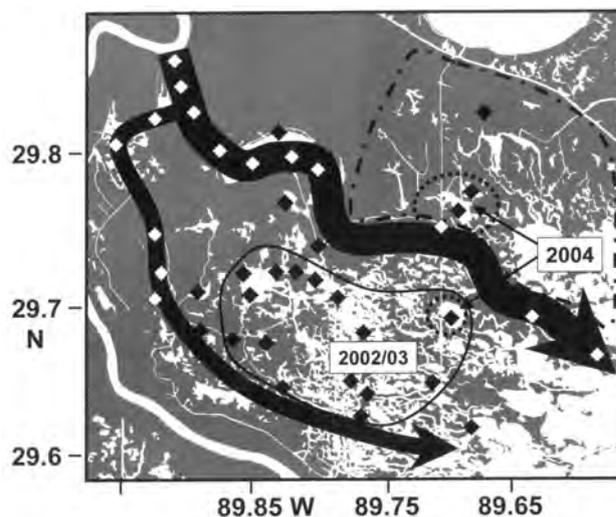
On 16 July 2002, *C. raciborskii* was detected for the first time in Louisiana. It was identified in a phytoplankton sample from the Caernarvon Breton Sound estuary (Grand Lake), which is a major area of re-introduction of river water into a Mississippi delta coastal basin (Day *et al.* 2003). Cell density in the sample was  $3 \times 10^6$  cells L<sup>-1</sup>. Since this first occurrence, the species was frequently encountered in the estuary during summer and fall 2002 -2004 at elevated water temperatures (>27 °C). The highest densities (max.  $24.5 \times 10^6$  cells L<sup>-1</sup>) were detected in Grand Lake on 10 July 2003 and exceeded the threshold level ( $20 \times 10^6$  cells L<sup>-1</sup>) for potential toxicity (McGregor and Fabbro 2000).

The species always occurred in the mid-southern part of the estuary, which is less influenced by the re-introduced Mississippi water (Fig. 1). The water column is here less disturbed and the species' akinetes are more likely to successfully overwinter in the upper sediment (Rick *et al.* in review). During fall 2004, the species was widespread in coastal Louisiana and especially abundant in the Atchafalaya Basin and in the Lake Fausse Pointe region. It was additionally

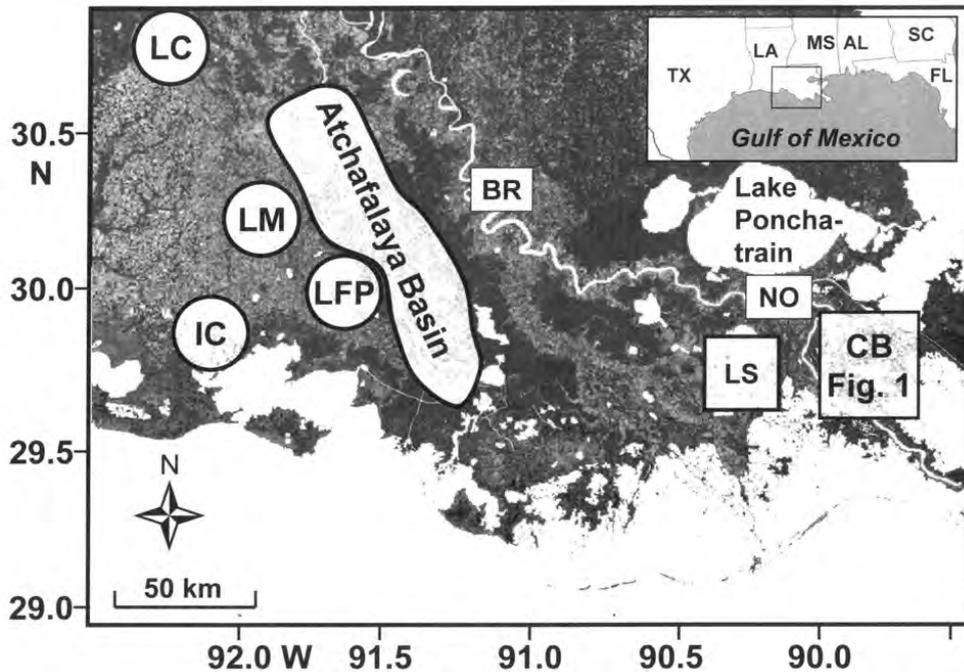
reported from Lafayette (Lake Martin), Ville Plate (Lake Chicot) and Intracoastal City areas. In summer 2003, it occurred in the upper Barataria estuary and in 2004 it was detected again in the lakes Cataouatche, Salvador and Allemands (Rabalais 2005; Ren *et al.* 2005; Fig. 2).

It is not known yet if the Louisiana strain of *C. raciborskii* is toxic. But our results, based on microscopic counting, indicate that the species has reached potentially hazardous densities in some areas, and since cyanotoxins are important environmental contaminants, public health management is dependent on emergency responses to specific poisoning events. Therefore, developing a real-time PCR assay to detect and quantify *C. raciborskii* in field samples is a valuable molecular tool to evaluate the potential for bloom formation and to respond to the situation rapidly to minimize or prevent human exposure to the toxins.

Our results indicate that primers and probe are specific for *C. raciborskii*, as the BLAST analyses revealed no significant hits with other microalgae. However, it is important to confirm the specificity of the assay using DNA from different species of microalgae. The optimal primer and probe concentrations (1000/250 nM forward/reverse primer, 50 nM Taq-Man probe) were determined based on the lowest Ct value detected in the serial dilution. This primer/probe combination successfully amplified DNA extracted from *C. raciborskii*, resulting in a reaction efficiency of ~96%.



**Figure 1.** Caernarvon Breton Sound estuary with stations sampled (2002 – 2004 time series). Black arrows show major paths of the diverted Mississippi water. Dash-dotted areas depict regions not affected by the diversion. Gray area surrounded by the solid line shows the region where *C. raciborskii* was detected in 2002 or 2003. Areas marked by dotted lines show 2004-detect areas.



**Figure 2.** *C. raciborskii* occurrence in southern Louisiana in fall 2004 (LC: Lake Chicot, LM: Lake Martin, LFP: Lake Fausse Pointe, Atchafalaya Basin, IC: Intracoastal City). The Caernarvon Breton Sound estuary (CB) was surveyed monthly from February, 2002. The square (LS) shows the occurrence of the species in the upper Barataria estuary (Rabalais 2005; Ren *et al.* 2005).

### Acknowledgements

Funding was provided by the Governor's Office of Coastal Activities - Governor's Applied Coastal Research and Development Program (GOCA-GACRDP: C190364) and the Water and Watershed Research Program of the EPA/USDA/NSF (EPA Grant Number: R828009). We would like to thank Dr. John Day, Dr. Robert Lane, and Dr. Robert Twilley for providing fieldwork logistics. Special thanks to the colleagues from ULL and LSU who helped with the fieldwork.

### References

- Briand, J.-F., Leboulanger C., Humbert J.-F., Bernard C. & Dufour P. (2004). *J. Phycol.* 40: 231-238.
- Chapman, A.D. & Schelske C.L. (1997). *J. Phycol.* 33: 191-195.
- Day, J.W., Ko, J., Cable, J., Day J.N., Fry B., Hyfield, E., Justic, D., Kemp, P., Lane, R., Mashriqui, H., Reyes, E., Rick, S., Snedden, G., Swenson, E., Templet, P., Twilley, R., Wheelock, K. & Wissel, B. (2003). First Interagency Conference on Research in the Watersheds, Oct. 27-30, US Dept. of Agriculture, Agricultural Research Service.
- Falconer, I.R. (2004). *Cyanobacterial Toxins of Drinking Water Supplies*. CRC Press, Boca Raton.
- Humpage, A.R., Fenech, M., Thoma, P. & Falconer I.R. (2000). *Mutation Research* 472: 155-161.
- McGregor, G.B. & Fabbro, L.D. (2000). *Lakes & Reservoirs: Research and Management* 5: 195-205.
- Ohtani, I., Moore, R.E. & Runnegar M.T.C. (1992). *J. Am. Chem. Soc.* 114: 7941-7942.
- Rabalais, N. (2005). *National Wetlands Newsletter* 27: 21-24.
- Ren, L., Mendenhall, W., Atilla, N., Morrison, W. & Rabalais N.N. (2005). [www.epa.gov/cyano\\_habs\\_symposium/abstracts/causes/poster/ren\\_abstract\\_cyano\\_in\\_lakes\\_in\\_louisiana.pdf](http://www.epa.gov/cyano_habs_symposium/abstracts/causes/poster/ren_abstract_cyano_in_lakes_in_louisiana.pdf).
- Rick, H.J., Rick, S., Fuentes, S. & Noel, J.L. *Gulf of Mexico Sci.* 25: 61-81.
- Thronsdon, J. (1978). In: Sournia, A. (ed.), *Phytoplankton Manual*, United Nations Educational, Scientific and Cultural Organization, Paris.
- Utermöhl, H. (1958). *Mitteilungen Internationale Vereinigung für theoretische und angewandte Limnologie* 9: 1-38.
- Xu, H.H. & Tabita F.R. (1996). *Appl. Env. Microbiol.* 62: 1913-1921.

## ***In vitro* interactions between several species of harmful algae and hemocytes of bivalve molluscs**

Hélène Hégaret<sup>1</sup>, Gary H. Wikfors<sup>2</sup> and Sandra E. Shumway<sup>1</sup>

<sup>1</sup> Department of Marine Sciences, University of Connecticut, Groton, CT 06340, Helene.Hégaret@uconn.edu and Sandra.Shumway@uconn.edu; <sup>2</sup> NOAA-NMFS, Milford, CT 06460, Gary.Wikfors@noaa.gov

### **Abstract**

Harmful algal blooms (HABs) can have noxious and sublethal impacts on shellfish. The northern quahog (= hard clam), *Mercenaria mercenaria*, can experience blooms of several HAB species, including *Prorocentrum minimum*, *Heterosigma akashiwo* and *Alexandrium fundyense*. To understand the possible roles of hemocytes, the immune-defense cells, in bivalve responses to HABs, and how the algal cells are affected by these responses, *in vitro* tests of interactions between those harmful algae and hemocytes of *M. mercenaria* were carried out. Possible differences in hemocyte parameters attributable to harmful algae and also the effect of hemocytes on the algae themselves were measured. Using microscopic and flow-cytometric observations, changes in hematology and physiology, including cell concentration, phagocytosis, adhesion, and oxidative burst response of hemocytes, were determined. Changes in the physiology and the characteristics of the algal cells, including mortality, size, chlorophyll fluorescence and internal complexity, were also determined. The results show a species-specific response of the hemocytes depending upon the harmful algae to which they were exposed. Thus, *in vitro* tests allow a better understanding of the role of the hemocytes and the hemolymph in the defense mechanisms protecting molluscan shellfish from harmful algal cells.

### **Introduction**

Harmful algal blooms (HABs) are increasingly recognized as having profound effects upon the ecology of coastal seas (Burkholder 1998) and upon the economics of fisheries and aquaculture (Shumway 1990; Anderson *et al.* 2000). Bivalve shellfish are routinely exposed to harmful algal blooms (HABs). In these suspension feeders, feeding and respiration are accomplished by the same physiological and behavioural activities of the gills, and thus avoidance of contact with HABs is possible for only short periods of time. Defense mechanisms in molluscan shellfish, protecting tissues from noxious or pathogenic agents, are attributable to an immune system (Cheng 1996) mediated by circulating cells called hemocytes that are similar to white blood cells in vertebrates. As bivalves have an open vascular system, hemocytes can be present everywhere in the body and, thus, come in contact with the harmful algae.

Previous experiments have shown effects of *in vivo* exposure of bivalve shellfish to HABs on hemocyte parameters. Hégaret *et al.* (2006) showed minimal effects of *Alexandrium catenella* on the hemocyte parameters of the oyster *Crassostrea gigas*; whereas, Hégaret and Wikfors (2005 a, b) demonstrated that *Prorocentrum minimum* significantly affects the hemocyte parameters of scallops (*Argopecten irradians irradians*) and oysters (*Crassostrea virginica*), and therefore likely their susceptibility to infection by

parasites or environmental stresses. Immuno-modulation by harmful algae has the potential to increase the susceptibility of bivalves to diseases and parasites, thereby impacting commercial production and ecosystem services of molluscan populations.

Experiments reported here demonstrate the interactions *in vitro* between hemocytes of the hard clam *Mercenaria mercenaria* and three harmful algal species. An understanding of these interactions *in vitro* can help identify the role of the hemocytes in defense mechanisms when bivalves are exposed to HABs in the environment.

### **Materials and Methods**

The algal species to which clams were exposed were obtained from the NOAA, Milford Laboratory (CT USA) collection: *Alexandrium fundyense* (strain BF2), *Prorocentrum minimum* (strain JA-98-01), and *Heterosigma akashiwo* (strain OL). *Rhodomonas* sp. was used as control. Algal species were cultured aseptically in 20-L carboys. Algal sizes varied from about 20 µm diameter for *A. fundyense* and *P. minimum* to 15 µm for *H. akashiwo* and *Rhodomonas* sp., which corresponds to the size of the hemocytes as well.

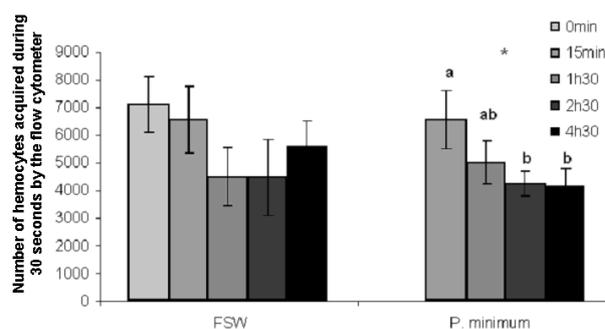
Northern quahogs (=hard clams) *Mercenaria mercenaria* were collected in Milford Harbor and kept in running seawater. Hemolymph was withdrawn from individual clams using a 5-ml syringe and

stored in microcentrifuge tubes on ice before use to limit clumping.

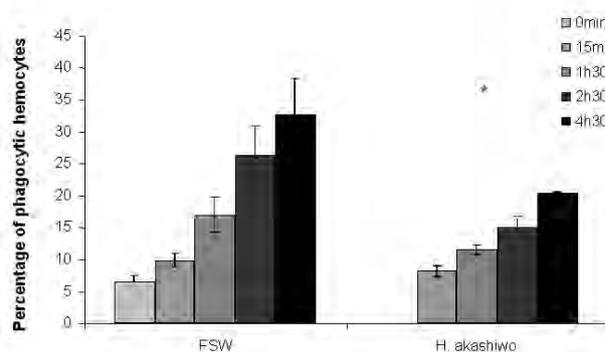
To analyze interactions between hemocytes and harmful algae, microscopic observations as well as flow-cytometric analyses, were done. Changes in shape, size and internal complexity of the algal cells, as well as variation in chlorophyll or cell numbers after exposure to hemocytes, were determined. Simultaneously, several hematological parameters were also assessed: internal complexity and size, but also adhesion, the percentage of phagocytic cells, production of reactive oxygen species, and percentages of apoptotic and dead cells. All analyses were done according to the protocols of Hégaret *et al.* (2003a, b). Clam hemocytes were exposed, in 5 replicates, to the three different species of harmful algae experimentally for 1, 2, 3 or 4 h in microplates for the test of adhesion and in flow cytometer tubes for the other assays, at cell densities ten times higher than a natural bloom, to simulate the concentration of cells that occurs during filtration:  $10^4$  cells  $\text{ml}^{-1}$  for *Alexandrium fundyense* (Shumway *et al.* 1988; Townsend *et al.* 2005),  $10^5$  cells  $\text{ml}^{-1}$  for *Heterosigma akashiwo* (Rensel and Whyte 2003),  $10^5$  cells  $\text{ml}^{-1}$  for *Prorocentrum minimum* (Hégaret and Wikfors 2005) and  $10^5$  cells  $\text{ml}^{-1}$  for *Rhodomonas* sp. as a control. Control analyses were also done on hemocytes in filtered seawater (FSW) only. Results were analyzed with Multifactor Analysis of Variance, with time and algal treatments as factors, using Statgraphics 5.0. The times of incubation often significantly affected the results and are presented with letters following One-Way ANOVA with time, but in this review, we focus mainly on the effects of the different algal treatments on the hemocytes, represented with an asterisk on the graphs, and only statistically significant results will be presented.

## Results

We observed species-specific effects of the algae on the hemocytes and vice versa. In the presence of *Prorocentrum minimum*, the number of hemocytes in the tube decreased after 4 h of incubation (Fig. 1). Microscopic observations showed the presence of large agglomerates of hemocytes and *P. minimum* cells. These agglomerates were not observed with the two other species of harmful algae. Phagocytosis of yellow, fluorescent microbeads by the hemocytes was inhibited by *Heterosigma akashiwo* (Fig. 2). In addition, microscopic observations showed chlorophyll fluorescence in some hemocytes. Concurrently, *Heterosigma akashiwo* cells incubated with hemocytes were



**Figure 1.** Effect of *Prorocentrum minimum* on the number of hemocytes from clams, *Mercenaria mercenaria*, over time (\* indicates significant difference between FSW and *P. minimum* treatments; letters indicate significant differences between the times of incubation).

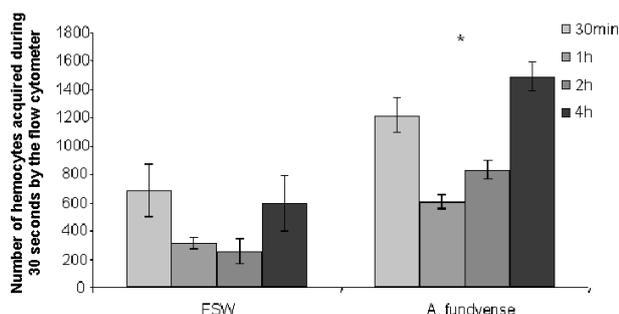


**Figure 2.** Effect of *Heterosigma akashiwo* on the percentage of phagocytic hemocytes from clams, *Mercenaria mercenaria*, over time (\* indicates significant difference between FSW and *H. akashiwo*).

eliminated and degraded (achlorotic and fragmented). Phagocytosis was not affected with the two other species of harmful algae. Hemocytes in the presence of *Alexandrium fundyense* cells had much lower adhesion than controls (Fig. 3), as many freely-circulating hemocytes were left in the tube, but *A. fundyense* did not seem to affect any other immune parameter tested. Microscopic observations showed the transformation of *A. fundyense* cells exposed to hemocytes into temporary cysts. The two other species of harmful algae studied did not affect adhesion of hemocytes. Most of these changes were observable after 1 or 2 h, but continued to increase up to 4 h of incubation.

## Discussion

The results demonstrate species-specific responses of the hemocytes to toxic algae and also that harmful algal cells are affected differently when exposed to hemocytes. Responses of hemocytes to harmful algal cells were very rapid; detectable within 1 h, but more extreme after 4 h. For future experiments, an incuba-



**Figure 3.** Effect of *Alexandrium fundyense* on the adhesion of clam, *Mercenaria mercenaria*, hemocytes over time (\* indicates significant difference between FSW and *A. fundyense*).

tion time of 4 h would probably be sufficient to detect the effects of hemocytes on algae and vice versa.

Fewer free, single hemocytes were present in the tube incubated with the harmful alga *Prorocentrum minimum* (Fig.1), suggesting that hemocytes are aggregating together or around the *P. minimum* cells. Wikfors and Smolowitz (1993, 1995) reported the presence of hemocyte aggregates in gill and mantle tissues of oysters and scallops exposed to *P. minimum in vivo*. Thus, the *in vitro* finding in the present study is consistent with a protective response of the hemocytes to isolate the harmful algal cells from other tissues. *In vivo* experiments (Hégaret and Wikfors 2005 a, b) showed that *P. minimum* triggered an initial, rapid increase in circulating hemocyte concentration, followed by a decrease, which may be attributable to aggregation around the *P. minimum* cells.

Microscopic observation of hemocytes incubated with *Heterosigma akashiwo* showed the presence of hemocytes with chlorophyll fluorescence, indicating phagocytosis of the algal cells by the hemocytes. This activity may have been responsible for depressed phagocytosis of plastic microbeads (Fig. 2), but release of an inhibitory metabolite by *H. akashiwo* cannot be ruled out. In the presence of hemocytes, *H. akashiwo* cells lost fluorescence and shape. Further experiments should be conducted to assess the ability of *H. akashiwo* to produce chemical compounds, such as toxins or proteases, affecting the hemocyte functions.

Microscopic observations showed transformation of some *A. fundyense* cells into temporary cysts when in contact with the hemocytes. Moreover, adhesion of hemocytes was affected by the presence of *A. fundyense* cells (Fig. 3). Previous, *in vivo* experiments exposing oysters to *Alexandrium catenella* showed no effect of the algae on hemocyte concentration, phagocytosis, or production of ROS (Hégaret *et al.* 2006),

consistent with the results of the present study. Unfortunately the previous study did not include measurements of hemocyte adhesion, the function affected *in vitro*.

Responses of hemocytes were very different according to the harmful algal species to which they were exposed. This could be attributable to different lectins on the surfaces of the different algal cells, which could elicit differences in hemocyte recognition and response. Protective hemocyte responses, such as phagocytosis, production of ROS, or adhesion and aggregation around the algal cells can follow. Hemocytes, however, also may release chemical compounds into the hemolymph (lysozymes); this response may explain degradation of *Heterosigma akashiwo* cells or transformation of *Alexandrium fundyense* into temporary cysts (Hégaret *et al.* this volume). Further analyses will be necessary to understand fully the functional roles of hemocytes in the defense mechanisms of bivalves exposed to HABs.

Finally, these *in vitro* assays are easier and much more efficient to perform than *in vivo* experiments, which require long-term exposure in the laboratory. The demonstration that *in vitro* responses correspond with *in vivo* interactions will facilitate future analyses of HAB-shellfish interactions.

### Acknowledgements

This work was supported by EPA/ECOHAB - GRANT 523792 to S.E. Shumway, G.H. Wikfors, and J.M. Burkholder and by NRAC/USDA GRANT to R. Smolowitz, D. Leavitt, S.E. Shumway and G.H. Wikfors. We also acknowledge the Lerner Grey Fund from the American Museum of Natural History, the Sigma Xi Grant of Aids, the Feng Student Activities Fund from University of Connecticut, and National Oceanic and Atmospheric Administration Center for Sponsored Coastal Ocean Research (NOAA/CSCOR) for funding.

### References

- Anderson, D.M., Kaoru, Y. & White, A.W. (2000). WHOI Technical Report WHOI-2000-11, Woods Hole, MA.
- Anonymous (2000). Food and Agriculture Organization of the United Nations, Rome, Italy, 142 pp.
- Burkholder, J.M. (1998). *Ecol. Appl.* 8 (Suppl.): S37-62.
- Cheng, T.C. (1996). In: *The Eastern Oyster Crassostrea virginica*, Kennedy, V.S., Newell, R.I.E., and Eble, A.F. (eds), Maryland Sea Grant, USA, pp.

- 299-333.
- Hégaret, H., Wikfors, G.H. & Soudant, P. (2003a). *J. Exp. Mar. Biol. Ecol.* 293: 237-248.
- Hégaret, H., Wikfors, G.H. & Soudant, P. (2003b). *J. Exp. Mar. Biol. Ecol.* 293, 249-265.
- Hégaret, H. & Wikfors, G.H. (2005a). *Harmful Algae* 4: 187-199.
- Hégaret, H. & Wikfors, G.H. (2005b). *Harmful Algae* 4: 201-209.
- Hégaret, H., Wikfors, G.H., Soudant, P., Lambert, C., Shumway, S.E., Bérard, J.B. & Lassus, P. (2006). *Mar. Biol.* 152: 441-447.
- Hégaret, H., Wikfors, G.H., Shumway, S. E. This volume.
- Shumway, S.E., Sherman-Caswell, S. & Hurst, J.W., (1988). *J. Shellfish Res.* 7: 643-652
- Shumway, S. E. (1990). *J. World Aquacult. Soc.* 21: 65-104.
- Townsend, D.W., Pettigrew, N.R. & Thomas, A.C., (2005). *Deep-Sea Research Part I-Topical Studies in Oceanography* 52: 2603-2630
- Rensel, J.E. & Whyte, J.N.C., (2003) In: *Manual on Harmful Marine Microalgae*, Hallegraeff, G.M., Anderson, D.M. & Cembella, A.D. (eds), UNESCO, Paris, pp. 693-722.
- Wikfors, G.H. & Smolowitz, R.M. (1993). In: *Toxic Phytoplankton Blooms in the Sea*, Smayda, T.J. & Shimizu, Y. (eds), Elsevier, New York, pp. 447-452.
- Wikfors, G.H. & Smolowitz, R.M. (1995). *Biol. Bull.* 188: 313-328.

## What's new in toxins?

Philipp Hess

Marine Institute, Rinville, Oranmore, County Galway, Ireland

philipp.hess@marine.ie

### Abstract

Toxins are at the heart of the problem of harmful algal blooms and therefore many disciplines around HABs and food safety rely on chemical data to facilitate different approaches to address the HAB/toxin problem. The recent discoveries of toxins and their analogues is reviewed; it is noted that no new chemical groups have been isolated from shellfish over the last 10 years. Analogues are classed into those produced by phytoplankton, those accumulated in shellfish and those relevant to human health. The usefulness of novel chromatographic and mass spectrometric techniques is discussed, at the example of UPLC-MS-MS, which enables rapid analysis of a large number of toxins (22 toxins/6.5 min). Studies investigating the feasibility of reference materials and their application in method validation are described at the example of azaspiracids. The use of passive sampling for clarifying the mechanisms of toxin accumulation in shellfish and the environmental behaviour of toxins is reviewed, as well as the behaviour of some shellfish toxins in the cooking process.

### Introduction

The study of marine microbes, and in particular algae, has a number of motivations. The most prevalent is probably our need to understand elemental cycles at global scale, such as carbon cycles and oxygen production. This understanding does not only drive our assessment of those resources necessary to survive, such as oxygen, but also our ability to distinguish between natural and anthropogenic processes, e.g. in the area of climate change. The next motivation is related to our knowledge on ecological systems and understanding of trophic levels and food chains, all of which impact on our evaluation of the sustainability of fisheries and aquaculture. Finally, we also need to understand certain species of algae that impact more directly on our health or economy, algae we classify as harmful. Harmful algae are most widely considered to be those algae that either adversely impact on our most important food resources from the oceans, i.e. fish and shellfish, or accumulate in these and cause human illness after consumption of fish or shellfish. Increasing evidence suggests that we may have arrived at a stage where anthropogenic impacts are becoming important at global scale, mainly through atmospheric emissions influencing our climate, emissions into the aquatic system causing eutrophication and through fisheries impacting on the balance of trophic levels in our oceans. Therefore, clearly, the subset of harmful algae as part of the total algal community is very likely to also be influenced by our activities and thus

all levels of motivation for studying algae become intertwined.

Harmful algae have been named thus to incorporate 2 major groups of algae: the ones causing fish kills or negatively impacting on fish and shellfish aquaculture (economic nuisance) and those that produce compounds that cause human illness when consuming fish or shellfish (public health and economic nuisance). This paper focuses on the latter group and will illustrate the role of chemical knowledge through the review of recent developments in structure elucidation, toxicology, analysis and ecological studies. Recently discovered compounds and their relevance to food safety will be described, with a particular focus on derivatives of known parent toxins. The potential of novel liquid chromatographic techniques for the fast and cost-effective analysis of shellfish toxins will be outlined at the example of ultra-high pressure liquid chromatography. Recent developments on tools for quality control, including standards and reference materials will be discussed as well as results from recent proficiency testing exercises. Finally, the usefulness of chemical analysis to field studies will be discussed at the example of the distribution of lipophilic toxins between the water column and shellfish during a toxic event in Ireland in 2005. The impact of this basic knowledge on the behaviour of toxins in shellfish tissues on risk assessment and management will be discussed at the example of a recent review of azaspiracids and other lipophilic toxins.

## Recent Discoveries in the Marine Toxin Area

No completely new toxin groups have been reported over the last 2 years, hence this review focuses on novel analogues reported from the main existing toxin groups. Due to the scope of the paper, the subject cannot be dealt with in an exhaustive manner, and the toxins from the freshwater and brackish water algae and cyanobacteria are not covered. Amongst the marine toxins, this review has focused on those related to shellfish poisoning, thus excluding also the toxins associated with *Gambierdiscus toxicus*, ciguatoxins, maitotoxins, gambierols and others. Recent developments in 9 toxin groups are discussed below in order of increasing lipophilicity.

### Saxitoxins

Saxitoxin and its analogues are amongst those toxins for which symptoms of paralytic shellfish poisoning (PSP) have been reported for the longest, symptoms at lower doses being completely reversible, however, high doses leading to death through paralysis of the respiratory tract. Interestingly, these most toxic compounds have been found to be produced both by marine dinoflagellates of the *Alexandrium* and *Gymnodinium* groups, and by cyanobacteria, thereby leading not only to fish and shellfish poisoning in humans (and fish kills) but also to problems in drinking water supply for man and farm animals. In the 1930s, Schantz *et al.* developed the PSP mouse bioassay (MBA), however, the structure of the main toxin was not elucidated until the late 1960s. By the mid 1980s, ca. 20 analogues were known in this group (Shimizu 1984). Subsequently, acetic acid esters were isolated from *Lyngbya wollei* (Onodera *et al.* 1997), and phenol-derivatives from *Gymnodinium catenatum* (Negri *et al.* 2004). Also, more hydroxy-metabolites were found in *Mytilus edulis* (Dell'Aversano *et al.* 2005).

### Domoic acids

At least 10 analogues are known in this compound group, all of which are stereo-isomers of domoic acid (DA), a potentially lethal toxin causing diarrhoea and vomiting at lower doses and permanent loss of short-term memory at higher doses in humans. However, not all analogues have been found in shellfish. Isodomoic Acid C was detected in New Zealand shellfish early in the new millennium but has been shown to not cause any problems of toxicity (Holland *et al.* 2005). DA and its diastereomer epi-DA are considered the only toxicologically relevant compounds and both risk assessment and risk management groups seem satisfied with the current EU limit of 20 mg.kg<sup>-1</sup>

in shellfish. One of the major discoveries in recent years is the fact that benthic diatoms are also capable of producing DA, thus potentially complicating the monitoring of this toxin group. This finding has also renewed the interest in the dynamics of DA accumulation in scallops (esp. *Pecten maximus*) where record concentrations of 2-3,000 mg.kg<sup>-1</sup> are regularly found in scallop digestive gland in most European countries, thereby leading to concentrations in whole scallops often a multiple above the regulatory limit. While a recent study in France (Nezan *et al.* 2006) was able to establish a reasonably good relationship between the occurrence of *Pseudo-nitzschia* species and DA accumulation in scallops, this has been much more difficult in offshore scallop production areas in Scotland and Ireland (Bogan 2006).

### Cyclic Amines and Imines

Several compound groups have been found in this category: gymnodimines, spirolides, pinnatoxins and pteriatxins, pinnaic acids and halichlorines, prorocentrolides and symbioimines (Molgo *et al.* 2007). Gymnodimines and spirolides are very controversial since their discovery is not related to human illness following shellfish consumption but due to their bioactivity in the intraperitoneal mouse bioassay. Both show similarly high potency (LD50 ca. 10-50 µg.kg<sup>-1</sup> bodyweight) and lead to mice dying very rapidly when injected with pure toxin or shellfish extracts, thus these toxins were also named "fast-acting toxins". Recent studies on gymnodimines demonstrated that oral potency in mice was virtually absent (Munday *et al.* 2004), thus leading to deregulation of this compound group in New Zealand (the only country which originally did regulate for this toxin group). More concern still exists about the spirolides, since oral exposure in mice still showed significant toxicity. These were initially discovered in Canadian shellfish and later shown to also occur in Europe, especially Norway (Aasen 2005), and France (Amzil 2006). The causative organism of spirolides, *Alexandrium ostenfeldii*, has also been found in other countries, including Ireland (Touzet and Raine, unpublished information) and Denmark (MacKinnon *et al.* 2006). A case study on spirolides in Norway showed that the concentrations occurring may reach levels of toxicity relevant to human intake. Also the monitoring of this group has been complicated by the discovery of fatty acid esters of spirolides in Norway since the ester formation delays the onset of symptoms in mice (Aasen 2005), due to the hydrolysis required.

Currently, risk assessment and risk management groups disagree on the need for regulation of this compound group: a recent EU risk assessment group (October 2005) suggested careful monitoring of the group while recent risk management groups (EU Member States, 16 March 2006 and Codex alimentarius CCFFP subcommittee, April 2006) suggest no need for regulation of this compound group. Ongoing evaluation in the EU under the auspices of the European Food Safety Authority's (EFSA) contaminants panel ensures that an independent evaluation is carried out taking into account most recent information.

#### *Yessotoxins*

This compound group is probably the most controversial amongst the marine toxins. Occurrence had been reported from Japan, New Zealand, Italy and Norway. Again, similar to cyclic imines, the main compound was discovered in 1985 in Japan due to unusual symptoms in the intraperitoneal MBA while other known toxins were not present. Although no human health effects could be shown anywhere, this toxin group was initially regulated indirectly worldwide through the prescription of the MBA as reference method in the EU. Since 2002, the toxin group was also regulated specifically in the EU (Anon. 2002), even at a level of 1 mg.kg<sup>-1</sup>, which is not easily implemented using the MBA reference method, since the "harmonised" protocol proposed in the EU detects levels of less than 1 mg.kg<sup>-1</sup>. Recent oral exposure studies in mice (Aune *et al.* 2002, 2006b) led to the FAO/IOC/WHO expert consultation in 2004 suggesting a safe increase of the limit to 12 mg.kg<sup>-1</sup>, thereby increasing the discrepancy between the current reference test and the level that would need to be regulated. Most recent risk management for YTX in the EU and Codex suggest complete deregulation of this compound group, which would result in it becoming an interference to the current reference test, adding to the interferences from gymnodimines and fatty acids.

Interestingly, it is this group in which most analogues were discovered over the last few years, again thanks to systematic studies of concentrated algal extracts using LC-MS techniques suited to the discovery of analogues (Miles *et al.* 2005). The complexity of the compound group includes noro-compounds, (di) hydroxylated, carboxylated and enone-derivatives as well as amides and arabinosides. Shellfish metabolism leads to hydroxy-, carboxy- and hydroxy-carboxy derivatives (Aasen *et al.* 2005). Despite several hydroxyl groups in the molecule, no fatty acid esters were reported for this structure. The review by Hess

and Aasen (2007) summarises the recent findings on structural analogues and the biogenetic origins including *P. reticulatum*, *L. polyedrum* and *G. spinifera*.

#### *Okadaic acid and Dinophysis toxins*

The okadaic acid group contains three major parent compounds, which seem to be produced in geographically distinct areas: okadaic acid (OA), dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2). DTX2 is a stereo-isomer of OA, and DTX1 can be described as a 35-methyl OA. The stereochemistry of DTX1 and -2 has recently been clarified by Larsen *et al.* (2007). OA is observed in most cases where the group of compounds are produced, however, DTX2 has only been reported from European shellfish, initially Ireland (1992), subsequently also Portugal, Spain, France, UK and more recently Norway. In Ireland, a clear relationship was established between OA produced by *Dinophysis acuminata*, and DTX2 plus OA being produced by *Dinophysis acuta*.

The discovery of fatty acid esters, mostly fatty acids esterifying at the 7-hydroxyl-group of OA, DTX1 or DTX2, as shellfish metabolites of the parent compounds (1984, Japan) has complicated the management of this problem since the observation time in mice needs to be increased to detect the presence of the esters, and the intraperitoneal model of exposure becomes a questionable tool for the evaluation of the overall toxicity present in a shellfish sample intended for human consumption. Physicochemical methods need to apply a hydrolysis step to transform the esters into the parent compounds. T. Yasumoto has isolated and synthesised some of the esters, however, analysis of shellfish samples has shown a wide variety of esters present and most risk managers agree on hydrolysis as a reasonable step to obtain a worst-case estimation of the total toxicity present in a shellfish sample. A notable addition to natural products was the discovery of the C16:0 ester of DTX1 in a sponge, where the fatty acid is attached at the 27-hydroxyl group of DTX1 (Britton *et al.* 2003). A number of analogues were also discovered in *Dinophysis* spp. and *P. lima*, mostly diol-esters where the carboxyl-group of the parent compound esterifies with the alcohol of diols or more complex compounds, such as DTX5 (Suarez-Gomez *et al.* 2005). Some of the compounds from *P. lima* (DTX4) have been shown to be water-soluble and it remains to be shown whether algae produce compounds related to OA for an ecological, e.g. allelopathic, reason. Some additional insight into this complex area can also be obtained from recent overviews by P. Vale (2006, 2007).

### *Pectenotoxins*

Pectenotoxins (PTXs) are another group of compounds that are controversial with respect to risk assessment. While large differences were observed between intraperitoneal and oral exposure in mice, most recent info on the histopathological effects of PTXs in the digestive tract of orally exposed mice suggests the need to carefully re-evaluate the risk from PTXs to humans (Ito *et al.* 2006). The assessment of PTXs is further complicated by their typical co-occurrence with toxins from the okadaic acid group, both being produced by *Dinophysis* spp. Recently reported analogues include PTX11 (Suzuki *et al.* 2006), PTX12, -13, and -14 (Miles *et al.* 2004, 2006). Fatty acid esters have not been reported for the parent compound (PTX2), however, fatty acid esters of the PTX2 secoacids were found in large quantities in Irish and Norwegian shellfish (Wilkins *et al.* 2006).

### *Azspiracids*

Azspiracids (AZAs) are another toxin group that were shown to cause diarrhoea and vomiting in humans, following consumption of contaminated shellfish (McMahon and Silke 1996; Satake *et al.* 1997). Initially only observed in Ireland, it became rapidly clear that AZAs also occurred in other European waters (James *et al.* 2002). A desmethyl-AZA1 (AZA3) and a methyl-AZA1 (AZA2) were discovered as analogues (Ofuji *et al.* 1999). Since then Ofuji *et al.* (2001), Brombacher *et al.* (2002), and James *et al.* (2003) have identified a further 8 hydroxy-analogues using mass spectrometric techniques. Initial evaluation of the compounds using ip MBAs suggests that the hydroxyl-analogues are less toxic than the parent compounds (Ofuji *et al.* 2001). Further compounds have been discovered (Rehmann *et al.* 2006, unpublished data), their full identification is currently underway. However, despite systematic search, no fatty acid esters of the AZA-group were discovered so far.

### *Brevetoxins*

Brevetoxins (BTXs), the causative agents of neurotoxic shellfish poisoning (NSP), are mostly associated with red tides (*Karenia brevis*) in the Gulf of Mexico, and very occasionally on the south east coast of the US, and once in 1993 in New Zealand. Although the illness is known since the mid 19th century, full structure elucidation was only possible during the late 1980s. Besides the transformation of BTX-B (PbTx-2) into the 10 times more toxic dihydro-BTX-B (PbTx-3), cystein conjugates of the parent compounds have been reported as major shellfish metabolites,

however, toxicity of all metabolites is not completely clarified (Plakas *et al.* 2004; Wang *et al.* 2004). More recently, Abraham *et al.* (2006) reported more polar metabolites from marine aerosols, in which the A-ring is opened, leading most likely to a reduced toxicity if the same structure activity relationship applies as found by Rein *et al.* (1994). Dechraoui *et al.* (2006) have studied the binding of analogues of this group to voltage-gated Na<sup>+</sup> channels, further contributing to potential knowledge on the relative toxicity of analogues.

### *Palytoxins*

This toxin group was traditionally associated with fish poisoning and aerosol problems in the tropics. More recently, *Ostreopsis ovata* and *Ostreopsis* spp. as well as palytoxin and related compounds were also found in Southern Europe (Spain, Italy, Greece), mostly causing problems to bathing people at beaches on the Italian coast of Genoa (Ciminiello *et al.* 2006) and in mouse assays in shellfish from Greece (Anon. 2005). Very little is known about safe levels of palytoxin(s) in shellfish and this group has only recently attracted the attention of risk evaluators in Europe, probably triggered by recent observations in southern Europe. An initial study of the uptake of *Ostreopsis* in shellfish was carried out in New Zealand (Rhodes *et al.* 2006, this conference), suggesting that the risk of human illness from shellfish is minimal. Recent reviews on palytoxins and ostreocins have been given by Katakou (2007) and Vale and Ares (2007) for chemical and biochemical aspects, respectively.

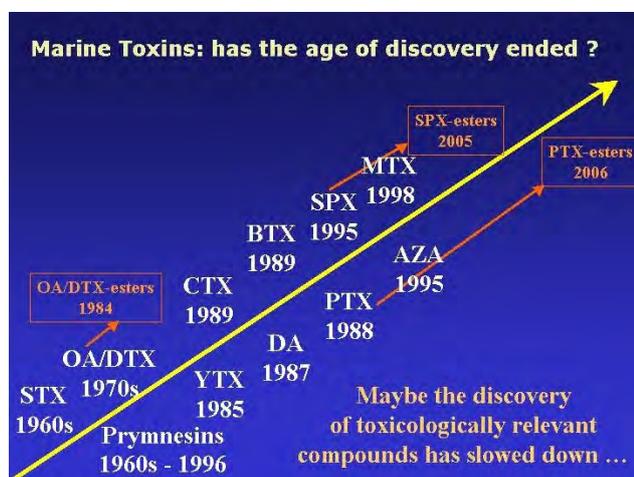
### *Summary*

The initial observation that no new toxin groups were discovered over the last few years led the author to a more comprehensive review of the discovery of most toxin structures and it is noted that most of the basic toxin structures of shellfish toxins known to date have been discovered from the mid 1970s to the mid 1990s, with the exception of STX which was already discovered during the late 1960s (Fig. 1).

The main reason for this sudden knowledge on toxin structures is probably a function of the better performance of recent NMR-spectrometers, increasing in power from the 60MHz in the 1960s to 300-400 MHz in the 1980s and currently 600-800 MHz. This increased NMR capability led to a better understanding of the molecules and the possibility to study smaller quantities of toxin. This discovery of novel compound groups has more or less ceased since 1995 (> 10 years) thus indicating that the combination of

bioassay guided fractionation and isolation of toxins has probably yielded most, if not all, base structures that are of toxicological relevance to the shellfish toxin field.

The discovery of analogues has flourished since the 1990s with the availability of good coupling techniques of liquid chromatography (LC) to mass spectrometry (MS). The discovery of these analogues is very much of a problem to the regulators in that it is neither clear whether the compounds are of toxicological relevance to shellfish consumers nor whether they can be appropriately detected using bioassays or physico-chemical analytical techniques. A pragmatic approach to managing toxin analogues has been proposed by Munday in 2004, considering both the concentration levels found for an analogue and its relative toxicity from animal studies if available (FAO/IOC/



**Figure 1.** Discovery of toxin groups or structure elucidation of causative agent.

WHO 2004). Further discussions on the principles of how to evaluate the toxicity of analogues, how to assign toxic equivalence factors and the current state-of-the-art on quantitative structure activity relationships are discussed in separate papers in the same proceedings (Hess *et al.* 2006d, e). The picture for the regulator has not only become more complicated because there are more toxin groups (nine described above) than the initial 3 groups (ASP, DSP, PSP) but also a multiple number of analogues. In fact, each group has been shown to contain a minimum of 10 analogues, often up to 30 or more. The discovery of this multitude of compounds coincided with the capability of monitoring for many analogues on a daily basis. This increased monitoring capability in turn has led to a widened knowledge base on co-occurrence of toxins thus posing the other principal difficulty of evaluating the effects of co-occurring toxin groups. While

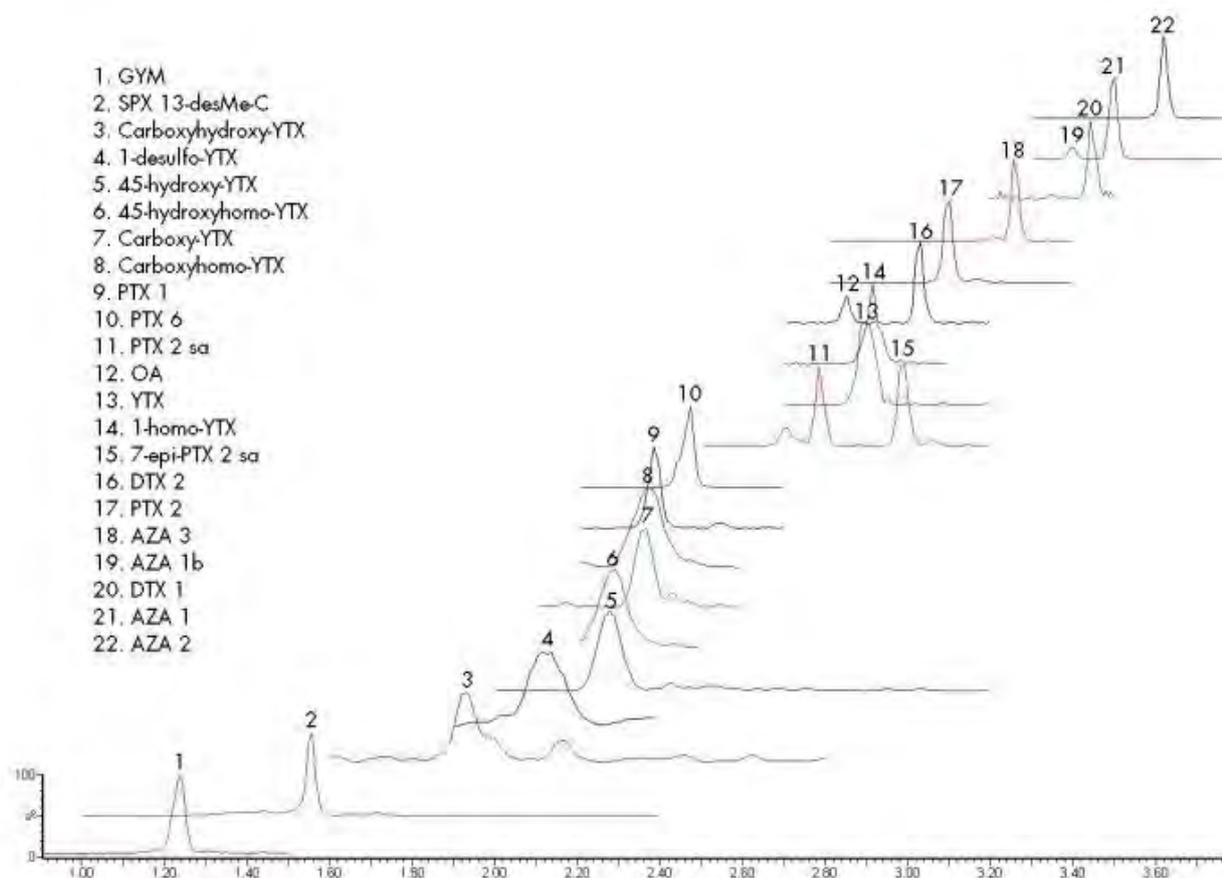
analogues within one toxin group can theoretically be dealt with (if a single mode of action can be established for a toxin group) it has yet to be shown if exposure to multiple different toxin groups can be sensibly regulated. This problem can only be handled after further research.

The following sections are again not intended as an exhaustive overview of recent developments, however, outline general trends at examples from studies carried out in the author's laboratory.

#### *Recent developments in the area of analytical methods*

LC-MS has been hailed as one of the most promising techniques for the analysis of phycotoxins thanks to the wide range of different compounds that can be detected. On the other hand, the technique is also limited by the number of compounds that can be detected at any given time during chromatography, and the number of compounds that can be separated sufficiently to avoid matrix components to affect the ionisation process that will in turn affect the response in the MS analysis. A combination of improvements has been reported presenting advances in both chromatography and mass spectrometry (Fux *et al.* 2007). The technique is referred to as Ultra-Performance Liquid Chromatography (UPLC) coupled to latest generation tandem mass spectrometry (MS). Thanks to the ability to support higher pressures (up to 15000 psi), the separation obtained in UPLC is superior both in speed and resolution, thereby allowing the separation of more compounds in a shorter time compared to traditional LC. The mass spectrometer developed by Waters/Micromass, Quattro Premier XE, also facilitates faster acquisition of multiple ion traces thereby supporting effectively the sharper peaks obtained in the UPLC, even with the ability to carry out fast switching between positive and negative ionisation, which increases the flexibility on the choice of separations. With such techniques it is easily possible to detect up to 25 compounds in less than 7 minutes, which represents typically an improvement of 3-4 fold compared to traditional LC-MS instruments (Fig. 2).

While triple stage quadrupole MS remains the tool of choice for quantitation of low levels of phycotoxins, the hybrid quadrupole time-of-flights (Q-ToF) instruments have also been successfully used in the author's laboratory for accurate and reproducible quantitation, albeit with a limited calibration range. Structural confirmation can be carried out confidently at relatively low levels (ng/mL) using either Q-ToF-MS or linear ion-trap-MS (Q-TRAP).



**Figure 2.** UPLC-MS separation of 22 lipophilic toxins (Fux *et al.* 2007).

#### *Validation of methods used to detect phycotoxins*

Very few methods for the detection of phycotoxins have been validated through interlaboratory trials, and hence a task force on method validation has been created by the AOAC (Anon. 2004). A mouse bioassay has been validated for STX (AOAC 2005a), and a HPLC method using pre-column oxidation and fluorescence detection has been validated for saxitoxin and related compounds (AOAC 2005b). For domoic acid, validated methods include a HPLC method based on UV detection (AOAC 2005c) and an ELISA method (AOAC 2006). However, for the large range of lipophilic toxins, including OA + DTXs, AZAs, YTXs, PTXs, SPXs, BTXs, CTXs, palytoxins and others, no methods have been validated in interlaboratory trials. A recent workshop co-hosted by the European Commission for public health and consumer protection (DG-SANCO) and the European Centre for the Validation of Alternative Methods (ECVAM) outlined possibilities for the replacement of animal testing and research priorities that need to be achieved to validate methods for a number of phycotoxins (Hess *et al.* 2006a).

The validation of all methods is to some extent hampered by the lack of appropriate validation tools, in particular appropriately contaminated test materials, but also certified calibration standards and certified tissue reference materials (CRMs). McCarron *et al.* (2007a, b, c) studied the feasibility of the preparation of homogenous and stable shellfish tissue RMs, using different preparation and stabilisation techniques, including the addition of antibiotics and antioxidants, gamma-irradiation and freeze-drying, for a range of toxins, including DA, OA, DTXs, AZAs, PTXs and YTXs. These studies have led to the production of in-house RMs (Hess *et al.* 2006b), method-validation RMs, shellfish materials used in proficiency testing (Hess *et al.* 2006b, 2007) and CRMs. Following studies by Satake *et al.* (1998), Quilliam *et al.* (2006) and Rehmann *et al.* (2003, 2006) investigated techniques for the isolation of lipophilic toxins. Continued efforts over the last 5 years in the author's laboratory, in collaboration with the National Research Council Canada (NRCC), have thus led to the development of both reference standards and reference mussel tissue for AZAs and DTX2, both of which should be commer-

**Table 1.** Reference materials (RMs) existing, under development and missing for phycotoxin standards and shellfish RMs.

Toxin Group	Existing		Under development		Missing	
	Standard	Matrix	Standard	Matrix	Standard	Matrix
AZA			AZA-1 AZA-2 AZA-3	AZAs		
BTX				x ?	x	x
DA	DA + epi-DA	DA				
OA	OA	OA, DTX-1	DTX-2	DTX-2	(DTX-3)	(DTX-3)
PaTX					x	x
PTX	PTX-2			PTX-2, PTX-2sa		
SPX	13desmethyl-C					x
YTX	YTX					x
STX	13	STX, dc-STX	5	Multi	(x)	(x)

x denotes areas where standard or matrix RMs are missing or the analogue for which they are under development is not known to the author at the time of publication.

cially available from NRCC in 2007. This will lead to a suite of standards and tissue CRMs being available for a large number of lipophilic toxin groups: OA + DTXs, AZAs, PTXs, YTXs, SPXs (see Table 1).

Unfortunately, standards and reference materials for brevetoxins and palytoxins are still direly needed and international efforts should focus on this area in the near future.

Several initiatives are currently undertaken for the validation of LC-MS methods for lipophilic toxins: the EU-project BIOTOX fosters research towards the validation of LC-MS methodology for 4 lipophilic groups (OA, AZA, YTX, PTX), and the EU-Community Reference Laboratory is organising a pre-validation study for the same toxins plus spirolides. American groups collaborate on the cross-validation of LC-MS and ELISA techniques, as well as the APHA mouse bioassay for the detection of brevetoxins, while Japanese scientists also plan intercomparison studies on LC-MS for the detection of a range of lipophilic toxins, including OA + DTXs, AZAs, YTX, PTXs, and brevetoxins. Recent interlaboratory trials on LC-MS methods for multiple lipophilic toxin groups point towards difficulties in achieving good comparability between laboratories when a fix protocol is prescribed. Hence, several official control laboratories have suggested that in-house validation based on the demonstration of performance criteria should be acceptable for official food control. This approach has been successfully implemented in the EU in the area of official control of veterinary residues and would allow to overcome the large differences between instruments from different manufacturers and different needs for and availabilities of calibration standards

and reference materials. In addition, the single-laboratory-validation (SLV) approach would facilitate incremental improvement of the area, allowing laboratories to become familiar with modern technologies step by step, and to implement novel methods as soon as novel toxins or analogues are discovered, a distinct advantage in the area of natural toxins.

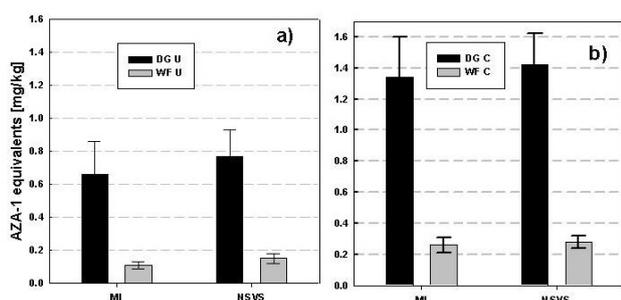
#### Distribution of Toxins in Seawater and Accumulation in Shellfish

The mechanisms leading to accumulation of toxins in shellfish are very likely multiple, and accumulation of toxins directly from the water could theoretically be a route of uptake. The passive sampling technique developed by McKenzie *et al.* (2004) has been used by Fux *et al.* (2006a) to study the distribution of lipophilic toxins in seawater following a bloom of *D. acuta* on the West coast of Ireland during 2005. Repeated replacement of passive samplers in 1-week cycles showed that up to 4 weeks after the bloom, large amounts of dinophysistoxins were present in the water (comparable with those during the bloom and significantly larger than prior to the bloom), however, these toxins were neither accumulated in uncontaminated shellfish regularly placed in this area at 1-week intervals nor in the shellfish growing locally. Although circumstantial, this study strongly suggests that shellfish do not significantly absorb lipophilic dinophysistoxin-2 directly from seawater, or particulate matter suspended in seawater. It remains to be clarified whether other mechanisms of accumulation through planktonic vectors may still play a role, or whether the uptake in shellfish can only be significant if the causative organisms are directly taken up by the

shellfish. The latter hypothesis is also supported by the observation that different shellfish species have different uptake of toxins, even when grown in the same production area. Passive sampling techniques should also be useful in establishing the uptake and partitioning behaviour of more water-soluble toxins such as DA, STX and YTX.

### Behaviour of Toxins During Different Heat Treatments

The behaviour of toxins during heat treatments of shellfish tissues was studied both for lipophilic AZAs (Hess *et al.* 2005) and hydrophilic DA (McCarron and Hess 2006). The overall concentrations of DA in whole mussel flesh did not change by more than ca. 20 % during steaming and harsher heat treatments, reflective of commercial processing (data not shown). This was attributed to a loss of toxin approx. equal to the loss of water from the matrix in the heat treatment. Such losses have been verified through the analysis of the fluids secreted during the process, and are also demonstrated by the partial re-distribution of DA from the hepatopancreas to the remaining tissues.



**Figure 3.** Increase in concentration of AZAs during heat treatment of shellfish (a) uncooked, (b) cooked. Dark bars are HP tissue, light bars are whole flesh including HP, error bars represent 1 SD, n=5. MI = Marine Institute, NSVS = Norwegian School of Veterinary Science.

However, a similar investigation on AZAs showed very different results, indicating that the concentrations of AZAs in either hepatopancreas or whole flesh can increase by a factor of up to 2 when heating shellfish (Fig. 3). Initially, we attributed this increase solely to the water loss from the tissue during heat treatment, since we did not detect any toxin in the fluids released from the tissue. Subsequently, we have shown that, in addition to water loss, the increase of AZA3 concentration upon heating may be related to either a release of toxin from the matrix or due to toxin conversion from another analogue into AZA3. These studies are important in the estimation of doses to which people have been exposed during

intoxication events. The Irish Food Safety Authority has just revised its initial risk assessment on AZAs, taking into account this information, thereby leading to a higher estimate of the intake in an event observed in 1997 (Anon. 2006).

### Discussion and Conclusions

Thanks to the recent developments in isolation and structure elucidation techniques, structure elucidation has become more rapid over the last 20-30 years. Also, thanks to the implementation of compound-specific analytical techniques, especially LC-MS, in routine monitoring of shellfish toxins, most compound groups responsible for human shellfish poisoning have probably been discovered to date. A rapid reporting of toxin profiles from around the globe will also establish over the next few years to what extent these toxin groups are currently distributed worldwide. The increased reports of toxin analogues over the past 10 years have been attributed to scientific enthusiasm, however, these discoveries are typically motivated by the need to explain which compounds are produced by the algae, and which ones are shellfish metabolites. Sometimes the compounds produced by algae are more toxic than the shellfish metabolites (AZA4, -5, PTX2sa, OH- and carboxy-YTX) but sometimes the shellfish metabolites may also be more toxic than the initially produced compound (DTX4-OA, PbTx3-PbTx2). Therefore, a clear understanding of all the analogues occurring in a group seems of utmost importance. Passive samplers are likely to play a pivotal role in clarifying the biogenetic origin and the environmental behaviour of toxins as well as their uptake into shellfish.

Compound-specific detection techniques appear to be a useful – if not imperative – technique both to increase our knowledge on the global distribution of toxin profiles and for the effective surveillance of shellfish pointed at those toxins that have been shown to be of importance in public health, including the description of their accumulation, transformation and depuration behaviour in shellfish. Compound-specific LC-MS detection has also been used in the studies described here to highlight the fate of toxins during home cooking or commercial processing thereby producing important information for risk assessors. Both interesting developments in LC-MS methodology and recent good progress in the availability of quality control tools such as certified standards, tissue materials, proficiency testing have been discussed above. Thanks to these factors it is likely that the initiatives

aimed at validation and standardisation of universal compound-specific LC-MS techniques will eventually accelerate the reduction and replacement of animal testing, currently in use in many shellfish surveillance programs.

### Acknowledgements

The author would like to acknowledge the enthusiastic efforts of all the staff and students in the Biotoxin Chemistry Team and the Biotoxin Unit at the Marine Institute, as well as the continued support by and collaboration with Profs. T. Yasumoto, T. Aune and M.A. Quilliam, Drs. G. Doucette, M. Twiner, M. Satake, E. Ito, C. Miles and J. Aasen, without their help this paper would not have been possible. The author also acknowledges the financial contributions of the Marine Institute, the Irish nationally funded project ASTOX (NDP ST-02-02) and the EU-FP6 funded project BIOTOX (contract no.: 514074), which have enabled the team at the Institute and our collaborators to conduct the research presented in this paper.

### References

- Aasen, J.A.B. (2005) PhD thesis: Identification and quantification of algal toxins in shellfish, and characterization of toxin profiles in Norwegian mussels. Norwegian School of Veterinary Science, Oslo, Norway.
- Aasen, J., Samdal, I.A., Miles, C.O., Dahl, E., Briggs, L.R. & Aune, T. (2005). *Toxicon* 45: 265-272.
- Abraham, A., Plakas, S.M., Wand, Z., Jester, E.L.E., EL Said, K.R., Granade, H.R., Henry, M.S., Blum, P.C., Pierce, R.H. & Dickey, R.W. (2006). *Toxicon* 48: 104-115.
- Amzil, Z., Royer, F., Sibat, M., Guimard, S., Neud-Masson, N. & Chiantella, C. (2006). Lipophilic toxins in French shellfish: first report on detection of pectenotoxin-2, spirolide-C and their isomers by liquid chromatography/mass spectrometry, poster 5.05 HAB XII.
- Anon. (2002). Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods Off. J. EC L75: 62–63.
- Anon. (2004). Marine and freshwater toxins community expresses need for modern methods. Inside Laboratory Management, Sept/Oct 2004: 9–10. Website (Accessed 17.01.2006): [http://www.aoac.org/marine\\_toxins/task\\_force.htm](http://www.aoac.org/marine_toxins/task_force.htm)
- Anon. (2006). Risk Assessment of Azaspiracids (AZAs) in Shellfish, August 2006. A Report of the Scientific Committee of the Food Safety Authority of Ireland (FSAI). Published on FSAI website: [www.fsai.ie](http://www.fsai.ie)
- AOAC (2005a). Off. Method 959.08. Paralytic Shellfish Poison, Biological Method. Official Methods of Analysis, 18th Ed., pp. 79–80. Gaithersburg, USA, AOAC Intl.
- AOAC (2005b). Off. Method 2005.06. Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. In: AOAC Official Methods of Analysis, 18th Ed., pp. 81–82. Gaithersburg, USA, AOAC Intl.
- AOAC (2005c). Off. Method 991.26. Domoic Acid in Mussels, Liquid Chromatographic Method. In: AOAC Official Methods of Analysis, 18th Ed., Gaithersburg, USA, AOAC Intl.
- AOAC (2006). Official Method 2006.01.
- Aune, T., Sørby, R., Yasumoto, T., Ramstad, H. & Landsverk, T. (2002). *Toxicon* 40: 77-82.
- Aune, T., Larsen, S., Aasen, J.A.B., Rehmann, N., Satake, M. & Hess, P. (2007). *Toxicon* 49: 1-7.
- Aune, T. *et al.* (2006b). In: Proc. 5th Intl. Conf. Molluscan Shellfish Safety, 14–18 June 2004, Galway, Ireland, B. Deegan, C. Butler, C. Cusack, K. Henshilwood, P. Hess, S. Keaveney, T. McMahon, M. O’Cinneide & J. Silke (eds), Marine Institute, ISBN 1-902895-33-9.
- Bogan, Y. (2006). PhD Thesis: Factors affecting the concentration of domoic acid in scallop, *Pecten maximus*. Letterkenny Institute of Technology, Ireland.
- Britton, R., Roberge, M., Brown, C., Van Soest, R. & Andersen, R.J. (2003). *J. Nat. Prod.* 66: 838-843.
- Brombacher, S., Edmonds, S. & Volmer, D. (2002). *Rap. Comm. Mass Spectrom.* 16: 2306-2316.
- Ciminiello, P., Dell’Aversano, C., Fattorusso, E., Forino, M., Silvana Magno, G., Tartaglione, L., Grillo, C. & Melchiorre, N. (2006). *Anal. Chem.* 78: 6153-6159.
- Dechraoui, M.Y.B., Wacksman, J.J. & Ramsdell, J.S. (2006). *Toxicon* 48: 702-712.
- Dell’Aversano, C., Hess, P. & Quilliam, M.A. (2005). *J. Chromatogr. A* 1081: 190-201.
- FAO/IOC/WHO advanced draft of Expert Consultation Oslo September 2004, on website: [http://www.fao.org/ag/agn/food/risk\\_biotoxin\\_en.stm](http://www.fao.org/ag/agn/food/risk_biotoxin_en.stm), accessed 2006-02-12.

- Fux, E., Biré, R. & Hess, P. (2006a). This volume.
- Fux, E., McMillan, D., Bire, R. & Hess, P. (2007). *J. Chromatogr.* 1157: 273-280.
- Hess, P. & Aasen, J.B. (2007). Ch. 10 in *Chemistry and Pharmacology of Marine Toxins*, L.M. Botana (ed.), Blackwell Publishing, ISBN 978-0-8138-2700-1.
- Hess, P., Nguyen, L., Aasen, J., Keogh, M., Kilcoyne, J., McCarron, P. & Aune, T. (2005). *Toxicon* 46: 62-71.
- Hess, P., Grune, B., Anderson, D.B., Aune, T., Botana, L.M., Caricato, P., van Egmond, H.P., Halder, M., Hall, S., Lawrence, J.F., Moffat, C., Poletti, R., Richmond, J., Rossini, G.P., Seamer, C. & Serratos, Vilageliu J. (2006a). *ATLA* 34: 193-224, EC-VAM workshop-report 55.
- Hess, P., Scurfield, J. & Wells, D.E. (2006b). In: *Proc.5th Intl. Conf. Molluscan Shellfish Safety*, 14-18 June 2004, Galway, Ireland, B. Deegan, C. Butler, C. Cusack, K. Henshilwood, P. Hess, S. Keaveney, T. McMahon, M. O'Connide & J. Silke (eds). Marine Institute, ISBN 1-902895-33-9.
- Hess, P. & Munday, R. (2006c). *Emerging toxins*. Meeting held as part of the 11th Intl. Conf. on Harmful Algae, Cape Town, November 2004. Draft manuscript available from authors.
- Hess, P. & Ramsdell, J.S. (2006d). *Symposium on the toxicity of toxin analogues*, Meeting held as part of the 12th Intl. Conf. on Harmful Algae. Draft manuscript available from authors.
- Hess, P., McCarron, P. & Quilliam, M.A. (2007). *Anal. Bioanal. Chem.* 387: 2463-2474.
- Holland, P.T., Selwood, A.I., Mountford, D.O., Wilkins, A.L., McNabb, P., Rhodes, L.L., Doucette, G.J., Mikulski, C.M. & King, K.L. (2005). *Chem. Res. Toxicol.* 18: 814-816.
- Ito, E., Suzuki, M. & Yasumoto, T. (2006). Poster 8.01, HABXII.
- James, K. J., Diaz Sierre, M., Lehane, M., Brana, Magdalena A. & Furey, A. (2003). *Toxicon* 41: 277-283.
- James, K.J., Furey, A., Lehane, M., Ramstad, H., Aune, T., Hovgaard, P., Morris S., Higman, W., Satake, M. & Yasumoto, T. (2002). *Toxicon* 40: 909-915.
- Katikou, P. (2007). Ch. 5 in *Chemistry and Pharmacology of Marine Toxins*, L.M. Botana (ed.), Blackwell Publishing, ISBN 978-0-8138-2700-1.
- Larsen, K., Petersen, D., Wilkins, A.L., Samdal, I.A., Sandvik, M., Rundberget, T., Goldstone, D., Arcus, V., Hovgaard, P., Rise, F., Rehmann, N., Hess, P. & Miles, C.O. (2007). *Chem. Res. Toxicol.* 20: 868-875.
- MacKinnon, S., Walter, J.A., Quilliam, M.A., Cembella, A.D., LeBlanc, P., Burton, I.W., Harstaff, W.R. & Lewis, N.I. (2006). *J. Nat. Prod.* 69: 983-987
- McCarron, P. & Hess, P. (2006). *Toxicon* 47: 473-479.
- McCarron, P., Kotterman, M., de Boer, J., Rehmann, N. & Hess, P. (2007a). *Anal. Bioanal. Chem.* 387: 2487-2493.
- McCarron, P., Burrell, S. & Hess, P. (2007b). *Anal. Bioanal. Chem.* 387: 2495-2502.
- McCarron, P., Emteborg, H. & Hess, P. (2007c). *Anal. Bioanal. Chem.* 387: 2475-2486.
- MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P. & Selwood, A. (2004). *Toxicon* 44: 901-918.
- McMahon, T. & Silke, J. (1996). *Harmful Algal News* 14: 2.
- Miles, C.O., Wilkins, A.L., Samdal, I.A., Sandvik, M., Petersen, D., Quilliam, M.A. Naustvoll, L.J., Rundberget, T., Torgersen, T., Hovgaard, P., Jensen, D.J. & Cooney, J.M. (2004). *Chem. Res. Toxicol.* 17: 1423-1433.
- Miles, C.O., Samdal, I.A., Aasen, J.A.G., Jensen, D.J., Quilliam, M.A., Petersen, D. Briggs, L.M., Wilkins, A.L., Rise F., Cooney, J.M. & MacKenzie, A.L. (2005). *Harmful Algae* 4: 1075-1091.
- Miles, C.O., Wilkins, A.L., Hawkes, A.D., Jensen, J.J., Selwood, A.I., Beuzenberg, V., MacKenzie, A.L., Cooney, J.M. & Holland, P.T. (2006). *Toxicon* 48: 152-159.
- Molgo, J., Girard, E. & Benoit, E. (2007). Ch. 18 in *Phycotoxins, Chemistry and Biochemistry*, L.M. Botana (ed.), Blackwell Publishing, ISBN 978-0-8138-2700-1.
- Munday, R., Towers, N.R., Mackenzie, L., Beuzenberg, V., Holland, P.T. & Miles, C.O. (2004). *Toxicon* 44: 173-178.
- Murata, M., Kumagai, M., Lee, J. S. & Yasumoto, T. (1987). *Tetrahed. Lett.* 28: 5869-5872.
- Negri, A., Stirling, D., Quilliam, M., Blackburn, S., Bolch C., Burton I., Eaglesham G., Thomas K., Walter J. & Willis, R. (2003). *Chem. Res. Toxicol.* 16: 1029-1033.
- Nezan, E., Antoine, E., Fiant, L., Amzil, Z. & Billard, C. (2006). *Harmful Algae News* 31: 1-3.
- Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y. & Yasumoto, T. (1999). *Nat. Toxins* 7 : 99-102.
- Ofuji, K., Satake, M., McMahon, T., James, K. J.,

- Naoki, H., Oshima, Y. & Yasumoto, T. (2001). *Biosci. Biotechnol. Biochem.* 65: 740-742.
- Onodera, H., Satake, M., Oshima, Y., Yasumoto, T. & Carmichael, W.W. (1997). *Nat. Toxins* 5: 146-151.
- Quilliam, M.A. (2006). Certified Reference Materials for Marine Biotoxins. In Proc. 5th Intl. Conf. Molluscan Shellfish Safety, 14–18 June 2004, Galway, Ireland, B. Deegan, C. Butler, C. Cusack, K. Henshilwood, P. Hess, S. Keaveney, T. McMahon, M. O’Cinneide & J. Silke (eds). Marine Institute, ISBN 1-902895-33-9.
- Rehmann, N., Hess, P. & Yasumoto, T. (2003). Development of a multitoxin isolation scheme for the marine biotoxins dinophysistoxin-2 and azaspiracid-1, -2 and -3 from mussels. Poster 11th Intl. Conf. Marine Natural Products, Sorrento, Italy, 4-8 Sept 2003.
- Rehmann, N., Kilcoyne, J., Yasumoto, T. & Hess, P. (2006). Isolation and purification of azaspiracids. In: Proc. 5th Intl. Conf. Molluscan Shellfish Safety, 14–18 June 2004, Galway, Ireland, B. Deegan, C. Butler, C. Cusack, K. Henshilwood, P. Hess, S. Keaveney, T. McMahon, M. O’Cinneide & J. Silke (eds). Marine Institute, ISBN 1-902895-33-9.
- Rein, K.S., Lynn, B., Gawley, R.E. & Baden, D.G. (1994). *J. Org. Chem.* 59: 2107-2113.
- Shimizu, Y. (1984). In: Herz, W., Grisebach, H., Kirby, G. W. (eds), *Prog. Chem. Org. Nat. Prod.* NY, Springer-Verlag, pp. 235-64.
- Suarez-Gomez, B., Souto, M.L., Cruz, P.G., Fernandez, J.J. & Norte, M. (2005). *J. Nat. Prod.* 68: 596-599.
- Suzuki, T., Walter, J.A., LeBlanc, P., MacKinnon, S., Miles, C.O., Wilkins, A.L., Munday, R., Beuzenberg, V., MacKenzie, L., Jensen, D.J., Cooney, J.M. & Quilliam, M.A. (2006). *Chem. Res. Toxicol.* 19: 310-318.
- Vale, P. (2006). *J. Chromatogr. A*, 1128: 181-188.
- Vale, P. (2007). Ch. 12 in *Chemistry and Pharmacology of Marine Toxins*, L.M. Botana (ed.), Blackwell Publishing, ISBN 978-0-8138-2700-1.
- Vale, C. & Ares, I.R. (2007). Ch. 6 in *Chemistry and Pharmacology of Marine Toxins*, L.M. Botana (ed.), Blackwell Publishing, ISBN 978-0-8138-2700-1.
- Wang, Z., Plakas, S.M., El Said, K.R., Jester, L.E., Granade, H.R. & Dickey, R.W. (2004). *Toxicon* 43: 455-465.
- Wilkins, A.L., Rehmann, N., Torgersen, T., Rundberget, T., Keogh, M., Petersen, D., Hess, P., Rise, F. & Miles, C. O. (2006). *J. Agric. Food Chem.* 54: 5672-5678.

## Development of a wide spectrum method for detection of cyanobacterial toxins by mass spectrometry

S. Hiller<sup>1</sup>, B. Krock<sup>2</sup>, A. Cembella<sup>2</sup> and B. Luckas<sup>1</sup>

<sup>1</sup>Friedrich Schiller University, Institute of Nutrition, Dornburger Str. 25, D-07743 Jena, Germany, susann.hiller@uni-jena.de; <sup>2</sup>Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

### Abstract

Methods based on liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) have been developed for detection of cyanobacterial toxins, including specific target compounds of most of the major known toxin groups. Tandem mass spectrometry analyses are commonly conducted in Multiple Reaction Monitoring (MRM) mode and such methods work well, provided that the target variants are known. Application of specific methods depends on knowledge of the prior occurrence of a known toxigenic organism or at least its potential contribution to the toxin content of the sample, as well as some insight into the expected toxin profile. If this information is lacking, toxin analysis can be very tedious. We developed an LC/MS-MS method, with which typical representatives of all known classes of cyanotoxins, including even undescribed derivatives, can be qualitatively monitored. With this method, diagnostic mass fragments of characteristic compounds of the different classes are detected in the Precursor Ion mode, thus allowing for monitoring of a wide spectrum of cyanotoxins in samples for which the toxin composition is undefined.

### Introduction

Cyanobacterial toxins that occur in brackish and freshwater and occasionally marine ecosystems may be categorized into the following groups: microcystins, nodularins, paralytic shellfish poisoning (PSP) toxins, anatoxins, and cylindrospermopsins. Each class includes a large variety of derivatives, comprising a total of over 100 known toxins.

The development of analytical methods for qualitative and quantitative detection of cyanobacterial toxins has been necessary to monitor these compounds in drinking water and in various other aqueous, plankton, and animal tissue matrices. For example, alternative liquid chromatography (LC)-based methods with mass spectrometric (MS) detection exist for simultaneous determination of various cyanobacterial toxins extracted from phyto-plankton (Dahlmann *et al.* 2003; Dell' Aversano *et al.* 2004; Bogialli *et al.* 2006). All these methods are based on detection by Multiple Reaction Monitoring (MRM). Yet it is impossible to detect all structural variants in this mode because only common transitions and target variants are considered. In contrast, scanning in Precursor Ion mode using a specific fragment molecule and additionally scanning over a mass:charge ( $m/z$ ) range that includes all molecular masses allows the detection of the entire spectrum of related toxins. This tandem MS/MS mode was first employed in 1993 for detection of mi-

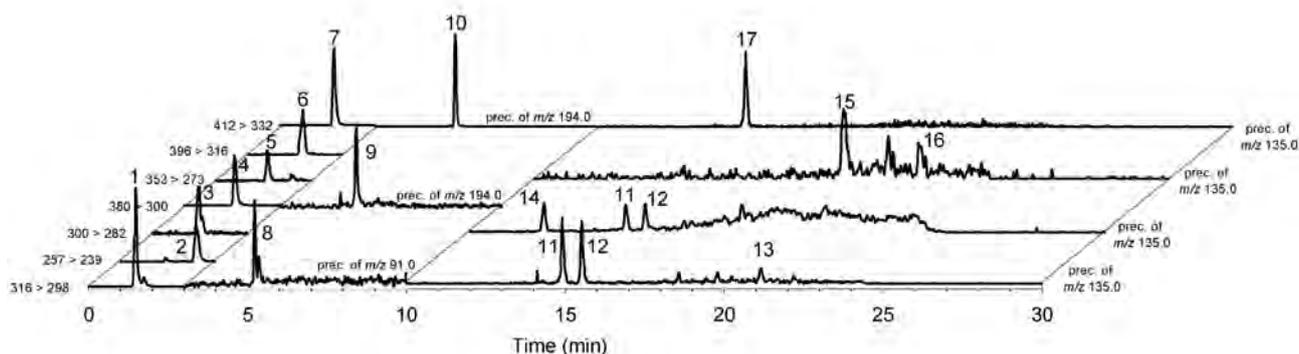
crocystins (MCs), but no further work followed (Edwards *et al.* 1993).

The aim of this method development was to provide a tool for comprehensive toxicity assessment of cyanobacterial samples by qualitative screening for all known classes of toxins. This was achieved in the Precursor Ion mode, which allows the detection of even undescribed toxin variants. The method will be particularly helpful for potentially toxic plankton samples for which the cyanobacterial composition has not been taxonomically characterized.

### Material and Methods

Standard solutions of PSP toxins, microcystins (MCs), nodularin (NOD), anatoxin-a (ANA), and cylindrospermopsin (CYN) were obtained from commercial sources. A sample of *Lyngbya wollei* containing deoxy-cylindrospermopsin (doCYN) was kindly provided by Geoff Eaglesham, Australia.

The analyses were performed on an Agilent Series 1100 HPLC system coupled via Turbo ion-spray source to a Sciex 4000 Q Trap mass spectrometer (ABI Sciex, Darmstadt, Germany). The chromatographic separation was carried out on a Luna column (3  $\mu$ m, 150 mm x 3.0 mm; Phenomenex, USA) using two eluents containing 50 mM formic acid and 2 mM ammonia formate in water (eluent A) or methanol/water (95/5, eluent B) and gradient elution (0 % B



**Figure 1.** Multi-compound chromatogram for cyanobacterial toxins. First period: MRM chromatogram of PSP toxins – NEO (1), dcSTX (2), STX (3), B1 (4), dcGTX 2/3 (5), GTX 2/3 (6), GTX 1/4 (7); Second period: Precursor Ion spectrum of m/z 91.0 for ANA (8), m/z 194.0 for CYN (9) and doCYN (10); Third period: Precursor Ion spectrum of m/z 135.0 for microcystins (MCs) and nodularins (NODs) – scan range m/z 400-550 with 17 eV CE for MC-YR (11), MC-LR (12), and MC-LW (13), scan range m/z 400-550 with 35 eV CE for MC-RR (14), m/z 900-1100 with 60 eV CE for MC-LA (15) and MC-LF (16), m/z 800-850 with 90 eV CE for NOD (17).

for 1 min, switched to 50 % B, held for 4 min, then further to 90 % B in 10 min, held for 5 min, then re-equilibrated).

Although this MS-MS method was only developed for qualitative screening, the limits of detection (LOD) were estimated for reference standards of cyanobacterial toxins, namely for PSP toxins: neosaxitoxin (NEO), decarbamoyl-saxitoxin (dcSTX), saxitoxin (STX), sulfo-carbamoyl toxin B1 (B1), decarbamoyl-gonyautoxins 2/3 (dcGTX 2/3), gonyautoxins 2/3 (GTX 2/3), gonyautoxins 1/4 (GTX 1/4), as well as for CYN, ANA, MC-LA, -LW, -LF, -LR, -YR, -RR, and NOD. Quantitative determination was carried out in Precursor Ion mode from chromatographic analysis of pure standard solutions of known concentrations. The peak height to averaged background noise ratio (S/N) was measured, whereby the background noise was defined as the baseline near the analyte peak. The LOD was calculated as a S/N of 3:1. For quantification of the epimeric pairs GTX1/GTX4, GTX2/GTX3, dcGTX2/dcGTX3, having the same molecular weight but different fragmentation patterns, an additional analysis yielding complete chromatographic separation of the epimers by hydrophilic interaction liquid ion chromatography (HI-LIC) (Dell' Aversano *et al.* 2004) was performed to determine the toxin response factors for quantification.

## Results and Discussion

The specific chromatographic gradient profile was chosen to keep all cyanotoxins belonging to each group within a limited range of retention times, in order to ensure inclusion of all occurring variants. A baseline separation of all toxin derivatives was there-

fore not achieved, but this is not necessary for such a qualitative method.

Based on the retention times of all cyanobacterial toxins (Table 1), three time periods were defined to optimize detection in different modes and with alternative parameters. For example, during the first three minutes, PSP toxins were detected in MRM mode, but there is no typical daughter ion that could be used in Precursor Ion mode to discriminate these compounds. The PSP toxins were all eluted between 1.4 and 1.7 min on the C18 column, showing no chromatographic separation of NEO, dcSTX, STX, B1, dcGTX 2/3, GTX 2/3, GTX 1/4, but resulting in a positive signal in case of occurrence in sample material (Fig. 1).

In contrast, for ANA, homo-anatoxin (HANA), CYN, doCYN, MCs, and NODs, a characteristic fragment for each toxin group is known and was utilized to analyse the compounds in Precursor Ion mode (Edwards *et al.* 1993; Dell' Aversano *et al.* 2004; James *et al.* 2005). Within the second period (3-10 min), ANA was detected by selecting m/z 91.0 as the precursor ion; CYN and doCYN were analysed based on m/z 194.0 as the daughter ion. Scan ranges for all the anatoxins was m/z 100-300, thereby including ANA, HANA, and degradation products. To include CYN, doCYN, epicylindrospermopsin (EpiCYN), and possible undescribed variants among the cylindrospermopsins, the mass range was set at m/z 300-500.

Microcystin and nodularin variants were analysed within the last period (until 30 min). The MC derivatives containing arginine (Arg) in position 2 and 4 of the peptide ring, such as MC-RR, show doubly charged ions as the base peak in a mass spectrum, in contrast to MCs with only one Arg residue, as for

**Table 1.** Retention times, MRMs, precursor ions and LOD of cyanobacterial toxins.

period 1				period 2				period 3			
Toxin	Retention time (min)	mass transition (m/z)	LOD (pg) S/N = 3	Toxin	Retention time (min)	Precursor ion (m/z)	LOD (pg) S/N = 3	Toxin	Retention time (min)	Precursor ion (m/z)	LOD (pg) S/N = 3
NEO	1.4	316 > 298	1.0	ANA	5.2	91.0	700	MC-RR	12.3	135.0	20
dcSTX	1.4	257 > 239	9.0	CYN	5.4	194.0	9	MC-YR	14.9	135.0	15
STX	1.4	300 > 282	10.0	doCYN	5.5	194.0	no standard	MC-LR	15.5	135.0	15
B 1	1.5	380 > 300	3.0					MC-LA	19.7	135.0	18
dcGTX 2/3	1.6	353 > 273	25.0					MC-LW	21.0	135.0	100
GTX 2/3	1.6	396 > 316	12.0					MC-LF	22.0	135.0	100
GTX 1/4	1.7	412 > 332	6.0					NOD	14.6	135.0	6

MC-LR or MC-YR. In the mass spectrum of the latter compounds,  $[M+H]^+$  ions are observed as the main peak and the  $[M+2H]^{2+}$  ions give only weak signals. On the other hand, MCs without Arg, such as MC-LA or MC-LF, form only singly charged ions (Yuan *et al.* 1999). Hence, for MCs both mass ranges (i.e.,  $[M+H]^+$  (m/z 900-1100) and  $[M+2H]^{2+}$  (m/z 400-550)) had to be covered using different fragmentation parameters, including the declustering potential (DP) and collision energy (CE). To achieve good sensitivity among the doubly charged ions for the MCs with two Arg residues, milder fragmentation conditions had to be chosen in comparison to conditions for derivatives containing only one Arg. Three different experiments were therefore required for detection of all MCs. In the case of MC-LW, without an Arg residue, a signal of  $[M+2H]^{2+}$  instead of  $[M+H]^+$  was observed. Nodularins form singly charged base ions, within the scan range m/z 800-850. The derivatives MC-RR and MC-LF, as representative MCs that elute early and late, respectively, on a C18 column, were employed to set elution time constraints to cover the entire range of microcystins (Hiller *et al.* 2007).

## Conclusions

We developed an effective analytical tool for the rapid, qualitative detection of cyanobacterial toxins in phytoplankton samples from fresh, marine or brackish waters. This MS-MS method includes detection of all major classes of cyanotoxins, including PSP toxins, ANAs, CYNs, MCs, and NODs, yielding a distinctive positive or negative signal for each toxin or toxin group. If a cyanotoxin peak is detected, a more sensi-

tive and definitive analytical method must be applied to ensure the toxin identity and for quantification.

The application of the Precursor Ion mode instead of the commonly used MRM mode for MS-MS detection of cyanotoxins enables detection of a wide range of structural variants, resulting from the presence of different functional groups and/or amino acid residues in the molecule.

An advantage of this qualitative method is in the ability to detect uncommon and undescribed cyanobacterial toxin variants in a rapid, cursory analysis of putatively toxic samples. Moreover, this capability is maintained even for samples for which both the taxonomic status of the cyanotoxigenic organisms and the toxin profile are unknown.

## References

- Bogialli, S., Bruno, M., Curini, R., Di Corcia, A., Fanali, C. & Laganà, A. (2006). *Env. Sci. Technol.* 40: 2917-2923.
- Dahlmann, J., Budakowski, W.R. & Luckas, B. (2003). *J. Chromatogr. A* 994: 45-57.
- Dell'Aversano, C., Eaglesham, G.K. & Quilliam, M. A. (2004). *J. Chromatogr. A* 1028: 155-164.
- Edwards, C., Lawton, L.A., Beattie, K.A., Codd, G.A., Pleasance, S. & Dear, G.J. (1993). *Rapid Comm. Mass Spectrom.* 7: 714-721.
- Hiller, S., Krock, B., Cembella, A. & Luckas, B. (2007). *J. Mass Spectrom.*, submitted.
- James, K.J., Crowley, J., Hamilton, B., Lehane, M., Skulberg, O. & Furey, A. (2005). *Rapid Comm. Mass Spectrom.* 19: 1167-1175.
- Yuan, M., Namikoshi, M., Otsuki, A., Watanabe, M. F. & Rinehart, K. L. (1999). *J. Am. Soc. Mass Spectrom.* 10: 1138-1151.

## Evidence of yessotoxins in Alfacs Bay- toxic effect evaluation by cell-based assays and toxin profile determination by liquid chromatography

E. Mallat<sup>1,2</sup>, E. Cañete<sup>1,2</sup>, A. Caillaud<sup>1,2</sup>, M. Fernández<sup>1,2</sup>, I. Bravo<sup>2</sup>, B. Paz<sup>2</sup>, J.M. Franco<sup>2</sup> and J. Diogène<sup>1,2</sup>

<sup>1</sup>IRTA, Centre d'Aqüicultura, Ctra. Poble Nou s/n, St. Carles de la Ràpita, 43540 Tarragona, Spain, elena.mallat@irta.es; <sup>2</sup>CRA Centre de Referència en Aqüicultura, CIRIT-Generalitat de Catalunya, Spain

<sup>3</sup>Unidad Asociada (CSIC-IEO) Fitoplácton Tóxico, Instituto Español de Oceanografía, Centro Oceanográfico de Vigo, 36200 Vigo, Spain, jose.franco@vi.ieo.es

### Abstract

In July 2005 a bloom of the dinoflagellate *Protoceratium reticulatum* at cell concentrations of 1600 cells.L<sup>-1</sup> was detected in Alfacs Bay, located in the northwestern Mediterranean, concomitant with the presence of *Dinophysis sacculus*. Positive DSP mouse bioassay measurements from samples from the bay indicated the presence of diarrhetic toxins in mussels. Analytical procedures have been optimised and applied to phytoplankton and mussel extracts. Mussel samples were first extracted in methanol/H<sub>2</sub>O (80:20), further percolated through an octadecylsilica cartridge, derivatized using the dienophile fluorescence reagent, DMEQ-TAD, and subsequently analysed by LC-FD. Yessotoxins in mussels were detected during this event at concentrations of 16-50 µg.kg<sup>-1</sup>. Phytoplankton samples were extracted by sonication with methanol followed by a clean-up step. Okadaic acid concentration levels in these samples were also studied to confirm the toxic effects recorded along the bloom of *P. reticulatum* and *D. sacculus*. Evaluation of cell-based toxicity of field samples through estimation of cell viability and morphological effects was studied and compared to the analytical measurements.

### Introduction

Yessotoxin and its derivatives constitute a group of polyether toxins produced by marine dinoflagellates, such as *Protoceratium reticulatum* and *Gonyaulax polyedra*, which have been traditionally included in the diarrhetic shellfish poisoning (DSP) group of toxins, although they do not inhibit protein phosphatases and do not lead to diarrhetic symptoms. The effects of these disulphated polyethers are not precisely described, but there are some indications that have an effect on calcium homeostasis of human lymphocytes (De la Rosa 2001). Apoptogenic activity of yessotoxins has also been reported (Leira *et al.* 2002; Suárez Korsnes *et al.* 2004). One of the major drawbacks of the presence of these compounds in mussel samples comes from their interference in DSP mouse bioassay measurements. Recently, the development of mass spectrometric techniques has permitted isolation and identification of several yessotoxin derivatives. Thus, hydroxyYTX, carboxyYTX, 1a-homo YTX, trinorYTX, 41a-homoYTX, 9-methylYTX, hydroxylamideYTX, 32-O-mono-hydroxyl-amideYTXs, diglycosylYTXs, 32-O-mono- and diarabinosides of 1a-homoYTX and up to 90 different analogues have been identified by Ciminiello *et al.* (2003), Miles *et al.* (2005), Paz *et al.* (2004), Finch *et al.* (2005), Satake *et al.* (1999), and Souto *et al.* (2005). *Protoceratium reticulatum* was previously detected in phytoplankton

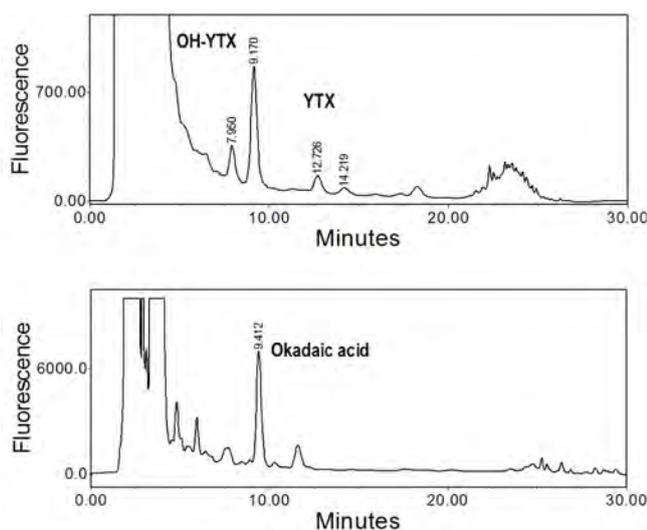
samples from the Ebro Delta embayments in summer 2001, and concentration levels of yessotoxin and homoyessotoxin were confirmed in mussel samples.

### Method Optimization and Results

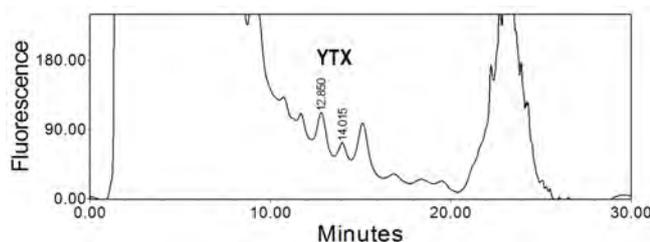
#### Chromatographic analysis

An extraction procedure for phytoplankton and mussel samples was carried out. Digestive glands from mussels were extracted using MeOH/H<sub>2</sub>O (80:20) under sonication, centrifuged and further filtered through a 0.45-µm nylon filter. Phytoplankton samples were first filtered through a GF/F filter, extracted twice in methanol under sonication and subsequently extracted with butanol. Clean-up of the extracts, both phytoplankton and mussel extracts, was done using an octadecylsilica cartridge. The eluates from the solid phase extraction were collected and evaporated under nitrogen. Derivatization of the extracts was accomplished using the dienophile reagent, DMEQ-TAD after 2 h of reaction at room temperature under dark conditions. An aliquot of methanol was then added to quench the excess of reagent, and the remaining extract was evaporated and reconstituted in methanol. Samples were injected in the HPLC using an octadecylsilica column and a methanolic/water mobile phase and detected fluorimetrically at  $\lambda_{exc}=370$  nm,  $\lambda_{em}=440$  nm. Okadaic acid was determined using a modification of Lee's method (Lee *et al.* 1987). An

aliquot of the above mentioned methanolic/aqueous mussel extracts was percolated through a Diaion HP-20 hand-packed column previously conditioned and eluted by methanol. The eluate was evaporated to dryness and derivatized using ADAM (9-anthryldiazomethane) for 2 h under dark conditions to obtain the okadaiate-ADAM derivatives. A silica clean-up was carried out and the eluate was further evaporated. Samples were reconstituted in methanol and injected into the HPLC with fluorimetric detection. Results are presented in Figs 1 and 2 and Table 1.



**Figure 1.** LC-FD chromatogram corresponding to the determination of YTX and okadaic acid (OA) in digestive glands from a mussel sample (Alfacos Bay, July 2005).



**Figure 2.** LC-FD chromatogram corresponding to the determination of YTX in a filter sample collected from the Alfacos Bay and filtered through GF/F.

#### Cytotoxicity measurements

The toxicity of yessotoxin on *in vitro* cultured fibroblasts (Buffalo Green Monkey, BGM) was assessed using a cell-viability assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium, MTT test, Manger *et al.* 1993). A standard of YTX tested up to 2000 ng.mL<sup>-1</sup> showed slight toxicity and therefore the IC<sub>50</sub> could not be determined. The low cytotoxic activity of YTX on BGM fibroblasts suggests the need to use other cellular models like the mouse fibroblast NIH3T3 cell line (Malagoli *et al.* 2006) or L6 y BC3H1 myoblast

**Table 1.** Yessotoxin and okadaic acid concentration in mussel samples collected during the *P. reticulatum* event in Alfacos Bay (Summer 2005) and densities of most relevant phytoplankton species (cells.L<sup>-1</sup>).

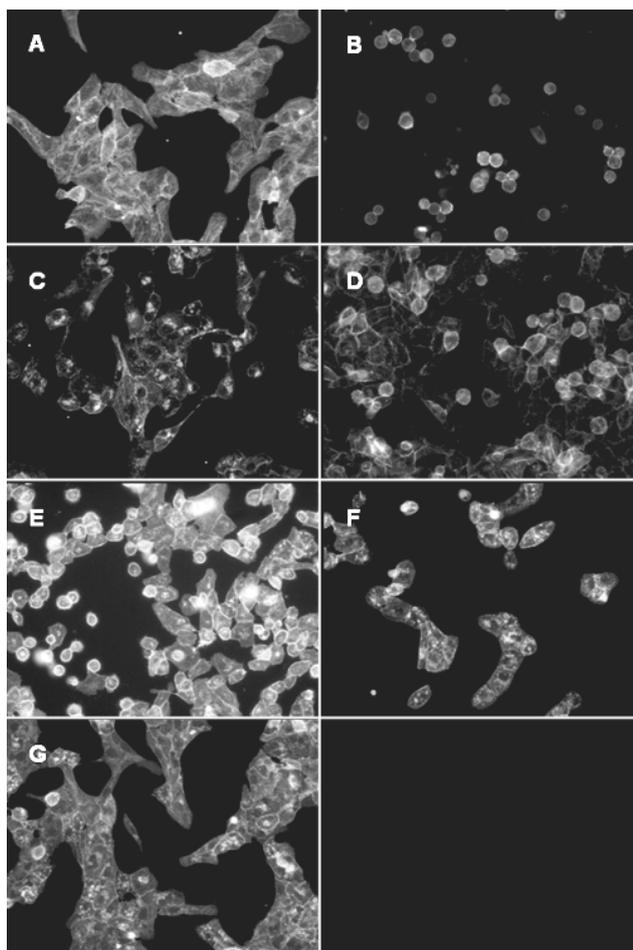
Sample	µg YTX.kg <sup>-1</sup> mussel	µg OA.kg <sup>-1</sup> mussel	<i>P. reticulatum</i> cells.L <sup>-1</sup>	<i>Prorocentrum</i> sp. cells.L <sup>-1</sup>	<i>Dinophysis caudata</i> cells.L <sup>-1</sup>
AM84140705	50.0	11.6	1920	0	40
AM47140705	38.1	4.6	1260	80	280
AM40140705	15.9	4.0	100	40	680

cell line (Suárez Korsnes *et al.* 2006) on which YTX is reported to be cytotoxic.

Fibroblasts previously exposed to toxin standards and natural extracts were stained with fluorescent phalloidin that specifically binds the F-actin microfilaments. Treatments included a standard of YTX, OA, mussel samples collected during two *P. reticulatum* events (2005 and 2006) and a phytoplankton sample containing 1394 *P. reticulatum* cells.L<sup>-1</sup> seawater. Control fibroblasts showed organization of the F-actin filaments as elongated fibres in the cytoplasm (Fig. 3A). When cells were treated with YTX and OA standards, both treatments induced F-actin disorganization in the cytoplasm. YTX induced granulation of actin in the cytoplasm (punctuated staining, Fig. 3B) in cells that preserved a “fibroblastic” shape, whereas OA induced shortening and loss of the F-actin fibres (Fig. 3C) in rounded cells. Mussel samples collected during the *P. reticulatum* events induced actin granulation in the cytoplasm (Figs 3F, 3G) similar to YTX. This alteration was not observed in cells exposed to a mussel sample collected when *P. reticulatum* was absent (Fig. 3D). For this last extract, we observed cell alterations similar to those obtained with the OA standard, suggesting that OA could be present in this sample. Cells exposed to a phytoplankton sample collected during a *P. reticulatum* event (Fig. 3E) showed actin granulation in fibroblastic-type cells and actin accumulation in rounded cells. These results confirmed the previously reported effect of YTX on the actin cytoskeleton disruption during the apoptotic process induced by YTX (Suárez Korsnes *et al.* 2007).

#### Conclusions

YTX, OH-YTX and OA were detected and quantified by LC-FD at ppb concentration levels in natural sam-



**Figure 3.** Fibroblasts BGM stained with the fluorescent phalloidindine-TRITC after 24 h exposure to the different treatments : A: control, B : OA standard ( $100 \text{ ng.mL}^{-1}$ ), C : YTX standard ( $200 \text{ ng.mL}^{-1}$ ), D : mussel extract ( $60 \text{ mg.mL}^{-1}$  MEM), E : phytoplankton sample ( $424 P. reticulatum$  cell eq. $\text{mL}^{-1}$ MEM), F : mussel extract obtained during the 2005 *P. reticulatum* event ( $60 \text{ mg.mL}^{-1}$  MEM), G : mussel extract of a 2006 *P. reticulatum* event ( $60 \text{ mg.mL}^{-1}$  MEM).

ples from Alfacs Bay (mussels and phytoplankton). Further experiments using LC-MS-MS should be performed to characterize YTX and YTX derivative profiles in phytoplankton and mussel and to detect possible metabolites and transformation products. In this study, alterations in the organization of the F-actin cytoskeleton have been used as a marker of the presence of YTX in natural samples. More work is needed to investigate the action mechanism of yessotoxin and derivatives and to develop valid detection methods.

In reference to the monitoring programme for marine toxins, the presence of YTX in Alfacs Bay could interfere with DSP toxicity evaluation assessed by the mouse bioassay. Therefore, the presence of YTX should be taken into account when assessing DSP risk.

## Acknowledgements

We kindly acknowledge the support received by the technical staff from IRTA-Sant Carles de la Ràpita. This study was supported by the project ACU-02-005, INIA, Ministry of Education and Science, Spanish Government/Centre de Referència en Aqüicultura, DURSI, Generalitat de Catalunya.

## References

- Botana, L.M. (2001). *Biochem. Pharmacol.* 61: 827-833.
- Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, S., Guerrini, F., Pistocchi, R. & Boni, L. (2003). *Toxicon* 42: 7-14.
- De la Rosa, L.A., Alfonso, A., Vilariño, N., Vieytes, M.R., Finch, S.C., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., MacKenzie, A.L., Beuzenberg, V., Quilliam, M.A., Olseng, C.D., Samdal, I.A., Aasen, J., Selwood, A.I., Cooney, J.M., Sandvik, M. & Miles, C.O. (2005). *Toxicon* 46: 160-170.
- Lee, J.S., Yanagi, T., Kenma, R. & Yasumoto, T. (1987). *Agric. Biol. Chem.* 51: 877-881.
- Leira, F., Alvarez, C., Vieites, J.M., Vieytes, M.R. & Botana, L.M. (2002). *Toxicol. in Vitro* 16: 23-31.
- Malagoli D., Marchesini E. & Ottaviani E. (2006). *Toxicol. Lett.* 167: 75-83.
- Manger R.J., Leja L.S, Hungerford J.M. & Wekell M.M. (1993). *Anal. Biochem.* 214: 190-194.
- Miles, C.O., Samdal, I.A., Aasen, J. Jensen, D.J., Quilliam, M.A., Petersen, D., Briggs, L.R., Wilkins, A.L., Rise, F., Cooney, J.M. & MacKenzie, A.L. (2005). *Harmful Algae* 4: 1075-1091.
- Oshima, Y. (1999). *Nat. Toxins* 7:147-150.
- Paz, B., Riobó, P., Fernández, M.L., Fraga, S. & Franco, J.M. (2004). *Toxicon* 44: 251-258.
- Sandvik, M. & Miles, C.O. (2005). *Toxicon* 46: 160-170.
- Satake, M., Ichimura, T., Sekiguchi, K., Yoshimatsu, S., Souto, M.L., Fernández, J.J., Franco, J.M., Paz, B., Gil, L.V. & Norte, M. (2005). *J. Nat. Prod.* 68: 420-422.
- Suárez Korsnes, M., Hetland, D.L., Espenes, A., Tranulis & M, Aune, T. (2004). *Book of Abstracts, The 5th Int. Conf. Molluscan Shellfish Safety, Galway, 14-18 June 2004*, p. 163.
- Suárez Korsnes, M., Hetland, D.L., Espenes, A., Tranulis M.A. & Aune T. (2006). *Toxicology in Vitro* 20: 1077-1087.
- Suárez Korsnes, M., Hetland, D.L., Espenes, A. & Aune T. (2007). *Toxicology in Vitro* 21: 9-15.

## Analysis of phycotoxins in hand-picked plankton cells by micro-column liquid chromatography-tandem mass spectrometry

M.A. Quilliam<sup>1</sup>, N.I. Lewis<sup>1</sup>, J. Aasen<sup>2</sup> and W. Hardstaff<sup>1</sup>

<sup>1</sup>National Research Council of Canada, Institute for Marine Biosciences, Halifax, NS, Canada

<sup>2</sup>The Norwegian School of Veterinary Science, Department of Food Safety and Infection Biology, Oslo, Norway

### Abstract

The toxin concentration and profile present in plankton can vary considerably between different geographical areas and even within a region and between seasons due to the presence of different species and strains. Traditional methods of analysis require substantial sample sizes (e.g., thousands to millions of cells) making it difficult to attribute the presence of toxins or variations in toxin profiles to individual species. A new method has been developed for the analysis of toxin content of single cells from both cultures and field samples. This sensitive method uses a micro-sampling and extraction procedure coupled with micro-column liquid chromatography-tandem mass spectrometry (LC-MS). LC-MS analyses of spirolides (SPX), pectenotoxins (PTX) and yessotoxins (YTX) from intact cells picked from field samples are provided. Variations in toxin profiles were observed in single cells of *Alexandrium ostenfeldii* from a sample collected at Ship Harbour, where we have observed considerable variability in toxin profile in previous years. In clonal cultures of *A. ostenfeldii* (NRC-AOSH1) similar toxin profiles were observed, however there was a strong correlation between cell size and toxin concentration.

### Introduction

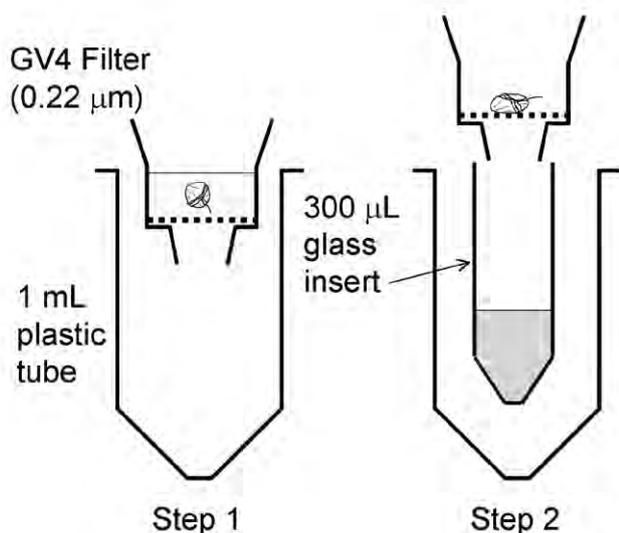
Early detection and identification of potentially toxic species is an important part of efforts to mitigate the effect of harmful algal blooms. The chemistry of phytotoxins can be complicated with many different analogues of varying degrees of toxicity being produced by the algae. Considerable variability occurs in toxin profiles of plankton concentrates from natural populations and may be due to the presence of different species and strains, or to the influence of environmental conditions and the age of a bloom. LC-MS has emerged as an important tool for detecting and analysing these toxins. A particular strength of this method is its ability to measure multiple toxins simultaneously. Traditional methods of analysis require thousands to millions of cells making it difficult to attribute the presence of toxins or variations in toxin profiles to individual species. A new highly sensitive method using micro-sampling and extraction procedures coupled with micro-column liquid chromatography-tandem mass spectrometry for analysis of lipophilic toxins has been developed. With this technique samples as small as single cells can be analysed.

### Materials and Methods

Field samples were collected from Ship Harbour, NS by vertical net tow. Clonal isolates of *Alexandrium ostenfeldii* and *Protoceratium reticulatum* were estab-

lished by micropipette isolation. Cultures were incubated in L1 medium (Guillard & Hargraves 1993) at 14 °C, photon flux density 50 – 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (*A. ostenfeldii*), or at 16 °C, photon flux density 90 – 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . (*P. reticulatum*) Motile *A. ostenfeldii* cells were photographed at 200 $\times$  magnification using a Leica DMRE light microscope. Estimations of cell volume and measurements of cell diameter were made using Simple PCI image analysis software (Compix Inc. Imaging Systems, Sewickley, PA). Occasionally cells stick to the glass surface of the pipette resulting in blank samples. To avoid transfer of more than one cell, a fresh glass micropipette was used at each stage of the procedure. Excess seawater was removed from the cell(s) by centrifugation at 2000  $\times$  g for 2 min at room temperature.

Sterile filters (Millex GV4, 4 mm, 0.22  $\mu\text{m}$ ; Millipore) were soaked in methanol (MeOH) overnight then flushed with 500  $\mu\text{L}$  MeOH and air-dried. Immediately prior to use the filters were washed with 100  $\mu\text{L}$  MeOH, centrifuged at 2000  $\times$  g for 5 min, then rinsed with 100  $\mu\text{L}$  of filtered seawater (FSW; 0.22  $\mu\text{m}$ ) and re-centrifuged. Cells were transferred by micropipette to the equilibrated GV4 filters which were then placed into 300  $\mu\text{L}$  micro-volume glass inserts in a microcentrifuge tube for extraction with 75  $\mu\text{L}$  of 80 % MeOH (see Fig. 1). Spirolides were extracted with 80 % MeOH with 0.1 % formic acid. Filters were allowed to stand for 5 min, and were then centrifuged



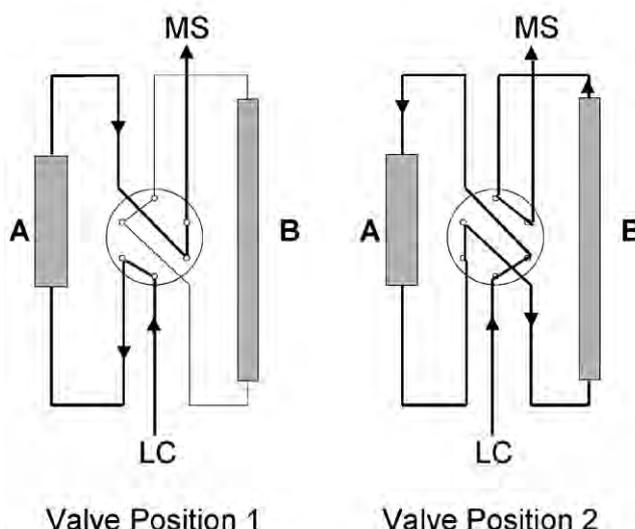
**Figure 1.** Micro-filtration system designed for extracting single cell samples.

at  $2000 \times g$  for 5 min to collect the filtrate. The inserts containing the single cell extracts were stored at  $-12^\circ\text{C}$ .

Liquid chromatography (LC) separation was performed using an Agilent 1100 series HPLC (Palo Alto, CA) equipped with an OASIS-HLB ( $10\ \mu\text{m}$ ,  $20 \times 2\ \text{mm}$  i.d.) and Hypersil-BDS-C8 ( $10\ \mu\text{m}$ ,  $150 \times 1\ \text{mm}$  i.d.) columns connected through a switching valve (see Fig. 2). Mobile phases for spirolides and okadaic acid-group toxins were (A)  $\text{H}_2\text{O}$  and (B) 95 % acetonitrile, both with 50 mM formic acid and 2 mM ammonium formate. For YTX analysis 5 mM ammonium acetate (pH 7) was used as an additive in both A and B. The OASIS column was equilibrated for 15 min with 10 % B at  $75\ \mu\text{L}/\text{min}$  (valve position 1) then sample extracts ( $50\ \mu\text{L}$ ) were injected and the flow increased to  $200\ \mu\text{L}/\text{min}$  for 2 min. Analytes were eluted by back-flushing from the OASIS cartridge onto the Hypersil column (valve position 2) at  $75\ \mu\text{L}/\text{min}$ . Toxins were eluted with a multi-step gradient as follows: 1) 10 to 30 % B for 0.5 min; 2) 30 – 100 % B for 10 min; then, 3) 18 min at 100 % B. YTXs required the following gradient: 1) 10 to 40 % B for 0.5 min; 2) 40 – 100 % B for 7.5 min; then 3) held at 100 % B for 18 min. Selected reaction monitoring (SRM) of major transition ions was performed in positive ion mode with an API 4000 triple quadrupole MS system (PE-SCIEX, Concord, ON) equipped with a Turbo IonSpray source (SCIEX, Streetville, ON, Canada).

## Results and Discussion

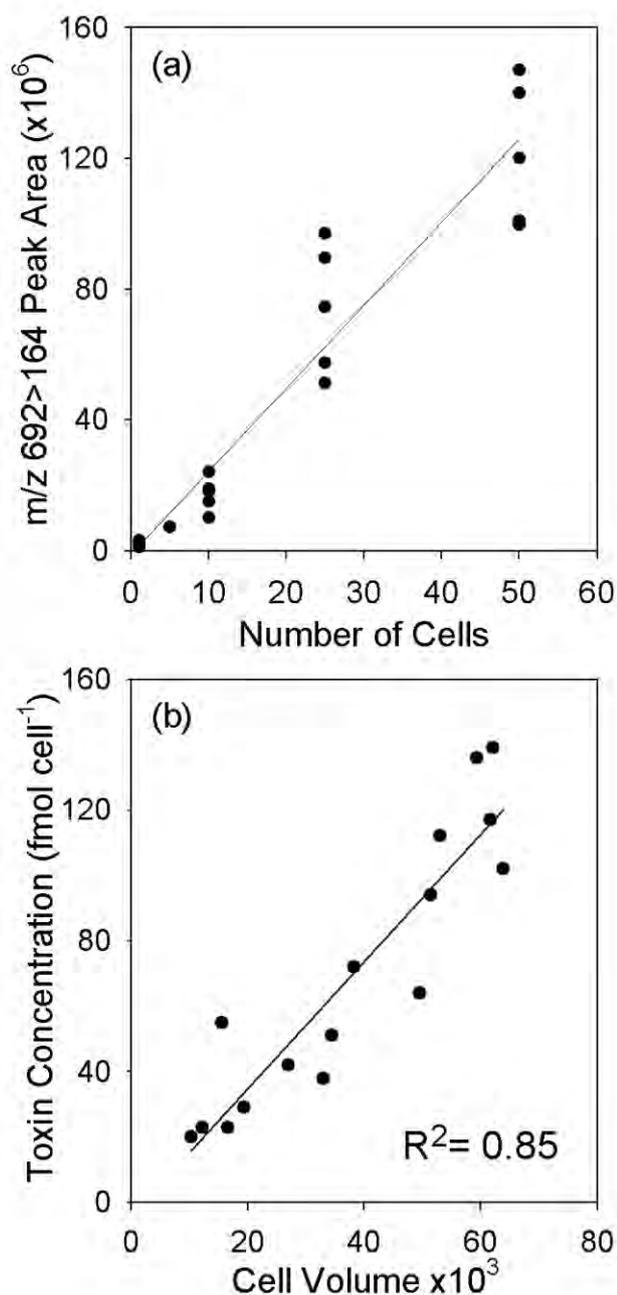
Extraction of toxins from a single cell required a micro-volume apparatus to ensure minimal loss and



**Figure 2.** Column switching system used for analysis. Column A: OASIS-HLB, and Column B: Hypersil-BDS-C8.

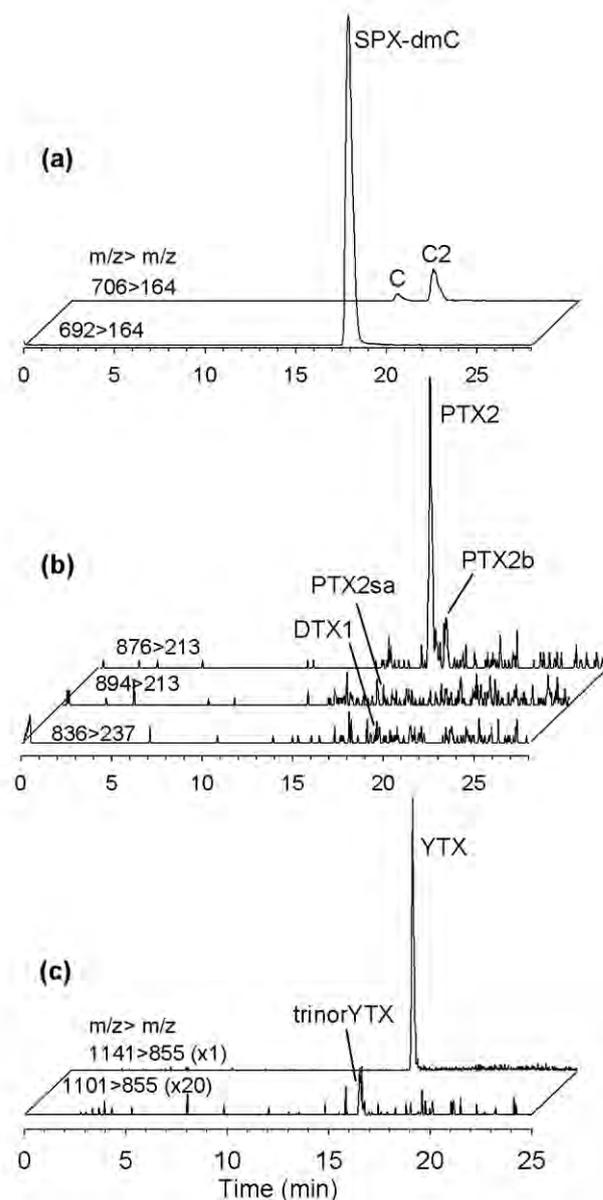
maximum yields. The Millex-GV4 filters served this purpose by allowing single cells to be trapped on the surface of a  $0.22\text{-}\mu\text{m}$  membrane and allowing subsequent removal of excess seawater. Collection of the extract was done directly into a micro-volume insert, minimizing sample handling and reducing loss of toxins to surfaces. Preliminary experiments confirmed that soaking the cells for 5 min in  $75\ \mu\text{L}$   $\text{MeOH}:\text{H}_2\text{O}$  (8:2) allowed complete extraction and good recovery after centrifugal filtration. On this basis  $\text{MeOH}:\text{H}_2\text{O}$  (8:2) was selected as the extraction solvent since it facilitated cell rupture and was compatible with direct injection into the LC-MS system.

The challenge was to attain enough sensitivity in order to quantitatively detect the very low toxin concentrations in the extract. The microbore ( $1\ \text{mm}$  i.d.) Hypersil column used in these studies, operates with a low flow rate of  $40\text{--}75\ \mu\text{L}/\text{min}$  permitting an LC-MS signal approximately five times higher than with a  $2\ \text{mm}$  i.d. column at  $200\ \mu\text{L}/\text{min}$  flow or 15–25 times higher than with a  $4.6\ \text{mm}$  i.d. column at  $1\ \text{mL}/\text{min}$  with the same injection size. Since ESI MS responds as a concentration-dependent detector, a low flow rate results in less dilution of a given quantity of analyte injected. The injection volume that a  $1\ \text{mm}$  i.d. column can tolerate in order to maintain chromatographic performance is limited and depends on the strength of the solvent in which the sample is dissolved. When 80 % MeOH was used as an extractant only  $1\ \mu\text{L}$  could be injected directly onto the column without sacrificing peak shape. The use of an OASIS-HLB cartridge column ( $2\ \text{mm}$  i.d.) provided a high sample injection capacity that was required for maximal sensitivity. It



**Figure 3.** (a) Concentration of spirolides showing a linear relationship with number of cells isolated and extracted from an *Alexandrium ostenfeldii* (AOSH1) culture. (b) Correlation of toxin concentration (fmol cell<sup>-1</sup>) with cell volume of isolated AOSH1 cells.

was then possible to inject up to 50  $\mu$ L of a solution of lipophilic toxins in 80 % MeOH (in H<sub>2</sub>O) without significant breakthrough. After washing the column to remove excess MeOH, salts and early eluting compounds, a gradient elution at low flow (75  $\mu$ L/min) was conducted with the OASIS column reversed from the injection position so that the analytes were back-flushed onto the micro-bore Hypersil column (1 mm i.d.). Using this system, it was possible to sharpen analyte peaks from a 50- $\mu$ L injection volume and pro-



**Figure 4.** Analysis of single cell extracts of (a) *Alexandrium ostenfeldii* (AOSH1) (b) *Dinophysis acuminata* from field samples and (c) *Protoceratium reticulatum* (PRSH6).

duce excellent sensitivity. Serially diluted standard solutions of 13-desmethyl spirolide C showed excellent linearity ( $r^2=0.99$ ), a near zero intercept and a detection limit of 2 pM (5 fmol injected).

When multiple cell extracts were examined, a linear relationship between cell number and toxin concentration was observed ( $r^2 = 0.91$ , Fig. 3a). Increasing scatter with a greater number of cells in the extract may be due to different extraction yields or to variations in toxin content per cell.

Toxin concentrations in single *A. ostenfeldii* cells picked from a field sample ranged from 0 – 37 fmol cell<sup>-1</sup> and did not correlate well to cell volume ( $r^2 = 0.23$ ). Undetected or low concentrations of spirolides

may be due to lost or broken cells or to cells being incorrectly identified as *A. ostenfeldii*. *In situ* populations of *A. ostenfeldii* are likely to be exposed to varying environmental conditions, which could influence growth rate and thus toxin production. Other factors such as life history phase may also influence spiroside cell quota (John *et al.* 2000) in natural populations. Variation in toxin profiles suggests that different strains of this species exist within the population, which explains the considerable variability in toxin profiles from bulk plankton samples observed over several years at this site (data not shown).

The volume of single cells isolated from an exponentially growing isolate of *A. ostenfeldii* (NRC-AOSH1) was  $10 - 64 \times 10^3 \mu\text{m}^3$  and the toxin concentration increased with cell volume from 20 to 140 fmol cell<sup>-1</sup> ( $R^2 = 0.85$ , Fig. 3b). Although total spiroside concentration varied, the toxin profiles were consistent (Fig.4a).

This method was also applied to the analysis of pectenotoxins from single-cell extracts (Fig. 4b) and extracts obtained from a pool of 25 cells of *Dinophy-*

*sis acuminata*. Pectenotoxins including PTX2 and PTX2b were observed in both samples and trace levels of dinophysistoxin (DTX1) were detected in the 25-cell extract, but were below the detection limit in the single cell analysis. Some PTX2 seco acid (PTX2sa), produced by enzymatic hydrolysis of the PTX2, was observed in the 25-cell sample but was either below the detection limit or was not present in single cells because faster isolation times reduced hydrolysis.

Levels of 20 – 40 fmol/cell of YTX were easily detected in single cells of *P. reticulatum* (Fig. 4c). Results of the analysis of the 25 pooled cells (36 fmol/cell) were comparable to those of the individual cells.

## References

- Guillard, R.R.L. & Hargraves, P.E. (1993). *Phycologia* 32: 234-236.
- John, U., Quilliam, M.A., Medlin, L. & Cembella A. (2001). In: *Harmful Algal Blooms*, Hallegraeff G.M., Blackburn S.I., Bolch C.J. & Lewis R.J. (eds), IOC UNESCO, Paris, pp. 299-302.

## Evaluation of the enzyme inhibition assay for diarrhetic shellfish toxins and the ELISA assay for yessotoxins by LC-MS

R. Sekiguchi<sup>1</sup>, M. Suzuki<sup>1</sup>, N. Takahashi<sup>1</sup>, M. Yamamoto<sup>1</sup>, M. Watai<sup>1</sup>, T. Suzuki<sup>2</sup> and T. Yasumoto<sup>1</sup>

<sup>1</sup>Japan Food Research Laboratories Tama Laboratory 6-11-10, Nagayama, Tama-shi, Tokyo, 206-0025, Japan, :sekiguchir@jfri.or.jp, <sup>2</sup>Tohoku National Fisheries Research Institute 3-27-5, Shiogama-cho, Shiogama, Miyagi, 985-0001, Japan, tsuzuki@affrc.go.jp

### Abstract

In our ongoing project to develop screening methods suitable for on-site use, we evaluated the performance of the PP2A inhibition assay kits developed by using a catalytic subunit of PP2A from whelks. More than 500 samples of scallops and mussels were assayed and the resultant OA contents showed good correlation with those obtained by LC-MS analysis ( $R^2=0.9797$ ). Next, we examined the ELISA microplate assay for YTXs that used polyclonal anti-YTX antibodies. After optimizing the assay conditions, we assayed more than 500 samples and compared the results with those of LC-MS. A good linearity was observed between the ELISA and LC-MS results ( $R^2=0.740$ ). However, the values obtained by ELISA were 8 times higher than those calculated by LC-MS for YTX, indicating a higher cross-reactivity of the antibody to 45-OHYTX and possibly to other analogues. Nevertheless, the good linearity observed between ELISA and LC-MS pointed to the usefulness of the ELISA assay as an on-site screening method for YTXs.

### Introduction

One of our projects is to develop biochemical methods suitable for use at the production sites or in the laboratories that do not use mice or expensive instruments.

First, as a functional assay for toxins with specific modes of action, we chose the PP2A inhibition assay for on-site monitoring of okadaic acids (OA).

Secondly, we chose an ELISA method for YTXs, which are difficult to detect using functional assays because of the lack of knowledge about specific pharmacologic targets.

Thirdly, we employed liquid chromatography coupled with mass spectrometry (LC-MS) using fourteen toxin standard.

In this paper, we focus on the PP2A inhibition assay kits for OA and on an ELISA method for YTX.

### Materials and Methods

#### Material

The catalytic subunit of PP2A (PP2Ac) was purified from muscle of the whelk, *Neptunea arthritica*, by the method of Takai (1998).

Both the PP2A inhibition and YTX-ELISA assay kits were developed in JFRL financed by Marino Forum 21. The specifications and protocol for the former were reported in the Project Report to Marino Forum 21 (2003) and briefly by Sato *et al.* (2003). Those for the latter will be reported in 2007 to the Ministry of

Agriculture, Forestry and Fisheries upon completion of this project. Details are not given here due to limited space. Toxin standards for data calibration and antibodies for ELISA were prepared in JFRL.

A total of 531 extracts of digestive glands (DG) collected as part of the national shellfish monitoring project conducted in various parts of Japan during 2003 and 2004 were used in this study. The shellfish species included scallops (*Pectinopecten yessoensis*), blue mussels (*Mytilus galloprovincialis*) and native mussels (*M. corsicum*).

#### Sample preparation for PP2A inhibition assay and YTX-ELISA

Toxins were extracted from the DG homogenate (2 g) of shellfish with 18 mL of methanol-water (9:1). After centrifugation, supernatants were kept in a freezer (-20 °C) until use for PP2A inhibition and YTX-ELISA assays.

#### PP2A inhibition assay

Solutions of the sample extract, substrate (p-nitrophenyl phosphate, pNPP) and PP2Ac were added to each well of the assay plate in this order. The plate was placed on a plate mixer for 1 min and then incubated for 30 min. The developed colour was measured with a microplate reader at 405 nm using a reference at 492 nm.

To assess the effect of ester toxins, we performed assays on both intact and hydrolyzed extracts. Hydrolysis was carried out by adding 100 µL of 1.25 N

NaOH to 500  $\mu\text{L}$  extract and heating the solution for 20 min at 100  $^{\circ}\text{C}$ . The colour was measured after neutralization with 1.25 N HCl.

#### Antigens and coating conjugates for YTX-ELISA assay

Polyclonal antibodies were prepared to construct the competitive ELISA kits for YTXs. We prepared two types of protein conjugate for immunization: one from YTX by ozonization and another from carboxyYTX using carbodiimide. Two YTX-BSA conjugates were dosed to rabbits, respectively, and the antisera were collected. A YTX-OVA conjugate was prepared from YTX by the ozonization method and used for coating plates.

#### YTX-ELISA assay procedure

Using the rabbit polyclonal anti-YTX antibody we prepared a prototype microplate assay kit for YTXs and optimized assay conditions. The assay procedure followed that employed routinely for ELISA assays. Briefly, the microplates were coated with the YTX-OVA conjugate a day prior to use and the anti-YTX antibodies bound to the conjugate during the assay were measured using an HRP-enzyme-labelled donkey anti-rabbit-IgG antibody (Chemicon International, Inc.). The assay took about 5 h to perform.

#### LC-MS analysis

The LC-MS analysis was carried out on unhydrolyzed extracts at TNFRI by M. Suzuki using calibrants of OA, DTX1, DTX3, YTX, and 45-OHYTX.

## Results and Discussion

#### Use of the *Neptunea* PP2Ac (NPP2Ac) for ELISA

We chose to use NPP2Ac because *N. arthritica* showed the highest enzyme content among the animal species tested, yielding as much as 800 units of PP2Ac from 1 kg of muscle. The stability and sensitivity of the catalytic subunit exceeded those of commercial enzymes that were hetero dimers of A/C subunits. PP2Ac activity remained unchanged for over 1 yr at 5  $^{\circ}\text{C}$  or -20  $^{\circ}\text{C}$  and enabled quantification of OA at a level equivalent to 0.1  $\mu\text{g/g}$  edible tissue, which is below the proposed EU action level (0.16  $\mu\text{g/g}$ ) for regulation. An inter-laboratory validation study involving 7 laboratories in Japan produced a maximum variation of 30 %.

#### Comparison of inhibition rates of shellfish toxins against NPP2Ac

DTX3 did not inhibit NPP2Ac by itself but quantitatively inhibited the enzyme after hydrolysis to DTX1. Two other structurally different toxins, YTX and pec-

tenotoxin-6, did not inhibit the enzyme at concentrations 200 times higher than for OA.

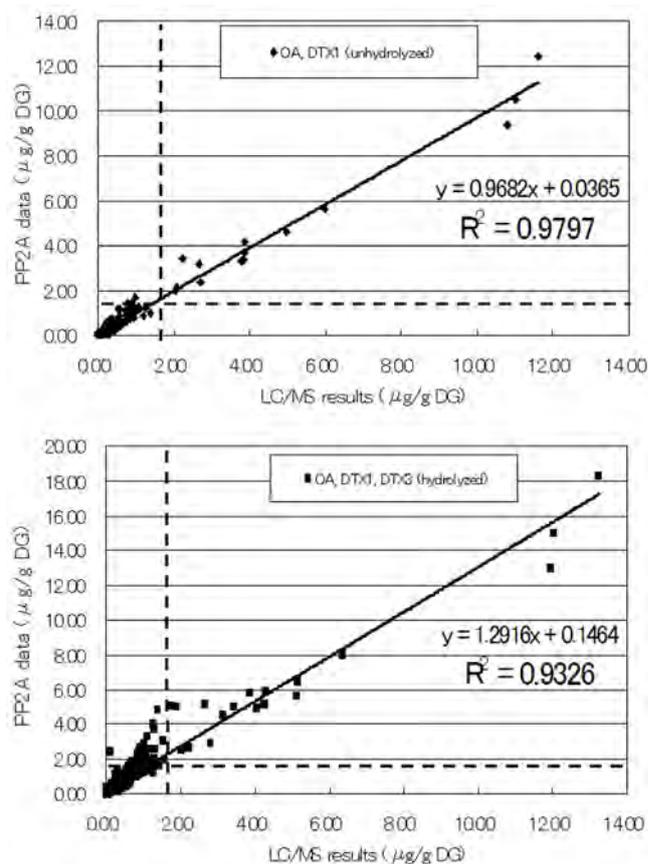
#### In-house correlation study on the PP2A assay and LC-MS (OA, DTX1, and DTX3)

We compared the PP2A inhibition assay kit with the LC-MS method using six samples: five samples spiked with known amounts of OAs to non-toxic mussels (0.1~0.5 ppm) and one naturally contaminated mussel sample. Good correlations ( $R^2=0.9379$ ) were observed between the two methods (data not shown).

#### Comparison of PP2A assay and LC-MS data

Samples were assayed before and after hydrolysis to estimate, respectively, free and ester type toxins. Results with unhydrolyzed samples showed a good agreement ( $R^2=0.9797$ ) between the PP2A and LC-MS methods (Fig. 1, upper). The results obtained on hydrolyzed samples are shown in Fig. 1 (lower).

The seemingly good correlation ( $R^2=0.932$ ) was questioned, however, due to the occurrence of samples in which OA levels were much higher by PP2A



**Figure 1.** Comparison of PP2A assay and LC-MS results on 531 samples from 2003 and 2004. PP2A results on unhydrolyzed samples (upper) are compared with LC-MS data obtained only on unhydrolyzed samples. -----: Equivalent to EU regulatory level for whole body.

inhibition assays than by LC-MS. Because the LC-MS data used for comparison were obtained on unhydrolyzed extracts and by using only a DTX3 (7-O-palmitoylDTX1) standard to estimate whole ester toxins, we hypothesized underestimation of ester toxins by LC-MS. We analysed by LC-MS four hydrolyzed extracts that showed wide discrepancies and found the data consistent with the PP2A data (C.V. <10%), supporting our hypothesis. In view of the simplicity of the procedures including hydrolysis, we propose the PP2A assay kit as a promising tool for on-site use.

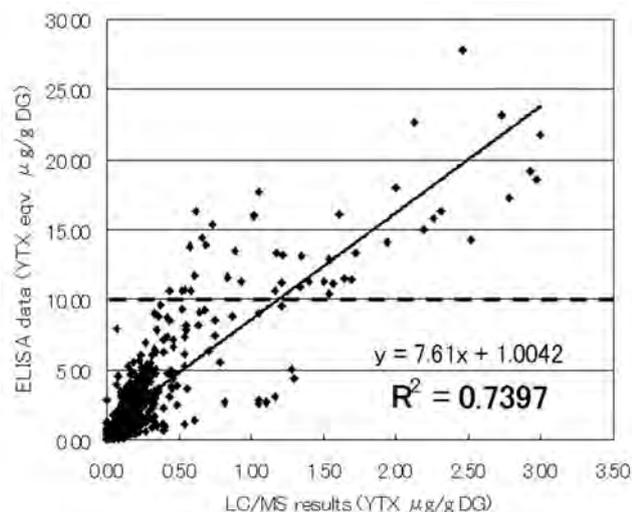
#### *The antigen and anti-YTX antibody specificity*

Comparison of two antigens for immunogenic potency indicated the carboxyYTX-BSA antigen to produce the highest titers. Thus, we used the anti-carboxyYTX antibodies in subsequent ELISA assays. The cross-reactivity with other YTX analogues (Briggs *et al.* 2004; Samdal *et al.* 2004, 2005; Aasen *et al.* 2005) could not be tested due to lack of standards. Nonetheless, OA, DTX1, DTX3, and PTX6 did not react to the antibodies at concentrations 20 times higher than for YTX.

#### *Comparison of YTX-ELISA assay data and LC-MS results*

We used YTX to prepare the standard curve. Data for other analogues cross-reacting to the antibody were calculated as the YTX equivalent. The kit could quantify YTX from 0.1  $\mu\text{g/g}$  tissue or greater.

We compared the results between the ELISA and LC-MS methods and observed good linearities for the two sample groups:  $R^2=0.875$  for 2003 and  $R^2=0.8063$  for 2004. The lower correlation in 2004 may be re-



**Figure 2.** Comparison of YTX-ELISA assay and LC-MS results. -----: Equivalent to EU regulatory level for whole body.

lated to the low YTX contents in the samples. The ELISA values were more than eight times higher than the LC-MS data, probably because of the presence of a variety of YTX analogues and metabolites in shellfish. Thus, use of an appropriate factor is required to avoid excessive false positives. False negatives were not observed, based on the current EU regulation level (roughly equivalent to 10  $\mu\text{g/g}$  DG).

Despite the dominance of low YTX samples, a linearity of correlation ( $R^2=0.74$ ) was obtained on 531 samples collected in 2003 and 2004 (Fig. 2). Because of its simplicity, the ELISA kits would be useful to screen YTX toxicity on production sites.

#### **Acknowledgements**

This study is a part of the research project of the Ministry of Agriculture, Forestry and Fisheries of Japan: Research for utilizing advanced technologies in agriculture, forestry and fisheries (No. 1504).

We acknowledge the assistance of Dr. K. Koike (Kitasato Univ.), Mr. T. Jin (Aomori Pref. Inst. of Public Health and Envir.), Mr. M. Okumura (Aichi Pref. Inst. of Public Health), Dr. K. Abe (Miyagi Pref. Inst. of Public Health and Envir.), Dr. T. Chonan (Hokkaido Inst. of Public Health), and Prof. Y. Oshima (Tohoku Univ.). The development of the PP2A and YTX-ELISA kits was supported financially by Marino Forum 21.

#### **References**

- Aasen, J., Samdal, I.A., Miles, C.O., Dahl, E., Briggs, L.R. & Aune, T. (2005). *Toxicol* 45: 265-272.
- Briggs, L.R., Miles, C.O., Fitzgerald, J.M., Ross, K.M., Garthwaite, I. & Towers, N.R. (2004). *J. Agric. Food Chem.* 52: 5836-5842.
- Japan Food Research Laboratory, The Project Report to Marino Forum 21 (2003).
- Samdal, I.A., Naustvoll, L.J., Olseng, C.D., Briggs, L.R. & Miles, C.O. (2004). *Toxicol* 44: 75-82.
- Samdal, I. A., Aasen, J., Briggs, L. R., Dahl, E. & Miles, C. O. (2005). *Toxicol* 44: 7-15.
- Sato, S., Igarashi, T., Sekiguchi, R., Watai, M. & Yasumoto, T. (2003). *HABTech 2003 Workshop Proceedings, Cawthron Report No. 906*: 38-39.
- Suzuki, T., Jin, T., Shiota, Y., Mitsuya, T., Okumura, Y. & Kamiyama, T. (2005). *Fish Sci.* 71: 1370-1378.
- Takai, A. (1997). *Jpn. J. Thromb. Hemost.* 8: 504-516.

## Preparation and simultaneous LC-MS analysis of fourteen shellfish toxins

M. Suzuki, R. Sekiguchi, M. Watai and T. Yasumoto

Japan Food Research Laboratories, 6-11-10 Nagayama, Tama, Tokyo, Japan, suzukim@jfrrl.or.jp

### Abstract

We prepared 14 shellfish toxin standards to be used as calibrants in LC-MS analysis: okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxins (PTX -1, -2, -3, -6), azaspiracids (AZA -1, -2, -3), yessotoxins (YTX, 45-OHYTX), brevetoxin-B2 (BTXB2), 7-O-palmitoylOA and 7-O-palmitoylDTX1. The former 11 toxins were purified from contaminated shellfish, except for YTX which was prepared from cultures of *Protocera-tium reticulatum*. 7-O-palmitoylOA and 7-O-palmitoylDTX1 were chemically prepared from OA and DTX1, respectively. The purities of the standard toxins were checked by <sup>1</sup>H-NMR, LC-DAD, and LC-MS. All toxins were quantifiable in 30 min in a single run by monitoring negative ions, except for AZAs that were detected on positive ions. We extracted digestive glands of scallops, *Patinopecten yessoensis*, and mussels, *Mytilus galloprovincialis*, spiked the extracts with standard toxins, and carried out recovery tests and reproducibility tests. Satisfactory results were obtained.

### Introduction

Worldwide a number of lipophilic toxins with varying structures and toxicological properties accumulate in bivalve shellfish and thus pose risks to human health. The representative toxins include okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), azaspiracids (AZAs), yessotoxins (YTXs), and brevetoxin-B2 (BTXB2) (Fig. 1). For protection of the public health, toxic shellfish are prohibited from the market by regulation. Hence proper methods for determining the toxins in shellfish are necessitated. Several research groups have proposed use of LC-MS methods to monitor the toxins in shellfish (Draisci *et al.* 1999; Goto *et al.* 2001; Quilliam *et al.* 2001; Quilliam 2003; McNabb *et al.* 2005; Holland *et al.* 2006). The methods both identify and quantify the toxins regardless of their structural and toxicological variations. Sample preparations are also simple and rapid. Because the lack of adequate supply of standard toxins lays

a serious obstacle to the use of LC-MS, our primary effort was directed to preparation of standard toxins. A total of 14 shellfish toxin standards were prepared; OA, DTX1, 7-O-palmitoylOA (palOA), 7-O-palmitoylDTX1 (palDTX1), PTX1, PTX2, PTX3, PTX6, YTX, 45-OHYTX, AZA1, AZA2, AZA3, BTXB2 (Fig. 1). Using these standards, we optimized conditions for a rapid and simultaneous analysis of all toxins.

### Materials and Methods

#### Materials

The standards of OA, DTX1, PTX1, PTX2, PTX3, PTX6, and 45-OHYTX were prepared from scallops, *Patinopecten yessoensis*, collected in Japan (Murata *et al.* 1986; Satake *et al.* 1996; Goto *et al.* 1997). Those of AZA1, AZA2, and AZA3 were supplied by Dr. M. Satake of Tohoku University, who had purified them from *Mytilus edulis* collected in Ireland (Satake *et al.*

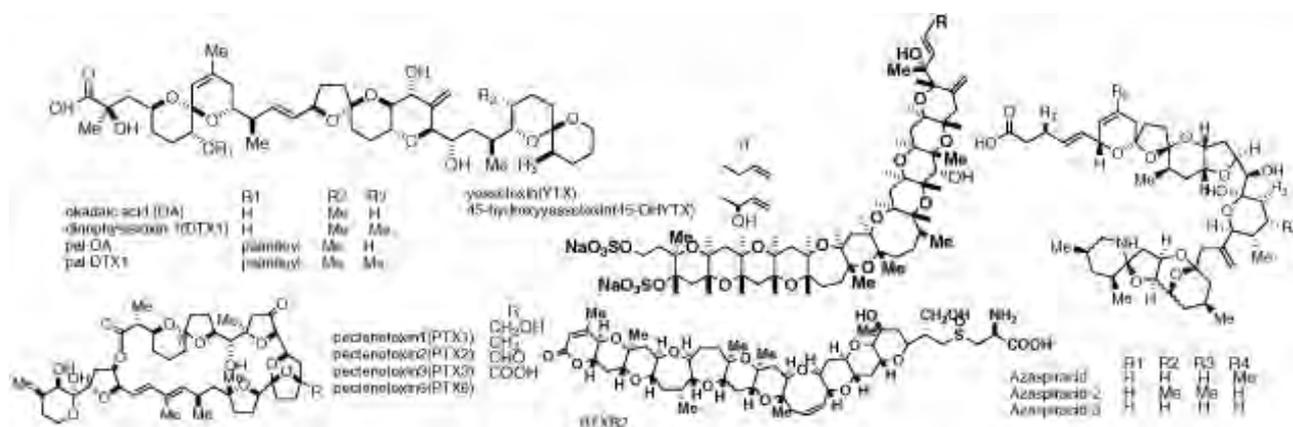


Figure 1. Structure of lipophilic shellfish toxins.

1998). *Perna canaliculus* collected in New Zealand was used to prepare BTXB2 (Murata *et al.* 1998). YTX was obtained from cultures of *Protocerratium reticulatum*. PalOA and palDTX1 were chemically prepared from OA and DTX1 respectively, following the method of Yanagi *et al.* (1989). Toxins were checked for purities by <sup>1</sup>H-NMR, LC-DAD, and LC-MS, weighed, dissolved in HPLC-grade methanol, and kept at -20 °C until used.

#### Preparation of extracts

Toxin extracts were prepared from digestive glands (DG) of naturally contaminated shellfish: scallops, *P. yessoensis*, collected in Aomori, Hokkaido, Iwate, and Miyagi, mussels, *M. galloprovincialis*, collected in Iwate and Mie, and the green-shelled mussel *Perna canaliculus* from New Zealand. To 1 g of DG was added 9 mL of methanol-water (9:1, v/v). The mixture was homogenized and centrifuged for 10 min at 1,098 × g. The supernatant (5 µL) was directly injected to the LC-MS instrument, otherwise stored at -20 °C until used. Nontoxic DG homogenates and nontoxic extracts thereof were prepared for use in spiking tests. Scallops and mussels commercially sold as safe were used. They were tested by LC-MS to prove no significant levels of the toxins were present. The DG homogenates were spiked with the toxins, except AZAs, at three different levels, 0.2, 0.5, and 1.0 µg/g homogenate. AZAs were spiked at 0.02 µg/g, because they could be detected at high sensitivity by monitoring positive ions. The spiked levels of the toxins in

the extracts were 0.02, 0.05, and 0.1 µg/ml, except for those of AZAs that were spiked at 0.002, 0.005 and 0.01 µg/ml levels.

#### Mass spectrometry

Mass spectrometry experiments were performed on FINNIGAN TSQ Quantum DISCOVERY triple stage quadrupole mass spectrometer (Thermo Electron Corp., MA, USA) equipped with an atmospheric pressure ionization source and an electrospray ionization (ESI) interface. ESI was effected by a spray voltage of 4.0 kV and heated capillary temperature was maintained at 325 °C. Liquid chromatography used was NANOSPAC (Shiseido, Tokyo, Japan). Toxins were separated with a Capcellpak C18 MGII column 1.5 mm ID x 250 mm (Shiseido, Tokyo, Japan) operated at 40 °C, 0.2 mL/min. Injection volume was 5 µL. Eluent A was water and B was methanol/acetonitrile (8:2 ;v/v) both containing 2 mM ammonium formate and 50 mM formic acid. The gradient condition changed solvent B to 100 % from 40 % in 10 min, and solvent B was kept to 100 % until 20 min. Toxins were detected by selected-ion monitoring (SIR): OA, m/z 803 [M-H]<sup>-</sup>; DTX1, m/z 817 [M-H]<sup>-</sup>; palOA, m/z 1041 [M-H]<sup>-</sup>; palDTX1, m/z 1055 [M-H]<sup>-</sup>; PTX1, m/z 919 [M-H+HCOOH]<sup>-</sup>; PTX2; m/z 903 [M-H+HCOOH]<sup>-</sup>; PTX3, m/z 949 [M-H+CH<sub>3</sub>OH+HCOOH]<sup>-</sup>; PTX6, m/z 887 [M-H]<sup>-</sup>; YTX, m/z 1141 [M-2Na+H]<sup>-</sup>; 45-OHYTX, m/z 1157 [M-2Na+H]<sup>-</sup>; AZA, m/z 842 [M+H]<sup>+</sup>; AZA2, m/z 856 [M+H]<sup>+</sup>; AZA3, m/z 828 [M+H]<sup>+</sup>; and BTXB2, m/z 1034 [M+H]<sup>+</sup>, respectively. For protection of the MS detector and column from contamination, we limited the injection volume to 5 µL and used the divert valve. Experiments to investigate the reproducibility of the results were performed in triplicate on methanol solutions of the standards and in quintuplet on spiked extracts. The recovery tests for spiked extracts were carried out in quintuplet.

#### Results and Discussion

The chromatograms for the 14 toxins in methanol solutions are shown in Fig. 2. PalDTX1 was the last to be eluted at 19 min. Thus, a simultaneous analysis of the 14 toxins could be carried out in a 30 min cycle. The standard deviations were less than 10 % for most toxins but increased at the lowest concentration of the toxins with the maximum of 15.1 % for palDTX1. The chromatograms of the toxins spiked to scallop extracts are shown in Fig. 3. Despite selected ions were monitored, many peaks arising from matrices appeared in the chromatograms. Identification and quantification of individual toxins were possible

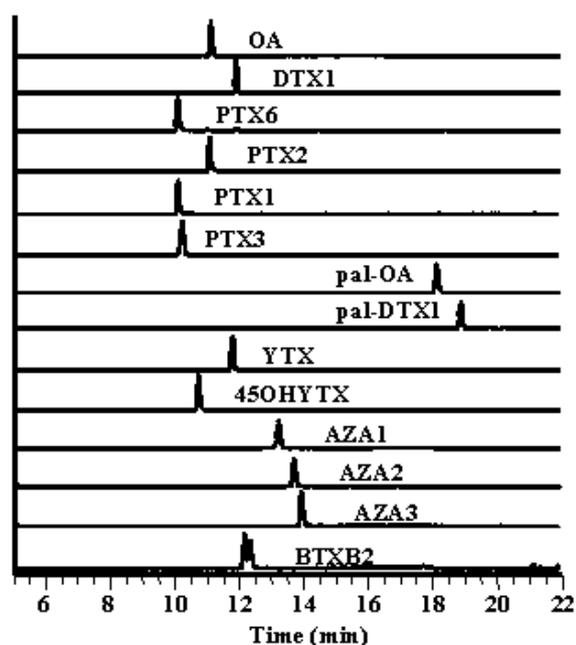
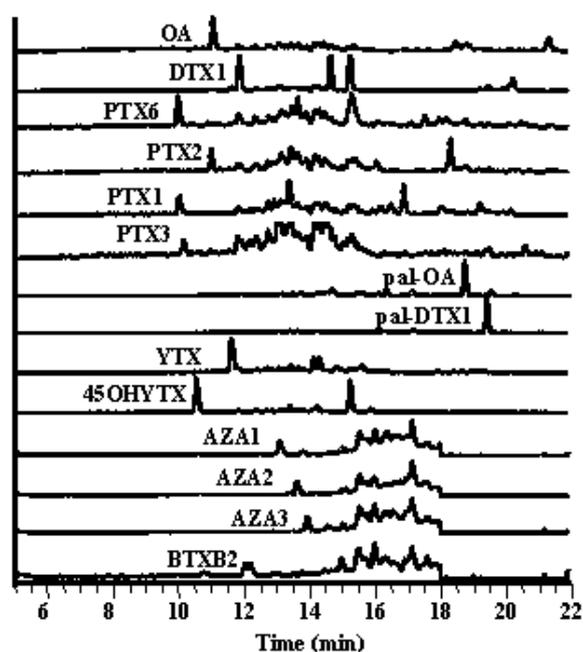


Figure 2. LC-MS Chromatograms for standard toxins.



**Figure 3.** LC-MS Chromatograms of the toxins spiked to scallop extracts.

when toxin standards were used, otherwise very difficult. The recoveries of the toxins from spiked extracts ranged from 80 to 110 %, and those from spiked DG from 70 to 110 %. The minimum detection limits for the toxins spiked to the DG extracts were 10 pg or lower, except those for PTX3 and BTXB2 (50 pg for both). The low sensitivity of PTX3 derives from its being a mixture of aldehyde, dihydrate, 43(S)- and 43(R)-methyl hemiacetals. BTXB2 also exists as a mixture of stereoisomers at the sulphoxide group, producing barely split peaks. The detection limit of 50 pg in 5  $\mu$ L of injected volume is equivalent to 100 ng/g DG of a toxin. If we assume that DG comprises 10–30 % of the whole meat by weight, the level of the toxin can be calculated to be 10–30 ng/g (= 10–30  $\mu$ g/kg). Thus the detection limits were well below the levels required by regulation.

Our LC-MS method enabled determination of 14 toxins in a single run for 30 min. From the point of harmonization, adoption of exactly the same analytical conditions in laboratories concerned seems desirable. However, problems may arise from the difference in the instrumental performance and different matrix effects. These problems can be overcome by the prop-

er use of the toxin standards and minor change in the LC conditions.

### Acknowledgements

This study was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labour and Welfare.

We are grateful to Dr. A. Yoshino of Tropical Technology Center in Okinawa for preparation of ester toxins.

We are grateful to Dr. L. Botana of University of Santiago de Compostela and Dr. K. Koike of Kitasato University for supply of raw material.

### References

- European Commission decision 2002/225/EC.
- Draisci, R., Palleschi, L., Giannetti, L., Lucentini, L., Lames, K., Bishop, A., Satake & M., Yasumoto, T. (1999). *J. Chromatogr. A* 847: 213-221.
- Holland, P., McNabb, P., Selwood, A. & Ginkel, R. (2006). IUPAC Symposium on Mycotoxins and Phycotoxins.
- Goto, H., Igarashi, T., Sekiguchi, R., Tanno, K., Satake, M., Oshima, Y. & Yasumoto T. (1997). *Harmful Algae*: 216.
- Goto, H., Igarashi, T., Yamamoto, M., Yasuda, M., Sekiguchi, R., Watai, M., Tanno, K. & Yasumoto T. (2001). *J. Chromatogr. A* 907: 181-189.
- McNabb, P., Selwood, A. & Holland, P. (2005). *J. AOAC Int.* 88: 761-772.
- Murata, K., Satake, M., Naoki, H., Kaspar, H. & Yasumoto, T. (1998). *Tetrahedron* 54: 735-742.
- Murata, M., Sano, M., Iwashita, T., Naoki, H. Yasumoto, T. (1986). *Agric. Biol. Chem.* 50: 2693-2695.
- Quilliam, M., Hess, P. & Dell'Aversano, C. (2001). *Mycotoxins and Phycotoxins in Perspective at the Turn of the Century*, pp. 383-387.
- Quilliam, M. (2003). *J. Chromatogr. A* 1000: 527-548.
- Satake, M., Terasawa, K., Kadowaki, Y. & Yasumoto, T. (1996). *Tetrahedron Lett.* 37: 5955-5958.
- Satake, M., Ofuji, K., Naoki, H., James, K., Furey, A., McMahon, T., Silke, J. & Yasumoto, T. (1998). *J. Am. Chem. Soc.* 120: 9967-9968.
- Yanagi, T., Murata, M., Torigoe, K. & Yasumoto T. (1988). *Agric. Biol. Chem.* 53: 525-529.

## Preparation of toxin standards for use in monitoring diarrhetic shellfish toxins by LC/MS

Atsushi Yoshino, Hideo Naoki and Takeshi Yasumoto\*

Okinawa Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, JST, 12-75 Suzaki, Uruma-city, Okinawa 904-2234, Japan, yoshino@ttc.co.jp

### Abstract

To promote wide use of LC-MS methods for monitoring of DSP toxins, we prepared DSP-toxin standards by different approaches. These were as follows: 1) okadaic acid (OA) and dinophysistoxin-1 (DTX1) were obtained from cultured *Prorocentrum lima*; 2) the black sponge *Halichondria okadai* was used as an alternative source of OA and DTX1; 3) purification procedures to separate DTX1 from DTX3 in the hexane fraction of scallops were optimized; 4) reaction conditions to synthesize corresponding 7-O-palmitoyl esters from OA and DTX1 were improved; and 5) other types of esters were synthesized to test their presence in the hexane fraction. Using reference OA, DTX1 and their esters, we expect to be able to semi-quantify the total OA/DTX1 amounts without doing hydrolysis.

### Introduction

In determining marine toxins in seafood, LC-MS is the most powerful tool because of its high sensitivity, specificity, and accuracy. To promote wide use of this method, however, an adequate supply of standard toxins is imperative. Though highly or moderately contaminated shellfish have been unavailable for years in Japan, we succeeded in preparing DSP-toxin standards by taking different approaches.

Firstly, we obtained okadaic acid (OA) and dinophysistoxin-1 (DTX1) by culturing *Prorocentrum lima*. Secondly, OA and DTX1 were extracted from the black sponge *Halichondria okadai*. Thirdly, in order to secure DTX1, which was of low occurrence in the preceding two sources, we improved purification procedures to enable purification of DTX1 from scallop extracts which were of very low DTX3 contents.

We here report the semisynthesis from OA and DTX1 to the corresponding 7-O-palmitoyl esters to be used in LC-MS analysis as standards for diarrhetic shellfish poisoning (DSP) toxins.

#### Isolation of OA and DTX1

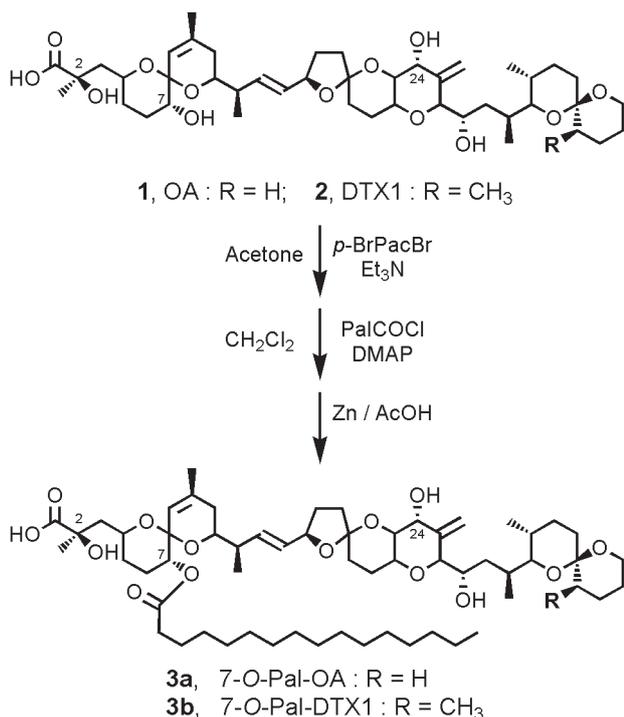
The dinoflagellate *Prorocentrum lima* was collected at Hamahiga Island, Okinawa, Japan, and grown for 25 days at 27 °C in a seawater medium enriched with ES-1 nutrients. Cells harvested by filtration were extracted with MeOH. Purification of OA and DTX1 was carried out by solvent partition and column chromatography. On average 100 L of culture produced 1.0 mg of OA and 0.15 mg of DTX1. OA and DTX1 were isolated from *H. okadai* as described in the literature (Tachibana *et al.* 1981).

DTX1 was further obtained by hydrolysis of DTX3 from scallops in ethanolic KOH solution.

#### Synthesis of 7-O-Pal esters

The synthetic procedure is a three-step method (Scheme 1). First, the carboxyl group of OA and DTX1 is protected with a p-bromophenacyl group. Then, acylation of 7-OH afforded the O-palmitoyl ester as a mixture of 7-O-Pal and 24-O-Pal esters. Finally, deprotection of the p-BrPac group gave 7-O-Pal-OA (3a) and 7-O-Pal-DTX1 (3b) in 31 % and 20 % total yield, respectively. The purification of products was by ODS chromatography and HPLC on a COSMOSIL 5C18-AR-II column using a gradient solvent system (A = H<sub>2</sub>O: AcOH, 100: 0.1, B = MeCN: AcOH, 100: 0.1, 90 % of B to 100 % for 10 min), yielding pure compounds 3a and 3b. During the acylation of OA, production of minor regioisomers such as 24-O-Pal-OA and 7,24-di-O-Pal-OA was observed by LC-MS analysis (data not shown). On the other hand, direct acylation of OA with palmitic anhydride led to formation of 2-O-Pal-OA and 2, 24-di-O-Pal-OA.

The structure and purity of the synthetic esters 3a and 3b were confirmed by HPLC-DAD, LC-ESI-MS and 1D- and 2D-NMR. The toxin quantity in a parent stock was determined by weighing. As an example, the negative mode LC-ESI-MS chromatogram and MS spectrum of 3b are shown in Fig. 1. To detect possible contaminants of minor quantities, solutions of high concentration, 3 µg/mL, were used for measurements. The chromatogram indicated that the synthetic product 3b has high purity. The MS spectrum showed

**Scheme 1. Synthesis of 7-O-Pal esters 3a and 3b**

an intense peak corresponding to the deprotonated form [M-H]<sup>-</sup> at *m/z* 1055.9.

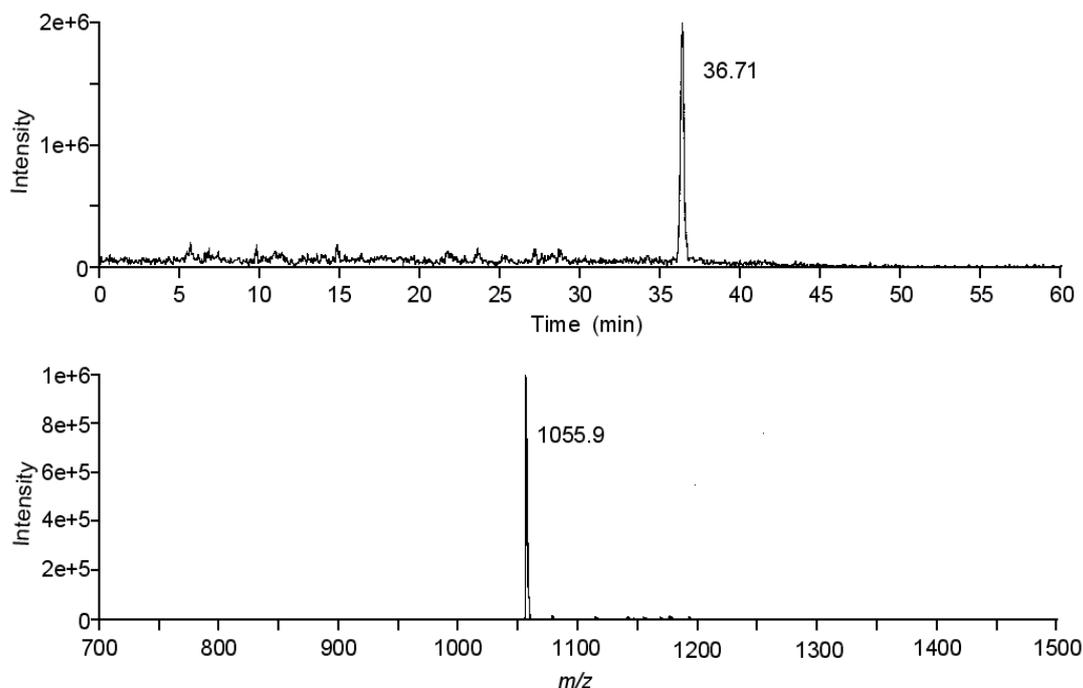
The <sup>1</sup>H-NMR spectrum of 3b is shown in Fig. 2. The 2D NMR spectra (DQF-COSY, HMBC) of 7-O-Pal esters allowed us to follow proton connectivities in partial structures. In accordance with the signal assignments in the literature, the 7-H signal in DTX1 (3.36) was shifted downfield to 4.76 in 3b.

**LC/MS measurements of 7-O-Pal esters**

Figure 3 shows an LC-DAD chromatogram at 200 nm and a SIM chromatogram by the ESI negative ion mode obtained on a mixture of OA and other toxins. The separation was performed with a COSMOSIL 5C18-AR-II column. The mobile phase was mixed from 0.1 % NH<sub>4</sub>COOH in water (A) and methanol (B) at a flow rate of 0.5 mL/min. There was a linear gradient elution from 90 % to 100 % B over 10 min. The sample concentration was 1 μg/mL for OA and DTX1, 3 μg/mL for other samples. All toxins of the OA group showed discrete peaks. The retention times (min) for OA, DTX1, 7-O-Pal-OA, 7-O-Pal-DTX1, 24-O-Pal-OA, and 2,24-di-O-Pal-OA monitored at 200 nm were 8.53, 9.30, 26.64, 28.53, 36.46, and 54.61 min, respectively. The negative [M-H]<sup>-</sup> ions for OA, DTX1, 7-O-Pal-OA, 7-O-Pal-DTX1, 24-O-Pal-OA, and 2,24-di-O-Pal-OA were detected at *m/z* 803, 817, 1041, 1055, 1041, and 1081, respectively. The detection limit under the conditions used was 0.5 ng/mL by the SIM analysis. It was therefore possible to detect esters down to ppb levels. By using the ester standards, approximation of the ester toxins in samples should be possible.

**Summary**

We produced OA, DTX1, 7-O-pal-OA, 7-O-pal-DTX1, 24-O-pal-OA, and 2,24-di-O-pal-OA of high purity. The semisynthetic method should enable the production of 7-O-pal-OA esters with different fatty



**Figure 1.** Full scan negative LC/MS chromatogram (top) and MS spectrum (bottom) of 7-O-Pal-DTX1

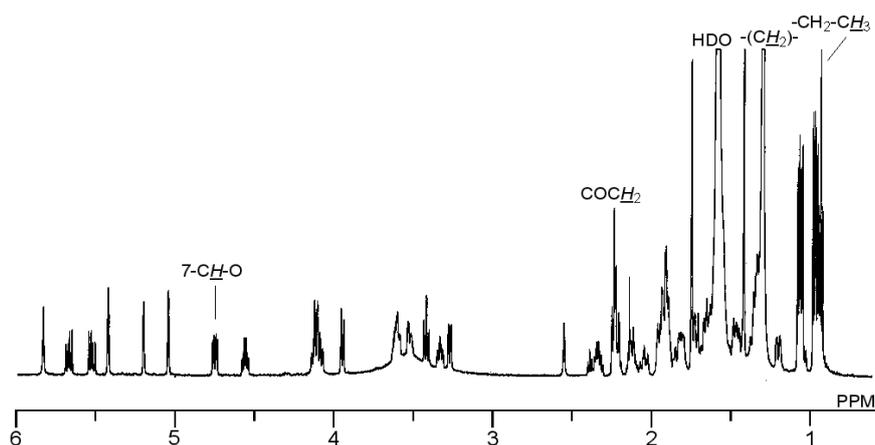


Figure 2.  $^1\text{H-NMR}$  spectrum of 7-O-Pal-DTX1

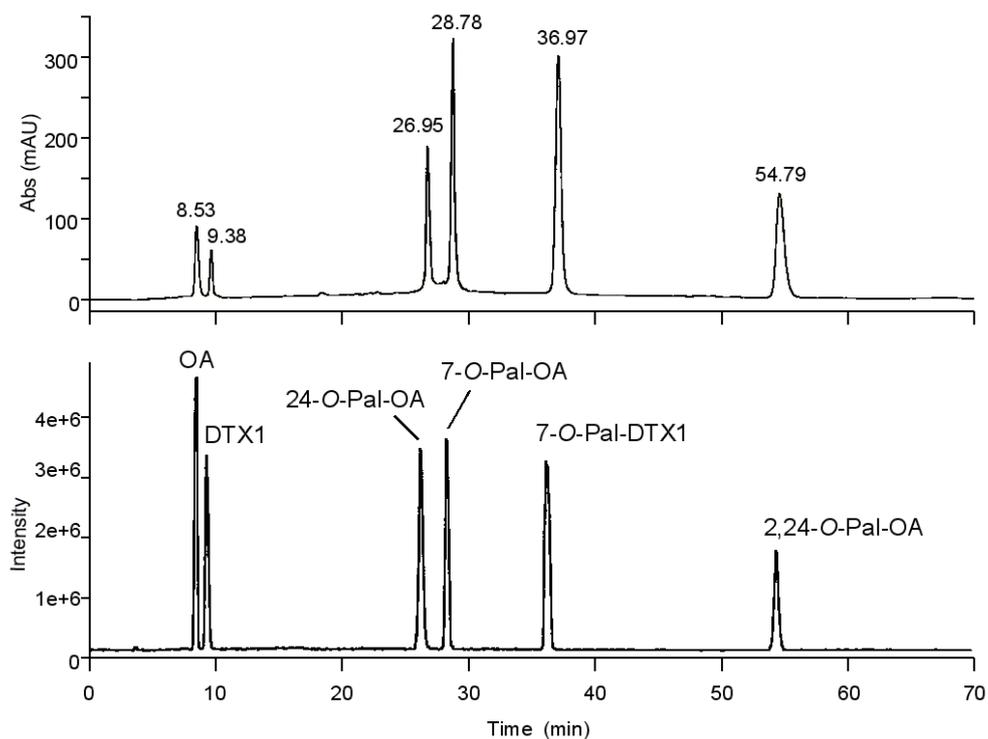


Figure 3. LC chromatogram (200 nm, top) and SIM chromatograms (bottom) of OA, DTX1 and Esters.

acid moieties. In LC/MS the toxins gave discrete peaks and were detectable with high sensitivity.

### Acknowledgements

This study was supported by Japan Science and Technology Agency and Okinawa Prefecture. We acknowledge the assistance of Japan Food Research Laboratories.

### References

- Hirata, Y. (1989). *Pure & Appl. Chem.* 61: 293-302.
- Murakami, Y., Oshima, Y. & Yasumoto, T., (1982). *Nippon Suisan Gakkaishi* 48: 69.
- Jørgensen, K., Scanlon, S. & Jensen, L.B., (2005). *Food Addit. Contam.* 22: 743-751.
- Tachibana, K., Scheuer, P.J., Yasumasa, T., Kikuchi, H., Engen, D.V., Clardy, J., Gopichand, Y. & Schmitz, F.J. (1981). *J. Am. Chem. Soc.* 103: 2469-2471.
- Yanagi, T., Murata, M., Torigoe, K. & Yasumoto, T. (1989). *Agric. Biol. Chem.* 53: 525-529.
- Yasumoto, T. & Torigoe, K., (1991). *J. Nat. Prod.* 54: 1486-1487.
- Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G.K. & Clardy, J., (1985). *Tetrahedron* 41: 1019-1025.

**AUTHOR INDEX**

- Aasen, J. 377  
 Adolf, J.E. 5, 55, 107, 135  
 Aguilera, A. 183  
 Ahmed, M.S. 162  
 Akers, R. 297  
 Akselman, R. 243  
 Al Abdessalaam, T.Z. 226  
 Al-Handal, A.Y. 246  
 Aligizaki, K. 219  
 Allis, O. 293  
 Alpermann, T.J. 51, 303  
 Alvarez, J. 334  
 Amorim, A. 273, 323  
 Andree, K. 62  
 Arin, L. 111, 334  
 Artigas, M.L. 273  
 Bachvaroff, T.R. 5, 55, 107  
 Backer, L.C. 297  
 Baden, D.G. 297  
 Baptista, M.S. 26  
 Barth, A. 96  
 Batoreu, M.C.C. 282  
 Bean, J.A. 297  
 Behrend, A. 22  
 Belas, R. 135  
 Benoit, E. 316  
 Bezteri, B. 51  
 Bianchi, L. 319  
 Bire, R. 187  
 Blanco, J. 279  
 Blasco, D. 111, 114  
 Blauw, A. 111  
 Borchert, J. 174  
 Boschetti, L. 319  
 Botelho, M.J. 273, 290, 338  
 Boyer, G.L. 341  
 Bravo, I. 374  
 Bresnan, E. 76, 165  
 Briggs, L. 326  
 Brotas, V. 171  
 Brown, C. 129  
 Brown, L. 165  
 Brownlee, E.F. 5, 148  
 Burchardt, L. 203  
 Bustillos, J. 86  
 Buzan, D. 103  
 Byrd, M. 103  
 Caillaud, A. 80, 114, 276, 279, 374  
 Calado, A.J. 190  
 Camp, J. 62, 111, 334  
 Campbell, L. 103  
 Cañete, E. 80, 114, 276, 279, 374  
 Cangini, M. 319  
 Cembella, A.D. 12, 51, 65, 114, 259, 303, 330, 371  
 Ceredi, A. 319  
 Cerejo, M. 290  
 Chang, F.H. 168  
 Chinain, M. 269, 306, 316  
 Chistoserdov, A. 353  
 Cho, Y. 59  
 Ciminiello, P. 344  
 Clement, A. 183  
 Cloete, T.E. 155  
 Collos, Y. 117  
 Costa, P.R. 171  
 Cox, A. 196  
 Cox, F. 174  
 Craveiro, S.C. 190  
 Culloty, S.C. 293  
 Curtis, B. 65  
 Cyronak, T. 249  
 Dalpra, D. 297  
 Darius, H.T. 306  
 Davenport, J. 293  
 Davies-Vollum, K.S. 196  
 De Fremicourt, I. 306  
 de la Iglesia, P. 347  
 Delgado, M. 80  
 Dell'Aversano, C. 193, 344  
 Di, B.P. 177  
 Dias, E. 282  
 Diener, M. 350  
 Diogène, J. 62, 80, 114, 279, 374  
 Doan-Nhu, H. 29  
 Du, W. 132  
 Dyble, J. 99  
 Edler, L. 246  
 Egerton, T.A. 203  
 Elandaloussi, L. 62, 114  
 Erler, K. 323  
 Esplund, C. 152  
 Estrada, M. 111  
 Evans, K.M. 262  
 Fahnenstiel, G.L. 99  
 Fattorusso, E. 344  
 Faust, M.A. 180, 269

- Fensin, E. 33  
Fernández-Tejedor, M. 62, 80, 114, 276, 279, 374  
Ferreira, M. 171  
Fiandrino, A. 117  
Fitridge, I. 168  
Fleming, L.E. 297  
Forino, M. 344  
Franco, J. 114, 276, 374  
Franzén, H. 152  
Fraser, S. 76  
Frost, B.W. 196  
Fuentes, C. 183, 353  
Fukami, K. 2  
Fukuyo, Y. 300  
Furey, A. 293  
Furio, E.F. 300  
Furones, D. 80  
Fux, E. 187  
Gao, Y. 132  
Garcés, E. 62, 111, 334  
Gago-Martínez, A. 347  
Garnett, C. 309  
Garrido, S. 171  
Gawel, J.E. 196  
Genovesi-Giunti, B. 117  
Giacobbe, M.G. 334  
Gilbert, H. 341  
Glibert, P.M. 40  
Göbel, J. 132  
Gol'din, E. 282  
Golubic, S. 306  
Gomes, S.S. 273, 290, 338  
Granéli, C. 152  
Granéli, E. 120, 152  
Greengrove, C.L. 196  
Grillo, C. 344  
Gumbo, J.R. 155  
Hanke, A. 206  
Hardstaff, W. 377  
He, F.L. 177  
Hégaret, H. 253, 356  
Heidemann, G. 103  
Heil, C.A. 96  
Hess, P. 187, 360  
Hiller, S. 68, 162, 371  
Hinzmann, M.F. 190  
Hiramatsu, K. 59  
Hiroishi, S. 47, 144  
Hirose, M.Y. 300  
Hoffer, S. 196  
Holland, W.C. 180, 269  
Holmes, M. 269  
Honsell, G. 193  
Horner, R.A. 196  
Hosoda, N. 144  
Ho-Van, T. 256  
Hubert, J. 196  
Huyen, N.T.M. 300  
Ibarra, D. 114  
Illoul, H. 334  
Inglis, G. 168  
Ishikawa, K. 47  
Ishimaru, . 59  
Jaekisch, N. 65  
James, K.J. 293  
Jarosch, A. 22  
Jenkinson, I.R. 126  
Jeong, H.J. 72  
John, U. 12, 51, 65, 259  
Juhel, G. 293  
Jordan, P. 282  
Karlson, B. 246  
Katayama, T. 300  
Katz, J. 135  
Kerbrat, A.S. 306  
Kibler, S. 72, 180, 269  
Kim, C.-H. 200  
Kim, D. 313  
Kim, Y.-S. 200  
Kirkpatrick, B. 297  
Kirkpatrick, G.J. 83  
Kloeppe, S. 259  
Kodama, M. 300  
Konopko, E. 341  
Kotaki, Y. 300  
Koukaras, K. 219  
Kraberg, A.C. 323  
Krause, E. 22  
Krock, B. 12, 303, 330, 371  
Krüger, T. 68  
Kumar-Roine, S. 316  
Laabir, M. 117  
Lagos, N. 330  
Lane, M. 203  
Larsen, J. 216  
Laurent, D. 306, 316  
Lawrence, J. 129  
Leggiadro, C. 309  
LeGresley, M.M. 206  
Legrand, C. 152  
Lekan, D.K. 36  
Lewis, N. 309, 377

- Li, J. 40, 177  
Licea, S. 86  
Lindberg, V. 44  
Lion, M. 243  
Litaker, R.W. 72, 180, 269  
Lohrenz, S.E. 83  
Lona, B. 174  
Lu, D. 40, 132, 239  
Lu, S. 40  
Luckas, B. 68, 162, 323, 350, 371  
Lü, S.H. 239  
Lundholm, N. 300  
Magnani, F. 319  
Magno, G.S. 344  
Malkiel, E. 135  
Mallat, E. 62, 114, 276, 279, 374  
Mann, D.G. 262  
Marshall, H.G. 203  
Martin, J.L. 206  
Martínez, B. 86  
Masó, M. 334  
Mason, P. 72  
Matsuyama, Y. 209  
Meave del Castillo, E. 212  
Melchiorre, N. 344  
Méndez, M.A. 330  
Milandri, A. 319  
Millie, D.F. 83  
Ming-Kei, W. 47  
Moestrup, Ø. 276  
Mohammad-Noor, N. 276  
Mohlin, M. 44  
Moita, M.T. 138  
Moline, M.A. 83  
Montanari, S. 319  
Morton, S.L. 256  
Moschandreou, K. 219  
Munday, R. 326  
Naoki, H. 300, 387  
Navarrete, A. 86  
Nelson, H. 90  
Neville, J. 196  
Nguyen-Ngoc, L. 216, 256  
Nguyen-Ngoc, T.G. 29  
Nguyen Thi, M.A. 29  
Nierenberg, K. 297  
Nikolaidis, G. 219  
Ní Rathaille, A. 223  
Noel, J. 353  
Nonogaki, H. 5  
O'Brien, N.M. 293  
Oda, T. 313  
Oelmüller, R. 68  
Ogawa, M. 59  
O'Halloran, J. 293  
Omura, T. 59  
O'Riordan, R.M. 293  
Oshima, Y. 59  
Page, F.H. 206  
Pastoureaud, A. 117  
Pauillac, S. 306, 316  
Paz, B. 114, 374  
Pereira, P. 282  
Pfitz, D.W. 135  
Pflugmacher, S. 19  
Pigozzi, S. 319  
Pitcher, G.C. 303  
Piumsomboon, A. 2  
Place, A.R. 5, 55, 107, 135  
Pompei, M. 319  
Postel, J.R. 196  
Poulton, N. 90  
Qi, Y. 132, 236  
Quijano-Scheggia, S. 62  
Quilliam, M. 309, 377  
Rafuse, C. 309  
Raine, R. 141, 223  
Rajan, A. 226  
Reger, R.N. 230  
Reguera, B. 243  
Reich, A. 297  
Reis, M. 323  
Relox, J.R. 300  
Rhodes, L. 326  
Riccardi, E. 319  
Rick, H.J. 353  
Riobo, P. 114  
Rodrigues, S.M. 273, 290, 338  
Rodríguez S., R. 212  
Romero, M.L. 300  
Rosa, R. 171  
Rubini, S. 319  
Rungsupa, S. 2  
Sampayo, M.A.M. 171  
Sampedro, N. 111, 330  
Sauviat, M-P. 316  
Scherp, P. 353  
Schofield, O.M. 83  
Sekiguchi, R. 391, 384  
Sellner, K.G. 5, 148, 158  
Sellner, S.G. 5, 148  
Sengco, M. 158

- Sequeira, M. 171  
Sheng, J. 135  
Shi, X. 40, 239  
Shumway, S.E. 253, 356  
Sieracki, C. 90  
Silva, M.J. 282  
Singh, R. 65  
Sivaipram, I. 2  
Skjevik, A.-T. 246  
Soasii, P. 2  
Sobrinho-Gonçalves, L. 138  
Songroop, C. 2  
Sorensen, K. 196  
Sosa, S. 193  
Soto, K. 330  
Spilling, K. 9  
Steidinger, K.A. 93  
Stewart, R. 168  
Stucken, K. 330  
Stumpf, R.P. 96, 99, 103  
Suzuki, M. 381, 384  
Suzuki, T. 381  
Takahashi, N. 381  
Takahashi, Y. 47  
Takashima, Y. 144  
Takata, Y. 300  
Tang, DL. 177  
Tartaglione, L. 193, 344  
Terenko, G. 233  
Terenko, L. 233  
Tester, P. 72, 99, 180, 269  
Thanh, T.D. 300  
Thu, P.T. 300  
Thuoc, C.V. 300  
Tillmann, U. 12, 51, 303  
Tomas, C.R. 36, 230, 249  
Tomlinson, M.C. 99, 103  
Touchette, B.W. 33  
Touzet, N. 141, 223  
Tubaro, A. 193  
Turrell, E. 76  
Tustison, J.A. 90  
Vale, P. 273, 290, 338  
Vandersea, M.W. 72, 180, 269  
Vaquer, A. 117  
Vargas M., M. 212  
Vasconcelos, M.T.S.D. 26  
Vásquez, M. 330  
Vassilakaki, M. 19  
Venail, R. 62  
Vernel-Pauillac, F. 316  
Vila, M. 111, 334  
Vilarinho, M.G. 290  
Villareal, T.A. 103  
Vincent, C. 117  
Vogelbein, W. 72  
Vuerich, F. 193  
Wang, Y.F. 239  
Wang, Z.H. 236  
Wanner, A. 297  
Watai, M. 381, 384  
Weissberg, R.H. 93  
Wiegand, C. 22  
Wikfors, G.H. 253, 356  
Wiles, K. 103  
Wiltshire, K.H. 323  
Wulff, A. 44  
Wyatt, T. 126  
Wynne, T.T. 99, 103  
Xia, P. 132  
Yamaguchi, K. 313  
Yamamoto, M. 381  
Yang, Y.F. 236  
Yasumoto, T. 300, 347, 381, 384, 387  
Yoshida, M. 144  
Yoshida, T. 47, 144  
Yoshino, A. 387  
Zhang, C. 40, 239  
Zhou, M.J. 239  
Zhu, D.D. 239  
Zhu, M.Y. 239  
Zou, J. 132



## SUBJECT INDEX

The index below refers to the abstracts of the articles. A CD-ROM is enclosed to allow for a search of the entire articles

- Adriatic Sea 193, 319  
 AFLP 51  
*Akashiwo sanguinea* 180  
 allelopathy 12  
*Alexandrium* 13, 51, 76, 141, 236, 246  
   *A. catenella* 117, 168, 183, 196, 200  
   *A. fundyense* 90, 206, 253, 356  
   *A. minutum* 80, 111, 141, 168, 223  
   *A. ostenfeldii* 51, 65, 309, 319, 377  
   *A. pseudogonyaulax* 233  
   *A. satoanum* 216  
   *A. tamarensis* 51, 59, 141, 168, 200, 223, 233  
   *A. tamutum* 51, 216  
*Amphidinium* 249  
   *A. carterae* 249  
   *A. massartii* 249  
 amphidinols 246  
 Arabian Gulf 226  
 ASP 193, 300  
 AZA, azaspiracids 187, 384  
  
*Bacillus* 2, 155  
 Baltic Sea 9, 68, 152, 246  
 Bangladesh 162  
 Bay of Fundy 206  
 Belize 180  
 brevetoxins 36, 297, 384  
 Black Sea 233, 286  
  
*Calanus helgolandicus* 138  
*Centropages chierchiae* 138  
 cephalopods 171  
*Ceratium furca* 2, 180  
*Chaetoceros* 2  
   *C. curvisetus* 2  
   *C. wighamii* 9  
*Chattonella* 246  
   *C. marina* 313  
   *C. subsalsa* 148  
 Chesapeake Bay 5, 55, 148, 203  
 Chile 183  
*Chrysochromulina polylepis* 13  
 chymotrypsin 22  
 ciguatera (CFP) 306, 316  
 clams 174, 273  
 clay treatment 148, 158  
*Cochlodinium polykrikoides* 180, 233  
 cockles 273  
 copepods 138  
 crabs 174  
*Crassostrea virginica* 5  
   *C. ariakensis* 5  
   *C. gigas* 114, 326  
 cyanobacteria 19, 22, 26, 44, 68, 99, 148, 152, 203, 293, 306, 341, 371  
 cyanopeptolin 22  
*Cylindrospermopsis raciborskii* 33, 330, 353  
  
*Daphnia magna* 22  
*Diatoma tenuis* 9  
 digital holography 135  
*Dinophysis* 114, 246  
   *D. acuta* 138  
   *D. norvegica* 246  
   *D. sacculus* 80, 279, 374  
 dinophysistoxins 187, 384, 387  
 dolphins 171  
 domoic acid 171, 174, 193, 300  
*Dreissena polymorpha* 293  
 DSP 80, 114, 279, 374  
  
 East China Sea 40, 132, 177, 236, 239  
 ebrideans 230  
  
*Fibrocapsa* 259  
   *F. japonica* 259  
 Flow-CAM® 90  
 forecasting 96, 103  
  
*Gambierdiscus* 180, 269, 306  
   *G. toxicus* 269  
 GIS technology (Geographic Information System) 93  
 glutathione 22  
*Gonyaulax*  
   *G. fragilis* 219  
   *G. hyalina* 219  
   *G. polygramma* 180  
   *G. spinifera* 180, 290, 303  
 Great Lakes region, USA 99  
 Gulf of Mexico 36, 83, 86, 93, 103, 297

- Gymnodinium*  
*G. aureolum* 233  
*G. catenatum* 168, 273, 338
- Gyrodinium*  
*G. instriatum* 33, 233  
*G. fissum* 286
- Halichondria okadai* 387  
*Hermesinum adriaticum* 230  
herring mortalities 206  
*Heterocapsa*  
*H. circularisquama* 13, 47  
*H. triquetra* 233  
*Heterosigma akashiwo* 13, 129, 253, 356  
*Hydrocoleum lyngbyaceum* 306  
hydrogen peroxide 19
- ImmunoStrip 341  
inhibition assay kits 381  
Ireland 187, 223
- Japan 47, 300  
jellyfish 86
- Karenia*  
*K. brevis* 13, 36, 83, 90, 93, 103, 297  
*K. mikimotoi* 13, 40, 132, 253
- Karlodinium* 80  
*Karlodinium veneficum* (syn. *K. micrum*) 5, 33, 55, 107, 135  
karlotoxins 5, 55, 107  
Korea 200
- Lingulodinium polyedrum* 168, 290, 323  
*Luciella* 72
- Mediterranean 62, 80, 111, 114, 117, 193, 219, 259, 279, 319, 334, 344, 374  
*Melosira arctica* 9  
*Mercenaria mercenaria* 5, 356  
microcystins 19, 22, 99, 144, 162, 282, 293, 341  
*Microcystis* 99, 155  
*M. aeruginosa* 144, 162, 282, 293  
mucilage 216  
*Mytilus*  
*M. edulis* 76  
*M. galloprovincialis* 114, 193, 290, 384
- Necora puber* 171  
New Caledonia 306  
New Zealand 168, 326
- nitric oxide 313, 316  
*Nitzschia navis-varingica* 300  
*Noctiluca scintillans* 2, 177  
*Nodularia*  
*N. harveyana* 68  
*N. spumigena* 44, 68  
nodularin 68  
North Carolina 33, 230
- Okadaic acid 187, 276, 279, 384, 387  
*Oscillatoria* 2, 226  
*Ostreopsis* 344  
*O. siamensis* 326  
*O. ovata* 344  
*Oxyrrhis marina* 107, 135
- Pacific coast of North America 174, 196  
Pacific coast of Central America 212  
palytoxin 326, 344  
*Patinopecten yessoensis* 384  
paua 326  
*Pecten novaezealandiae* 326  
pectenotoxins 279, 377, 384  
*Peridiniella catenata* 9  
*Peridinium quinquecorne* 180  
*Perna canaliculus* 326  
*Pfiesteria piscicida* 135  
Philippines 300  
phyco cyanin 341  
*Polybius henslowii* 171  
Portugal 22, 138, 171, 190, 273, 290, 323, 338  
probes 62, 141  
*Prorocentrum* 180, 276  
*P. arabianum* 256  
*P. donghaiense* 40, 132, 236, 239  
*P. belizeanum* 180  
*P. cassubicum* 190  
*P. faustiae* 276  
*P. lima* 190, 387  
*P. micans* 226  
*P. minimum* 33, 148, 226, 253, 356  
*P. rhathymum* 276
- Protoceratium* 290  
*P. reticulatum* 168, 180, 290, 303, 347, 374, 384
- Prymnesium parvum* 13  
*Pseudomonas* 2  
*Pseudo-nitzschia* 29, 114, 193, 246  
*P. australis* 165  
*P. calliantha* 62, 193  
*P. cuspidata* 29

*P. decipiens* 193  
*P. delicatissima* 62, 165, 233, 300  
*P. fraudulenta* 165  
*P. multiseriata* 300  
*P. pungens* 29  
*P. seriata* 165, 233  
PSP/PST 76, 80, 111, 183, 196, 200, 206, 212, 273,  
330, 338, 350  
*Pyrodinium bahamense* 86, 212

Reactive oxygen species (ROS) 19, 313  
rheology 126

*Sardina pilchardus* 171  
satellite imagery 103  
saxitoxins 212, 330  
Scotland 76, 165  
sea urchins 326  
SeaWiFS 103  
*Scrippsiella*  
    *S. hangoei* 9  
    *S. trochoidea* 132, 233  
*Skeletonema*  
    *S. costatum* 2, 9, 132, 177, 233  
South America 243  
SPATT (Solid Phase Adsorption Toxin Tracking)  
187  
species concepts 262  
spirolides 65, 309, 319, 377  
*Synechocystis* 19

Thailand 2  
*Thalassiosira*  
    *T. baltica* 9  
    *T. curviseriata* 132  
trypsin 22  
turtles 86

UV radiation 44

Vietnam 29, 216, 256, 300  
virus 129

*Woloszynskia halophila* 9

Yessotoxins 114, 187, 290, 303, 323,  
347, 374, 377, 381, 384



United Nations  
Educational, Scientific and  
Cultural Organization



Intergovernmental  
Oceanographic  
Commission