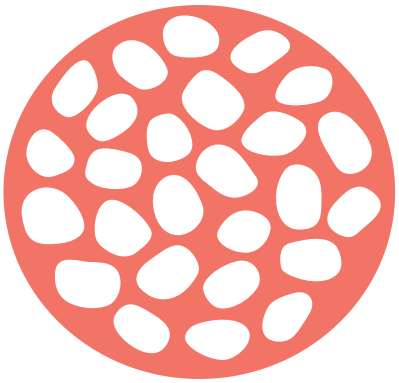

ICHA
BRAZIL2016

MARINE AND FRESH-WATER HARMFUL ALGAE

PROCEEDINGS OF THE 17TH INTERNATIONAL CONFERENCE ON HARMFUL ALGAE

9-14 October 2016 | Florianópolis, Brazil

Edited by: Luis A. O. Proença and Gustaaf M. Hallegraeff

International Society for the Study of Harmful Algae
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ICHA

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Conference on Harmful Algae
Brazil 2016

Hosted by

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PREFACE

The 17th International Conference on Harmful Algae (ICHA) occurred in Florianópolis, Santa Catarina State capital, South Brazil, at the Centro Sul Convention Center between 9 to 14th October 2016. This was the first time the ICHA was hosted in South America. The 17th ICHA idea was born as a regional initiative, and represents aspirations not only from the Brazilians but from the entire South American scientific community.

The proposal was supported by the IOC-FANSA Group, which forms the regional committee and includes scientists from Uruguay, Argentina, Chile, Peru and Ecuador. That was very convenient, since we share with our neighbors several economic, social, ecological issues and, of course, problems related to harmful algae.

Species of *Alexandrium, Gymnodinium, Dinophysis* or *Microcystis* do not know political borders and freely ride through marine and freshwater ecosystems within the South American continent. ICHA 17 was hosted by the Federal Institute of Santa Catarina (IFSC), a public governmental educational institution with more than 30.000 students. IFSC is home of LAQUA, one of the national laboratories for algal toxins analysis. The conference central theme was Harmful Algae, from cells to fisheries: species, toxins, ecology, management and new technologies. The conference was attended by 350 participants from 35 countries. There were 2 keynote speakers, 8 plenary speakers, 145 orals presentations, 20 fast-talks, 250 posters and a heavily attended round table to discuss the recent HAB events in Chile.

Conference activities started with the first ever Student Speed Networking mini-course, held on Sunday 9th October and mentored by Vera Trainer, Lisa Campbell, Adriana Zingone, Marina Montresor and Mohamed Abdul Baki. Main conference topics included: Climate anomalies, global change, and record blooms, heavily influenced by the dramatic salmon-kills and PSP events suffered by Chilean producers in 2016, advanced 'omics *in situ* sensors and over 60 contributions on Cyanobacteria were presented. The main topics were approached within 2 key notes, given by Gustaaf Hallegraeff - Progress in our understanding of fish- killing microalgae: implications for management and mitigation; Lora Fleming - Ecological public health, Harmful Algal Blooms and climate change; and 8 plenary talks, by Philipp Hess - Chemical and analytical sciences in a whirlwind of global change; Marina Montresor - The diatom genus Pseudo-nitzschia: life history and its relevance to species ecology and evolution; Satoshi Nagai - Metagenomic approach for HAB monitoring in Japanese coastal waters; Ichiro Imai - Environment-friendly strategies for prevention of harmful algal blooms using algicidal bacteria associated with seagrass beds; Raphael Kudela - Wiring the ocean to understand and predict Harmful Algal Blooms; Mark Wells - Harmful Algal Blooms and climate change: challenges and paths for moving forward; Janaina Rigonato - Mining cyanobacterial genomes for natural products and Nestor Lagos - Paralytic Shellfish Poison toxins: clinical applications. On Tuesday 11th October members of the program GlobalHAB organized a town meeting to present and discuss with the ICHA community the Program Scientific Implementation plan. Social activities included different city tours on Wednesday afternoon, a barbecue on Tuesday with samba and caipirinha, where Brazilians HAB experts paid homage to Edna Granéli. The farewell conference banquet closed the event in a Brazilian way: a carnival with a troup of drummers and dancers from local Escola de Samba. A full report and conference highlights can be found in Harmful Algal News (55 -December 2016). A poll of 110 participants from 35 countries indicated that the conference was overall very well evaluated. The credit for the success of the conference is to be shared with all attendees, and the Regional FANSA and the International Advisory committees. We thank all the colleagues who chaired the scientific session and helped to run the conference smoothly, by keeping the program on time and conducting the discussions. Thanks to ISSHA for the financial support to help the attendance of 27 students from 13 countries. Thanks also to all sponsors and exhibitors, especially to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, Conselho Nacional de Desenvolvimento Cientifico e Tecnológico - CNPq and Fundação para Amparo a Pesquisa e Inovação do Estado de Santa Catarina - FAPESC, for their funding contribution to the conference.

We are very grateful to Instituto Federal de Santa Catarina - IFSC for hosting the conference and providing support with personnel. Also our thanks go to Attitude Promo Events, especially to Alice Helena Silva and Michelle Rullier Cisneros.

This Proceedings volume contains 36 papers from contributors organized into seven topics covering HAB Ecology, Benthic HABs, Cyanobacteria, Toxicology, Genomics and Mitigation. Thanks are due to the referees who peer reviewed submitted manuscripts within their area of expertise.

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Are HABs and their societal impacts expanding and intensifying? A call for answers from the HAB scientific community

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Abstract

Hypotheses, evidence and questions about the global expansion and increasing impacts of HABs have been put forward since the first recognition of these phenomena by international scientific fora. After about four decades of ever intensifying research and monitoring activity, the HAB scientific community is called to put together the data on the occurrence of harmful species and on their impacts that have so far been collected at local scales and analyze them in a regional and global perspective. The Global HAB Status Report Initiative (GHSR) aims at producing an overview of HAB events and their societal impacts; GHSR will combine the effort of Regional Groups, International Agencies, ISSHA and individual scientists in a large scale exercise towards a worldwide appraisal of the occurrence of toxin-producing microalgae, along with an assessment of the status and probability of change in HAB frequencies, intensities, and range resulting from environmental changes at the local and global scale.

Keywords: Global HAB status; OBIS; HAEDAT

Introduction

Harmful algal blooms (HABs) are long known natural phenomena which have attracted increasing attention of scientists (Fig. 1), environmental agencies, fishermen and citizens over the years. The first surge in interest coincided with the 1st Conference on Toxic Dinoflagellates held in Boston (Ma, USA) in 1974 (LoCicero 1975), which was convened in recognition of the need to integrate different fields of research in order to study these events, understand their driving factors and manage their impacts. The names of scientists – and also of microalgal species – involved in HABs have dramatically changed since then, yet the Boston Conference marks the birth of the interdisciplinary scientific community which regularly keeps on meeting at ICHA conferences. In addition to this Conference series, over the years other important steps have been made towards coordinated research and management of HABs: the establishment of the IOC Intergovernmental Panel on HABs (in 1992), the International Society for the Study of Harmful Algae (1997) and the research programs GEOHAB (1998) and GlobalHAB (2015), as well as the publication of the newsletter Harmful Algae News (1992) and of the journal Harmful Algae (2002).

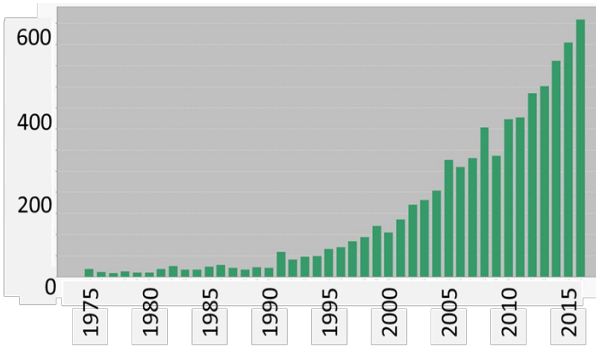

Fig. 1. Exponential increase in HAB publications in the period 1975 to 2016. Search in the Web of Knowledge for titles including the words “harmful blooms or harmful algal blooms” or ”red tides” or ”toxic algae” or ”toxic phytoplankton”. Indexes: SCI-EXPANDED, SSCI, A&HCI, ESCI.

Are HABs increasing and expanding?

Interestingly, the possible increase in frequency of red tides outbreaks is mentioned already in the

preface of the Boston meeting proceedings (Prakash 1975) in which some time series of HAB data were also explored (e.g., Baldridge 1975). In fact, whether HABs have increased and expanded and what are the possible reasons for the observed trends have been the most frequently asked questions about HABs ever since, with hypotheses and possible explanations debated in widely cited studies (e.g., Hallegraeff 1993; Smayda 1997; Heisler *et al.* 2008; Paerl and Huisman 2009; Wells *et al.* 2015). In these overviews, eutrophication, human-mediated introduction of alien harmful species, climate variations and aquaculture impacts were mentioned as possible reasons for an expansion and intensification of HABs.

On the other hand, many more toxic species are known today than in the past. For example, the discovery of new toxic members of *Alexandrium, Pseudo-nitzschia*, *Ostreopsis* and *Gambierdiscus*, as well as the new genera *Azadinium*, *Karenia, Karlodinium, Takayama* and *Vulcanodinium,* have contributed to widen considerably the reference list of known harmful species since it was first established in 2002 (Fig. 2).
The number of known toxins (Fig. 3) has increased in parallel with the discovery of the species producing them, or even preceding it as in the case of *Azadinium.* In addition, the need to protect human health and food resources for an ever increasing human population has resulted in increased monitoring and research activity. Therefore, capability and efforts to detect harmful species and harmful events have clearly grown over the years, which would by itself explain the increase in frequency and distribution of reporting of harmful events across the world's seas.

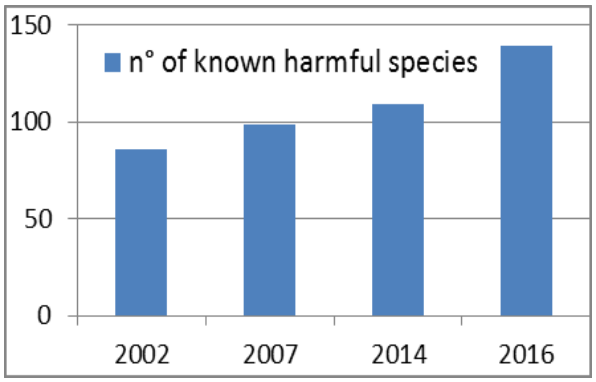

Fig. 2. Number of species known to produce toxins impacting on fish and humans, as listed in the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (Moestrup *et al.* 2009 onwards), accessed in different years.

Whether actually increasing or not, HABs will undoubtedly constitute a growing threat to human activities related to the sea, including the exploitation of wild or cultivated seafood, recreational activities and tourism. In this perspective, understanding long-term trends and large-scale distribution patterns of harmful species is a most relevant goal, as we need to predict whether, where and when we should expect changes in HAB frequency and intensity in order to plan effectively management operations and the use of the marine space.

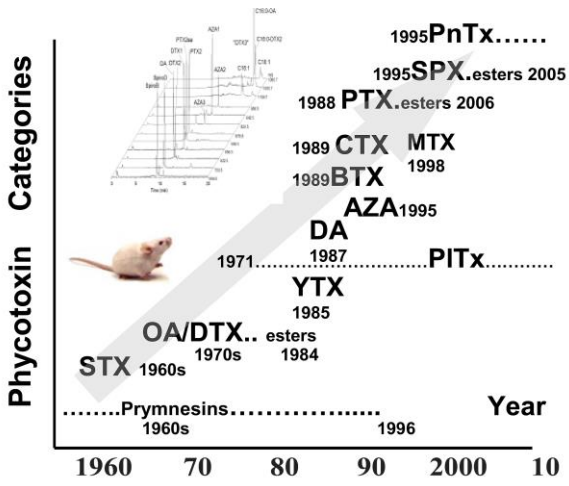

Fig. 3. Timeline of discovery of the major categories of phycotoxins (modified after Hess 2008). The inset images refer to the change in methodologies from the mouse bioassay to mass spectrometry. STX: Saxitoxin; OA: Okadaic Acid; DTX: Dinophysis Toxins; YTX: Yessotoxin; PlTX: Palytoxins; DA: Domoic Acid; AZA: Azaspiracids; BTX: Brevetoxin; CTX: Ciguatoxin; MTX: Maitotoxin; PTX: Pectenotoxin; SPX: Spirolides; PnTX: Pinnatoxins.

While long-term series of data concerning the diversity and abundance of harmful species and/or the presence of toxins in seafood are accumulating worldwide, signals of wide interannual variations and of intensification of harmful events caused by microalgae keep on emerging in different areas, along with records of new events in areas not affected before. Examples are the recent, exceptional *Pseudo-nitzschia* bloom along the east Pacific Canadian coasts (McCabe *et al.* 2016), the unprecedented *Alexandrium* blooms in Tasmania

(Hallegraeff this volume), the northward expansion of *Alexandrium* blooms in Chile (Hernández *et al.* 2016), and the range expansion of pelagophyte blooms (Zhang *et al*. 2012; Gobler *et al.* 2013). Less easy to track are signals of negative trends or lack of any trends, as this type of negative results are generally considered unattractive in the current scientific literature. Therefore, a sound appraisal of the global status of Harmful Algal Blooms is presently a difficult task. The turning point towards this goal would be to try and put together local information and start looking at it in a regional and global perspective.

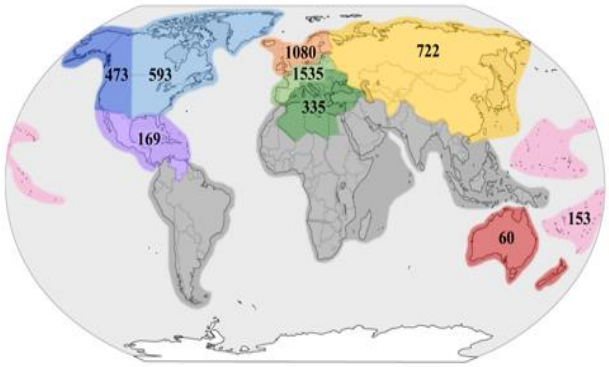

Fig. 4. Total number of HAEDAT records as of 1/3/2017 in different OBIS regions. The grey coloured regions of South America, Africa and South East Asia point to key missing data sets. Compiled by L. Schweibold and G. Hallegraeff.

A data-based HAB Status Report

To this aim, the first ever 'Global HAB Status Report' (GHSR) aims at producing an overview of HAB events and their societal impacts by providing a worldwide appraisal of the occurrence of toxin-producing microalgae and assessing the status and probability of change in HABs frequency, intensity, and range resulting from environmental changes at the local and global scale. Linkages will be established with the International Panel on Climate Change (IPCC) reporting mechanism which is increasingly focusing on the biological impacts of climate change. The GHSR will provide the scientific community as well as decision makers with a reference on HAB occurrence and impacts on ecosystem services. The development of a GHSR was decided at the tenth session of the IOC Intergovernmental Panel on HABs (IOC-IPHAB), in 2013, and partners included the International Atomic Energy Agency (IAEA), the International Council for Exploration of the Sea (ICES), the North Pacific Marine Science Organization (PICES) and the International Society for the Study of Harmful Algae (ISSHA). The project receives financial support from the Government of Flanders.

The Report will focus on several HAB datasets which are being gathered, including the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae (www.marinespecies.org/hab) and will build on newly collected data as well as data in the databases HAEDAT (http://haedat.iode.org) and OBIS (http://www.iobis.org), which are both components of the IOC International Oceanographic Data Exchange Programme IODE. The first GHSR will be launched at the 18th ICHA in October 2018, and there are plans to update it periodically thereafter.

Different regions and countries suffer from different types of HABs, and this is reflected in the way countries/regions enter their data in HAEDAT. North America and Europe operate highly sophisticated shellfish toxin monitoring programs, which often report high target species abundances even in the absence of toxin data or shellfish farm closures. The effectiveness of these programs is well reflected in the fact that only an estimated 1.5% of events involve human poisonings. On the other hand, Pacific HAEDAT data exclusively concern human ciguatera poisonings diagnosed by medical practitioners (99% human poisonings), without any associated microalgal or toxin data being collected. OBIS HAB species occurrence data are even more incomplete, and heavily biased by European records (Fig. 5). It is noted that only 18 *Gambierdiscus* records are included [as of 15/9/2017] for the whole world. Available data for the key target species *Alexandrium, Dinophysis* and *Pseudo-nitzschia* exhibit an increase in frequency over the past 30 years, undoubtedly reflective of increased awareness.

HAB scientists are expected to play a major role in the development of the GHSR, and many of them are already involved and contribute actively to the development of the above mentioned collective datasets.

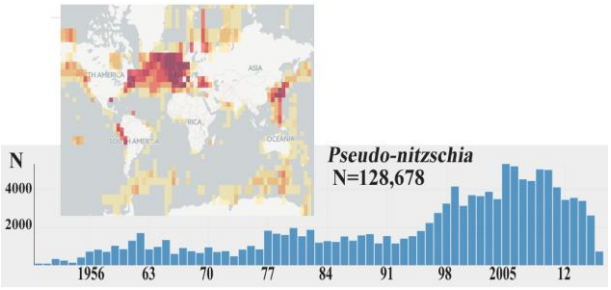

Fig. 5. OBIS species records of *Pseudo-nitzschia* diatoms between 1950 and 2014. Data as of 17/7/2017. Records are heavily biased towards Europe.

As a first step towards their full involvement, a call was launched shortly prior to the 17th ICHA for the participation in the compilation of a dynamic poster, to be prepared during the Conference, which would collect graphs showing decadal trends in harmful species abundance and impact. Several colleagues listed in the acknowledgements recognised the importance of this initiative, and expressed their willingness to cooperate. The relevance and implications of this new collective scientific challenge were also discussed during an information session at the 17th ICHA. Several examples of trends in species, toxic events or toxins, either published or unpublished, were provided from different sites of the world, demonstrating that appropriate data exist, which have seldom been exploited within this long-term change perspective and never at the global scale.

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Climate shift triggers shellfish harvesting bans in Uruguay (south-west Atlantic Ocean)

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Abstract

Harmful algal toxins accumulate in shellfish representing a human health hazard; therefore commercial shellfisheries should be controlled. In Uruguay, the National Direction of Aquatic Resources is the government authority responsible for shellfish health and commercialization. Harvesting and commercialization of shellfish are banned when their toxins exceed safety limits. We analyzed 36 years (1980-2015) of shellfish bans in Uruguay in the context of regional environmental changes, and anomalies in sea surface temperature (SSTA). Thirthy-two bans were registered and the two most frequent causative toxins were paralytic shellfish toxin (PST) and diarrhetic shellfish toxins (DST). The PST producer species were, mainly, *Gymnodinium catenatum* and *Alexandrium tamarense*, and the DST producer species was *Dinophysis acuminata* complex. Ban periods ranged from 7 to 189 days, with the longest ban caused by DST in 2015. We found that the bans caused by the cold-water species *A. tamarense* and by *G. catenatum* had stayed the same or decreased. On the other hand bans caused by the warm-water *D. acuminata* complex showed a significant increasing trend after the late 1990s, which was related with regional warming signals observed in the South Atlantic Ocean. Toxic outbreaks in Uruguay may increase with regional climate change as temperature increases.

Keywords: Dinophysis, DST, *Gymnodinium catenatum*, PST, *Alexandrium tamarense*, climate change.

Introduction

Certain marine algae produce toxins that accumulate in shellfish, and pose a serious threat to human health through the consumption of contaminated shellfish. The National Direction of Aquatic Resources (Dirección Nacional de Recursos Acuáticos, DINARA, is its acronym in Spanish) is the government authority responsible for sanitary control of shellfish and its commercialization in Uruguay. In the monitoring plan, established in 1980, toxins in shellfish are weekly determined, and when toxins exceed internationally established safety limits, harvesting and commercialization are banned. We analysed a 36 years period (1980-2015) of bans in Uruguay in the context of regional environmental changes and anomalies in sea surface temperature.

Material and Methods

Sea surface temperature anomalies (SSTA) was obtained and analysed as the cumulative sum of SSTA per year (csSSTA) following Ortega *et al*. (2016). Temperature and salinity *in situ* and phytoplankton samples (fixed with 4% neutralized formaldehyde) were obtained by the monitoring program (4 to 6 sites, weekly sampled, Fig. 1), detailed in Méndez (2006). Toxin presence and/or concentration in shellfish were determined by mouse bioassay standardised method (Industry Department, DINARA). Data from the monitoring program were adopted as mean (minimum-maximum). The normality and homogeneity of variances within the data were confirmed with the Shapiro-Wilk and Finger test respectively. Days of bans per year for the *D. acuminata* complex were transformed with natural logarithm adjusted to normal distribution. The trend of the days of bans per year, per species, was evaluated by linear regression. To evaluate the relation among csSSTA and bans of different species, linear regressions and Pearson correlations were performed; only statistically significant results are shown. The alpha value for statistical significance was 0.05 and all of the analyses were performed

Proença, L. A. O. and Hallegraeff , G.M.(eds). Marine and Fresh-Water Harmful Algae. Proceedings of the 17th International Conference on Harmful Algae. International Society for the Study of Harmful Algae 2017.

using an R platform (R Development Core Team 2008).

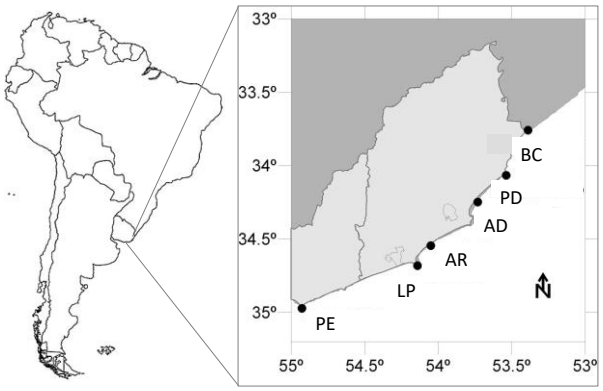

Fig. 1. Study area, showing the location of the sample station of the monitoring program in the South coast in Uruguay in this study: Barra del Chuy (BC), Punta del Diablo (PD), Aguas Dulces (AD), Arachania (AR), La Paloma (LP) and Punta del Este (PE).

Results and Discussion

Dinophysis acuminata complex bans had increased significantly in the last 20 years (R adj. = 0.949; $p < 0.001$), and were the most frequent and extended bans in Uruguay. These species produce diarrhetic shellfish toxins (DST), lipophilic compounds, that accumulate in shellfish tissues and cause diarrhetic illness in human consumers and are the principal problem for aquaculture in many countries (Reguera *et al* 2014). The other species causing bans were *Alexandrium tamarense and Gymnodinium catenatum*. *A. tamarense* had not been responsible of bans in the last 20 years and occurred in low frequency during the phytoplankton community analysis of the monitoring program. This cold water species (Balech, 1995), showed an optimal growth rate at 17ºC in laboratory experiments (Hamasaki *et al.* 2001), so its absence could be related to the increase in temperature. While *G. catenatum* outbreaks have remained unchanged through time, in the period of study it caused a total of 20 bans of 18 (7-182) days of bans per year. Regional SSTA showed a shift from a cold period (negative SSTA) to warm period (positive SSTA) after 1997, followed by increasing frequency of DST bans. A positive and highly significant correlation among csSSTA and *D. acuminata* complex bans after the regime shift in 1997 was found ($P = 0.988$; $p < 0.001$ $N = 18$, Fig. 2).

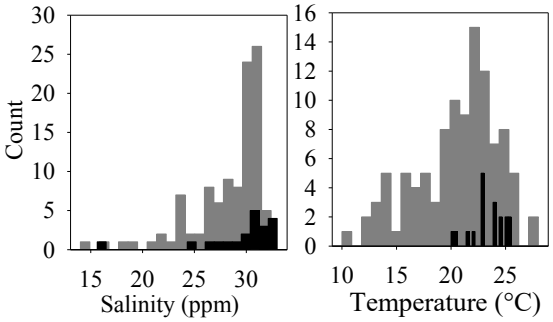

Fig. 2. Coastal salinity and temperature during the bans caused by *Dinophysis acuminata* complex before (black bars) and after (grey bars) 1997. Data from the monitoring program, determined *in situ* from the coast.

Temperature and salinity measured during DST bans were 23.4 °C (20 – 25.5 ºC) and 30 ppt (16 - 33 ppt) before the switch in 1997; and 20.5 (10 – 28.5) ºC and 28.3(14 – 32.4) ppt (Fig. 3). The *D. acuminata* beans during the period before 1997 shown a superior temperature mean than the posterior period, however, the number of observations and the maximum are superior after 1997 (Fig. 3). These relatively high values of temperature and salinity could be related to an increased influence of warmer oceanic waters advected by the Brazilian Current after 1997 in agreement with Ortega *et al.* (2016). *D. acuminata* is a cosmopolitan species with warm water preferences, and it had been seen that in batch culture, the maximum growth rates is at 20°C (Kamiyama, 2010), and therefore a warm-water regime may favour its proliferation. Furthermore, higher temperatures could delay the depuration capacity of shellfish. The toxin molecules move through the tissues of the mollusc, and they move faster at higher temperatures. The second tissues pool where the toxins are located, have a slower depuration rate than the first one (Nielsen *et al.* 2010). Therefore, warm regimes may favour blooms of *D. acuminata* complex and the frequency of bans.

Changes in ocean water currents and interannual climate variability associated with warming, could be related with the increase in frequency and duration of *D. acuminata* complex outbreaks in Uruguay. This warming trend in the area has been documented before (eg. Ortega *et al.* 2012; 2013) and the area was marked as a hot spot of warming

(Hobday *et al.* 2016). This problem not only threats human health, but also the economic sustainability of local seafood-dependent communities that had already been impacted by the consequences of warming (Hobday et al. 2016) caused by the closure of fisheries due to red tides.

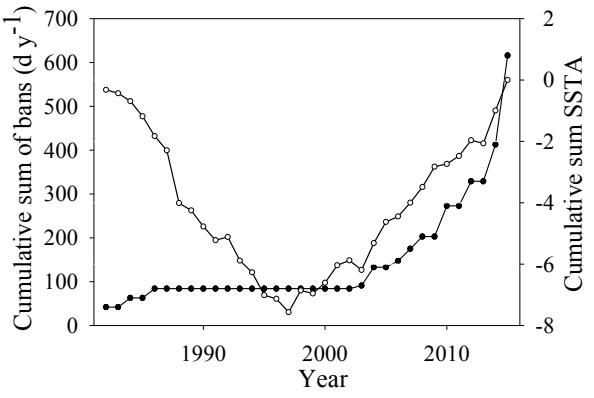

Fig.3. Cumulative sum of days of bans per year of *Dinophysis acuminata* complex (black dotted line) and cumulative sum of sea surface temperature anomalies (SSTA; white dotted line) in the period of study. The vertical line indicates the switch from cold to warm period in year 1997.

Acknowledgements

We thank Agencia Nacional de Investigación e Innovación (ANII) and Industry Department, DINARA.

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Extreme abundant bloom of *Dinophysis ovum* associated to positive SST anomalies in Uruguay

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Abstract
Blooms of *Dinophysis* of the acuminata complex have been reported in Uruguay as causative of Diarrhetic shellfish poisoning at austral summer (January to March) since 1992. An exceptional abundant and toxic bloom, was detected in summer 2015, The bloom initiated during a dry period with a consequent salinity increase, due to the advection of Brazilian warm water current to the Uruguayan coast. This species was identified genetically as *Dinophysis ovum* and the toxin production was quantified by Méndez *et al.* in this volume. This bloom caused the longest ban period due to lipophilic toxins in Uruguay (5 months in Maldonado and 6 months in Rocha Department).The bloom reached record concentration of $15x10^4$ cell.L^{-1} in La Paloma and $113x10^3$ cell.L^{-1} in Punta del Este stations. The description of population growth conditions, will be an advance forward the predictive of future toxic periods. The evolution of this exceptional bloom was tracked along six coastal stations. Cell densities above $30x10^4$ cell.L^{-1} were observed at a narrow range of salinity (31,4-32) and temperature (20-24 ºC).

Keywords: Dinophysis ovum, SST anomalies, Toxic bloom, Uruguay

Introduction

Dinophysis blooms have been reported in Uruguay since 1992. They have been associated to the austral summer, when salinity increases due to the advection of warm salty oceanic waters over the Uruguayan shelf. The contamination of bivalve molluscs due to *Dinophysis* blooms, represent a severe threat for the shellfish industry. The first shellfish closure due to DSP (Diarrheic Shellfish Poisoning) took place in 1992 (Méndez, 1993) and up to date *D. acuminata* complex have been the responsible of all the following DSP shellfish closures in Uruguay.

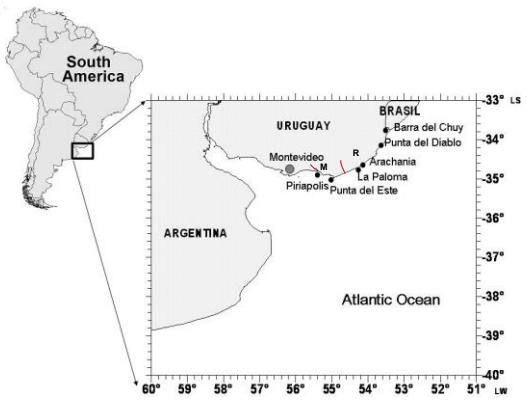

Fig. 1. Sampling stations along the uruguayan coast. Maldonado(M) and Rocha(R) Departments.

The aim of this work is describe the extreme *Dinophysis ovum* bloom which caused the longest ban due to lipophilic toxins in Uruguay and characterize its environmental conditions. The description of population growth conditions, will be an advance forward the predictive of future *D. ovum* toxic events.

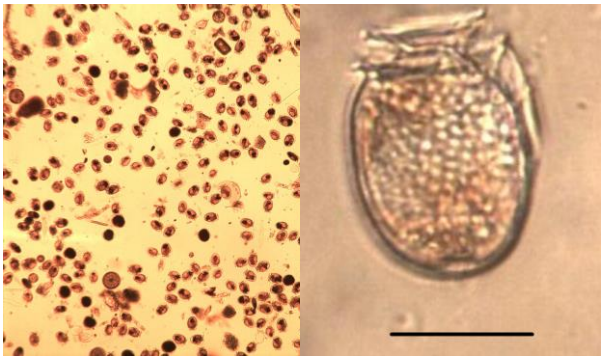

Fig. 2. Micrograph of the almost mono-specific bloom of *Dinophysis ovum* from Uruguay, March 2015 (Scale Bar=20µm)

Material and Methods

Field samples were taken weekly from January to September at 6 stations along the external Rio de la Plata and the Atlantic coast of Uruguay (Departments of Maldonado and Rocha) (Fig. 1).
YSI probe was used to record surface temperature

and salinity. Quantitative samples were preserved with Lugol´s iodine. 10 ml counting chambers were used to settled aliquots, identify and quantify the phytoplankton species by the Utermöhl method (1958), under an inverted microscope (Leitz Labovert FS). Micrographs were taken using an Evolution C camera coupled to the microscope. Data of historic DSP bans and *Dinophysis* records was provided by the National Monitoring Program on Harmful Phytoplankton and Toxins in Molluscs (DINARA-MGAP).

Results and Discussion

An exceptional bloom of *Dinophysis ovum* (Fig.2) was identified in March 2015. Maximum densities were found in Punta del Este (113 x 10^3 cells L^{-1} on March 9), at salinity 32 and temperature 20ºC and in La Paloma (156 x 10^3 cells L^{-1} on March 16) (Fig.3) at salinity 31.4 and temperature 23ºC. The ban imposed due to Lipophilic toxins detection in shellfish was the longest (189 days) in Uruguay since 1992. The number of cumulative days a year, due to DSP shellfish closures, has shown a noteworthy increase, particularly in the last 5 years (Fig. 4).

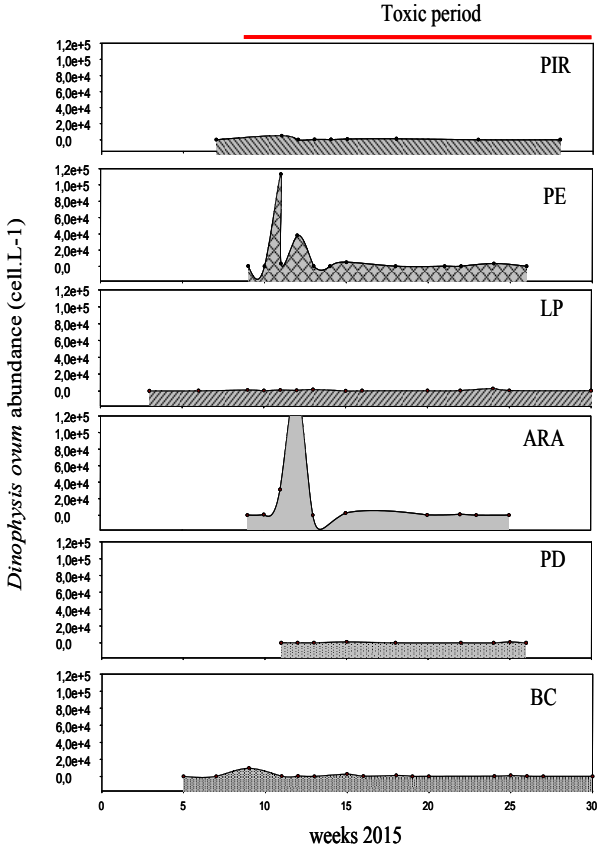

Fig. 3. Density of *D. ovum* at the six coastal locations: Piriapolis(PIR), Punta del Este (PE),La Paloma (LP), Arachania (ARA), Punta del Diablo (PD) and Barra del Chuy (BC).

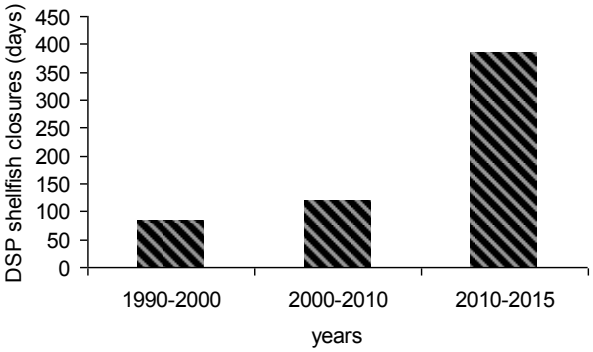

Fig. 4. DSP shellfish closures in Uruguay (1990-2015).

The higher abundances during the bloom of *Dinophysis ovum* were registered at higher salinities and temperatures (Fig.5).The bloom initiated during summer when warm salty waters of Brazil Current were advected over the Uruguayan shelf (Fig.6). In addition, 2015 summer was particularly dry and warm as it could be seen in the distribution of the sea surface temperature anomalies (SSTA) in the study area (Fig.7)

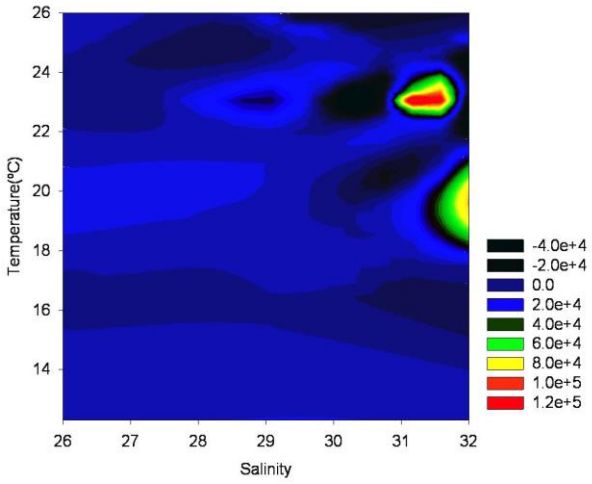

Fig. 5. Abundance of *D. ovum* vs Salinity and Temperature during the toxic event .

DSP associated to *Dinophysis acuminata* complex proliferations in Uruguay have been frequently reported during spring-summer (Ferrari *et al*. 2000; Méndez and Ferrari, 2002; Méndez, 2006), as in other parts of the world like France (Hongqin *et al*.2007), Brazil (Proenca & Mafra 2005), United States (Hattenrath-Lehmann *et al*. 2013) and Spain (Reguera *et al*. 2012).

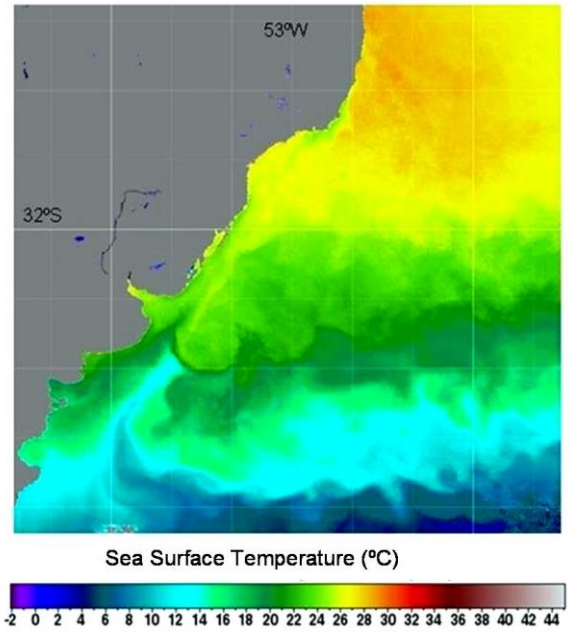

Fig. 6. Aqua MODIS image of sea surface temperature (SST) off the south western Atlantic Ocean (29º-36º S/51-56ºW). Shelf waters off Brazil, Uruguay and Argentina, in March 2015.

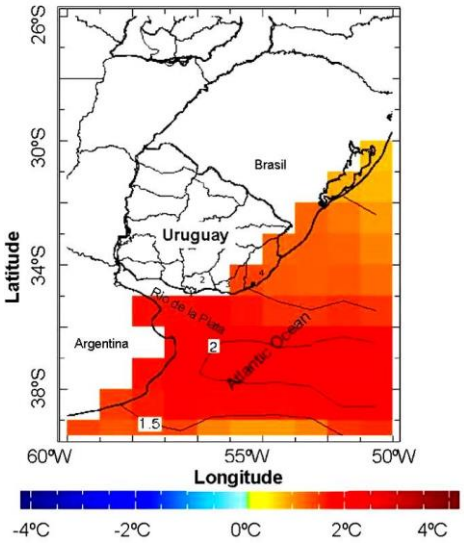

Fig. 7. Distribution of sea surface temperature anomaly, in Rio de la Plata estuary and shelf waters off Uruguay, March 2015, in relation with the 1971-2000 monthly mean. 1 = Montevideo, 2 =Canelones, 3=Maldonado and 4=Rocha. (http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NCEP/.EMC/.CMB/.GLOBAL/.Reyn_SmithOIv2/.monthly/.ssta).

Most of the historic DSP toxic blooms showed low cell densities of *D. cf acuminata.* This species has been observed at increased, during a 22 years period, reaching the previous record of 24300 cel,L^{-1} at Punta del Este, in 2014. The density of *D. ovum* registered in 2015, was exceptionally higher (156 x 10^3 cells L^{-1}), being the record value registered in South America until date. This could be showing a rising trend of the Dinophysis density. The same increasing trend could be observed in the days of ban, which are twice higher than two decades before.

Densities >30000 cel.L^{-1} were associated to salinity higher than 31 and temperature higher than 20°C, indicating a water upcoming from Brazil current. The positive SSTA observed, and previous research (Ortega *et al*. 2016) shows a warming trend in the Uruguayan coast. Because of that, and according to our results, the observed increment of Dinophysis toxic blooms could respond to the increasing incidence of the brazilian current on the uruguayan shelf.

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Characterization of *Dinophysis ovum* as the causative agent of the exceptional DSP event in Uruguay during 2015

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Abstract

Shellfish from the Atlantic coast of Uruguay have been contaminated by lipophilic toxins 13 times between 1992 and 2015. Events occurred mainly during spring-summer and always associated with blooms of the *Dinophysis acuminata* complex. An exceptionally dense ($15x10^4$ cells L^{-1}) and toxigenic bloom of *Dinophysis* developed in February 2015 (austral summer). Shellfish harvesting closures along the Atlantic coast of Uruguay started in February 26th and lasted for 6 months A size-fractioned (20-60 µm) sample was taken for toxin analysis at the peak of the bloom, composed almost exclusively of cells of *Dinophysis acuminata* complex. Individual cells were picked from an ethanol-fixed sample for genetic analysis. Only okadaic acid was found in the LC-MS analysis. Sequencing of the mitochondrial gene region encoding for the cytochrome c oxidase subunit 1 (*cox1*) showed the species was most closely related to *D. ovum*. The cells were small, with a smooth oval hypotheca and a narrow cingulum. Morphometric measurements (n = 100) showed the cells had a maximum length (L) of 34.2 ± 1.6 µm and a dorso-ventral depth (D) of 24.4 ± 1.2 µm. This is the first study on the morphology, phylogeny and toxinology of the main agent of DSP outbreaks in Uruguay.

Keywords: Diarrhetic Shellfish Poisoning, *Dinophysis* cf *ovum*, morphology, okadaic acid, cox1, Uruguay

Introduction

Dinoflagellates of the genus *Dinophysis* are the main agents of diarrhetic shellfish poisoning (DSP) events worldwide (Reguera *et al*. 2014). Since the first report of *Dinophysis* cf *acuminata* (Méndez 1993) and of DSP in 1992 (Ferrari *et al*. 2000) in Uruguay, DSP outbreaks affecting shellfish resources along the Uruguayan coast have been reported almost every year. DSP toxins are usually detected during spring-summer (Méndez and Ferrari 2002; Medina et al. 2003; Méndez 2006).

During late February (summer) 2015, a toxic DSP event with lipophilic toxins in mussels above regulatory levels started in the northern coast of Uruguay and spread to the south, affecting the entire oceanic coast. On March 12, coinciding with the bloom maximum, samples were collected for morphometrical, genetic and toxinological description of the local strains of *Dinophysis.*

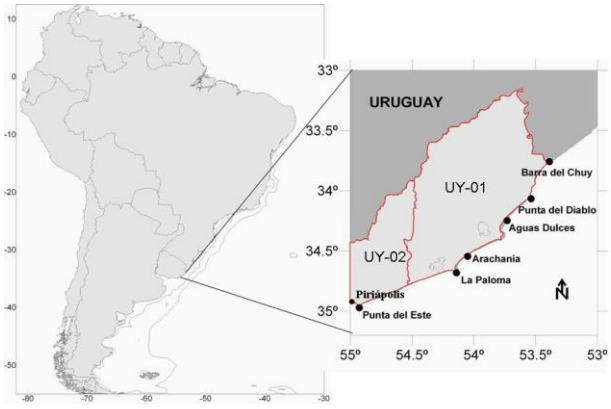

Fig 1. Sampling location, Punta del Este.

Material and Methods

Cell counting: Phytoplankton samples were collected from a coastal bridge in Punta del Este (Fig. 1) on March 12, 2015 during a harvesting ban

due to lipophilic toxins in mussels above regulatory levels according to mouse bioassays results from the monitoring programme. Cells density was estimated by the Utermöhl method (1958) after sedimentation of 25mL of a bottle sample fixed with Lugol and scanning the whole surface of the sedimentation chamber under an inverted microscope (Leitz Labovert FS). Images were taken with an Evolution C camera coupled to the inverted microscope.

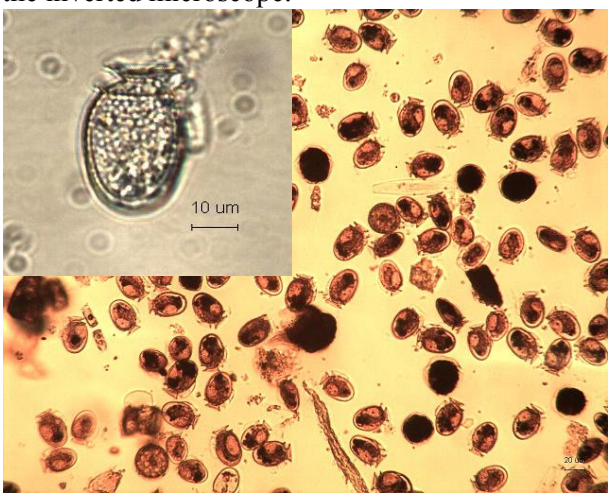

Fig. 2. A bloom of *Dinophysis ovum*, Micrograph taken from a bottle sample, fixed with Lugol, from Punta del Este, Uruguay, during the toxic event in March 2015. Inset: a single cell of *D. ovum* (scale bar = 10 µm)

Toxin analysis: 20 mL of a size-fractionated (20-60 µm) net haul sample were immediately filtered through a Whatman (GF/F) filter and transferred to a centrifuge tube with 3mL of 80% MeOH. An aliquot was taken and fixed with neutral formaldehyde (4% final concentration) to estimate cell density in the haul sample and calculate the amount of toxin per cell. Analysis of the extract by LC–MS was carried out following standard procedures for lipophilic toxins (Table 1) (Gerssen *et al*. 2009; EU-RL 2010).

Cells isolation, PCR amplification and DNA sequencing: Cells isolated from the net sample fixed with ethanol were washed with sterile mQ water on a glass slide, centrifuged in an Eppendorf tube (5 min, 13000 rpm, 4ºC), the supernatant discharged, the pellet resuspended in 10µl of mQ sterile water and divided in 2µl aliquots in 5 PCR micro-tubes. PCR was done with the primer DINOCOX1F/DINOCOX1R, as described in Raho et al. (2013). PCR products were purified with ExoSAP-IT (USB Corporation, OH, USA) and amplicons sequenced in both directions using the LightRun™ sequencing service (GATC Biotech AG, Germany).

Results and Discussion

The density of *D. acuminata* complex (1.13 x 10^5 cells L^{-1}) observed on March 12 at Punta del Este reached a record value for this location.

Table 2. List of toxins screened in samples of the *Dinophysis* bloom, Uruguay, March 2015

Compound	Molecule	m/z $[M+Na]^+$	Ref
OA	C44H68O13	827,4536	1
DTX1	C45H70O13	841,4689	1
DTX2	C44H68O13	827,4543	1
PTX2	C47H70O14	881	1
Belize.	C81H132O20	1447,891	2
Bel acid	C44H72O14	847,482	3
Metoka.	C45H70O13	841,4693	1
Noroka.	C43H66O11	781	1
5	C48H74O14	897	1
6	C50H76O14	923	1
7	C53H82O14	965	1
8	C53H82O14	965	1
9	C53H82O15	981	1
10	C53H82O16	997	1
DTX6	C51H76O14	935	1
12	C52H80O14	951	1
13	C54H82O14	977	1
OA-D8	C52H80O14	951,5422 m/z $[M+H]^+$	4
Prorocentrol.	C56H85NO3	980,6081	5

[1]Paz *et al*. 2007; [2]Napolitano *et al.* 2009; [3]Cruz *et al.* 2008; [4]Pizarro *et al.* 2008; [5]Torigoe *et al*. 1988. Belize.= Belizeanolide; Bel. Acid= Belizeanic acid; Metoka = Methylokadaate; Noroka = Norokadanone; Prorocentrol. = Prorocentrolide; OA-D8 = OA-D8 diol ester

Four days later, a density of 1.56 x 10^5 cells L^{-1} was observed at La Paloma (Fig. 1). This is a record value for Uruguay and for the southwestern Atlantic coast (Méndez *et al.* this volume). The official monitoring programme established a ban in this location, due to DSP above regulatory levels detected by mouse bioassay in wild mussels, from March to August 2015.

Morphological measurements of the Uruguayan specimens were: maximum length L: 34.2 $\pm$ 1.6µm; dorso-ventral depth D: 24.4 $\pm$ 1.2 µm; L:D = 1.4 (n = 100) (Fig 2). The size was in the range

of *D. ovum* observed in the Galician Rias Baixas, Spain (Raho *et al*. 2008), and smaller than the original description of *D. ovum* by Schüt (1895) and Schiller (1933).

Since *Dinophysis* was overwhelmingly dominant in the sample, the cells were morphologically identical and the sequence was very clean, we assumed the results are representative. The partial *cox-1* gene sequence from Uruguay was practically identical (3-4 different nucleotides along a total alignment of 865 nt) to those labelled as "*Dinophysis ovum/sacculus*" in Raho *et al*. (2013) and the sequences of Papaefthimiou *et al*. (2010). These authors, despite an initial morphological characterization of their strain as *D*. cf. *acuminata*, reported that based on the *cox-1*sequence the Greek strain was more related to *D. ovum*.

The sequence from Uruguay had 14 bp different from those of *D. acuminata* (AM931582), which is the reference of this species with morphological information (Fig 3). Therefore, the corresponding molecular phylogeny grouped *D. ovum* from Uruguay with the above mentioned sequences from Greek coastal waters (GU452507, GU452508 and GU452509) and from the NE Atlantic (AM931583) (Fig. 3). We thus designate the cells as *Dinophysis ovum*.

LC-HRMC analysis of a net-haul (20-60 µm fraction) overwhelmingly dominated by a single species of *Dinophysis* ($17x10^3$ cells mL^{-1}) showed OA as the only lipophilic toxin present in detectable levels. The estimated cell toxin quota was 7 pg OA $cell^{-1}$, which is similar to values reported for the Galician *D. ovum* (Raho et *al*. 2008).

Conclusions

The 2015 DSP outbreak in Uruguay was a record for the region in terms of *Dinophysis* cells density and persistence of DSP toxins in shellfish above regulatory levels. This is the first report and genetic characterization of *Dinophysis ovum* from Uruguay.

D. ovum cells contained ~7 pg OA $cell^{-1}$, which confirms this species as a major DSP toxin producer during this event.

Acknowledgements

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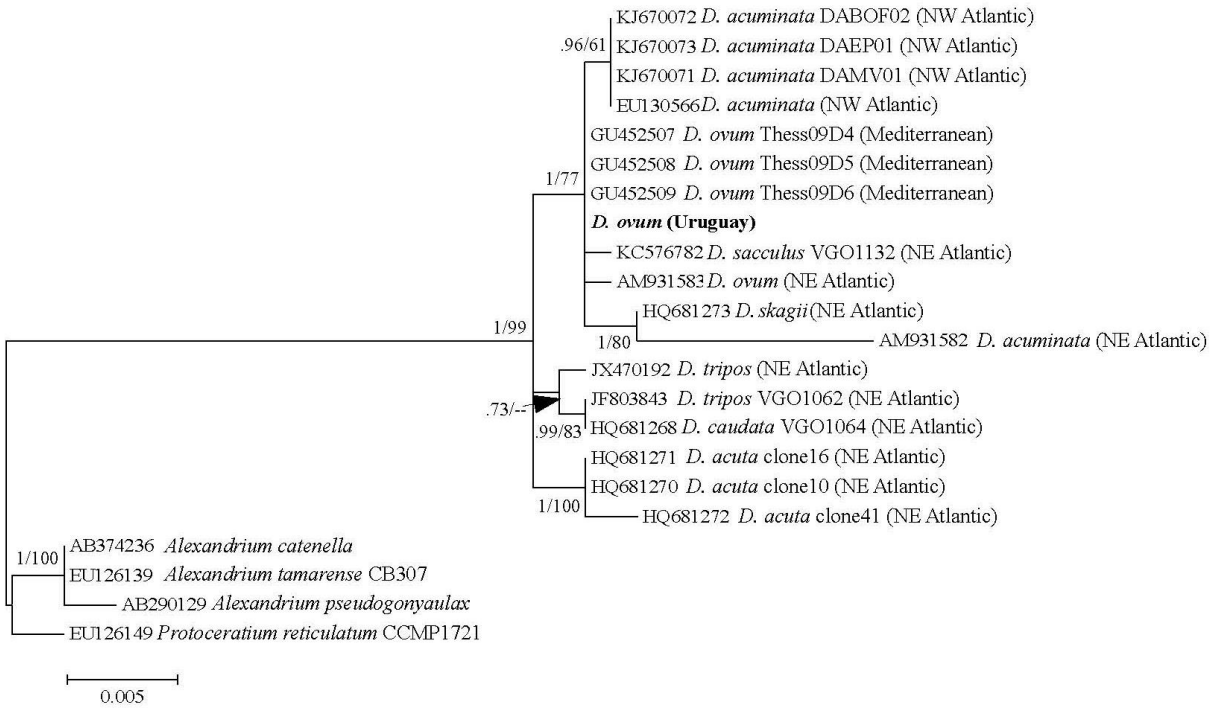

Fig.3- Phylogenetic tree based on *cox 1* gene sequences from Europe and the bloom of *D. ovum* found in Punta del Este, Uruguay, March 2015.

Watch out for ASP in the Chilean Subantarctic region

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Abstract

Domoic acid (DA) is one of the toxins monitored in the Magellan region (48° - 55° S) by LC-DAD since 1997, at levels ranging between non-detectable and trace. However, in November 2015, DA levels up to 20 µg g^{-1} were detected in ribbed mussels (*Aulacomya atra*) at a monitoring station in Ringdove estuary, (49°46' S, 74°19' W). A fortnight after this, DA was also detected by liquid chromatography with diode-array detector and liquid chromatography mass spectroscopy in fractionated plankton (20-200 µm), and below the regulatory limit in ribbed and blue (*Mytilus chilensis*) mussels (0.74 and 1.52 µg g^{-1}, respectively), both from Madre de Dios Island (50°20' S, 75°21' W), a more oceanic area. DA is commonly detected in scallop (*Argopecten purpuratus*) from low latitudes (27° S) and in blue mussels from the northern Chilean fjords (41°- 46° S). This finding of DA in areas with little or no human influence such as the Subantarctic channels and fjords, lead to consider that 2015-2016 was not only the year of dinoflagellates and Paralytic Shellfish Poisoning at the Chilean coast, but also suggested that these pennate diatoms may also have been involved in HAB events during the spring, but were unnoticed in the Magellan region mainly due to restricted access to these remote oceanic areas.

Keywords: Domoic acid, mussels, regulatory limit, Subantarctic channels-fjords

Introduction

Domoic acid (DA), which causes Amnesic Shellfish Poisoning (ASP), is one of the toxins that have been monitored in the Magellan region (48°- 55°S, southern Chile, Fig. 1) by liquid chromatography with diode-array detector (LC-DAD) in mussels from at least 43 stations over the last 3 years (Guzmán *et al*. 2016). Some stations have been monitored since 2011 (Guzmán *et al.* 2012). In addition, some DA analyses were carried out in 1997 and 2000 (Guzmán *et al*. 2000; Pizarro *et al*. 2000) due to the presence of the diatom *Pseudo-nitzschia australis* (Guzmán *et al*. 2000). The DA levels in bivalves were always in trace amounts, up to 0.66 µg g^{-1} (*e.g.* Pizarro *et al.* 2000), or non-detectable (*e.g.* Guzmán *et al*. 2000).

In 2015, for the first time, toxin levels close to the regulatory limit were found in ribbed mussels (*Aulacomya atra,* 19.75 µg g^{-1}) from a monitoring site, Ringdove estuary (49°46' S, 74°19' W; point M25N, Fig 1). During the following months, these levels decreased to non-detectable in the same species and sampling location.

A fortnight later and in samples from a more oceanic area, Madre de Dios Island (50°20' S, 75°21' W; points E13-15, Fig.1), DA was detected by LC-DAD and liquid chromatography with mass spectrometry (LC-MS) in the fractionated plankton (20-200 µm) and also at sub-toxic levels (*i.e.* below the regulatory limit) in ribbed (*Aulacomya atra*) and blue mussels (*Mytilus chilensis*). Madre de Dios Island is characterized by calcareous (western coast, E13-14) and siliceous (eastern coast, E15) formations.

In Chile, DA is common in low latitudes (27-30° S) where it has been detected in scallops (*Argopecten purpuratus*) since 1999 (Suárez-Isla *et al*. 2002), in a tunicate (*Pyura chilensis*), blue

and ribbed mussels (*M. chilensis, A. atra*) and clams (*Protothaca thaca*) (López-Rivera *et al*. 2009). In the northern sector of the Chilean fjords (41°- 46° S), the toxin has been detected since 1997 in blue mussels, clams (*Venus antiqua*), scallops (*Zigochlamys patagonica*) and oysters (*Ostrea chilensis)* (Suárez *et al*. 2002).

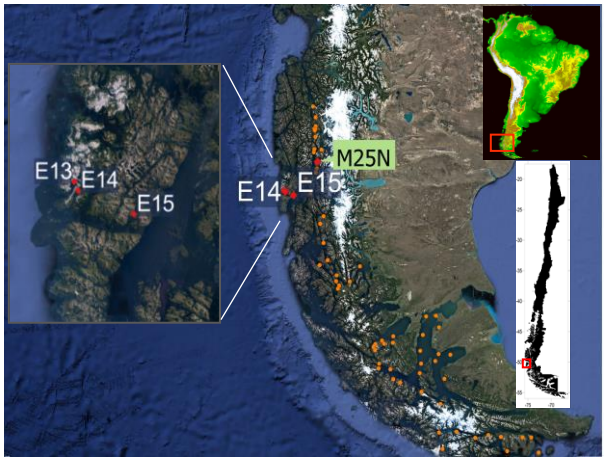

Fig. 1. Monitoring stations in the Magellan region. M25N station is highlighted. In the box map are shown the sampling stations in Madre de Dios Island.

Material and Methods

Plankton (20-200 µm), mussels (ribbed and blue) and clams (*Ameghinomya antiqua*) samples were collected during the Madre de Dios Island expedition (November 23 - December 3, 2015) in the Magellan region at three sampling stations: E13-E14-chalky and E15-siliceous stations (Fig. 1).
Water samples (plankton) were collected by bottle or pumping (5-10 min) from between 3 and 13 m depths (chlorophyll maximum). Size-fractioned plankton concentrates from water samples with a superimposed framed mesh system (200-100–20 µm mesh size) were obtained. Then each fractionated water sample was filtered through glass fiber filter (Whatman GF/F 0.7 µm, 25 mm) and the filter preserved in a centrifuge tube (1.5 mL). The bivalves were collected from the intertidal zone. Both filters and molluscs were frozen until analysis in the laboratory. Plankton extracts were obtained by adding 1 mL methanol to each centrifugation tube, manually homogenized with a plastic tip, and vortexed for 1 min before centrifugation at 10,285 × g for 10 min. The supernatant was recovered, filtered (Millex PVDF 0.45 µm, 13 mm), transferred to vials (1 mL) and dried at 37 ºC with a stream of nitrogen. Methanolic extracts of molluscs for DA analysis were obtained according to Quilliam *et al.* (1995). 2 g of homogenized meat was added to 15 mL centrifuge plastic tubes with 1 mL of aqueous methanol (50%, v/v) and mixed by vortexing for 1 min. The extracts were centrifuged at 2,656 × g for 10 min, the supernatant collected and the pellet resuspended in 1 mL aqueous methanol. This extraction was repeated using the same procedure. The supernatants were combined and mixed in 2 mL vials and dried at 37 ºC with nitrogen. For toxin analysis, each dry extract was re-suspended in 300 µL methanol and analyzed by LC-DAD at the Punta Arenas IFOP-red tide laboratory and by LC-MS/MS at the AWI-laboratory.

Results and Discussion

DA was detected in the fractionated plankton and at sub-toxic levels in ribbed (*A. atra*) and the blue (*M. chilensis*) mussels (Table 1).
Pseudo-nitzschia spp. (Fig. 2) as a percentage of the total phytoplankton was: 13-62, 4-35 and 2-28 % for E13, E14 and E15, respectively. Maximum concentrations of *Pseudo-nitzschia seriata* and *P. delicatissima* complexes were 2,500 and 10,400 cells L^{-1}, respectively.

The DA quota per cell (1.3-17.0 pg $cell^{-1}$; Table 1) was high compared to reports of maximally 1.7 pg $cell^{-1}$ for *P. australis* in culture and 0.01 pg $cell^{-1}$ for *P. calliantha*, both from the north of Chile (Álvarez *et al.* 2009). Reports from other parts of the world estimate DA cell quotas ranging from 0.15 to 33.6 pg $cell^{-1}$ in *P. seriata* (Fehling *et al.* 2004; Lundholm *et al*. 1994), up to 0.95 pg $cell^{-1}$ in *P. calliantha* (Besiktepe *et al*. 2008) and 67 pg $cell^{-1}$ in *P. multiseries* (Bates *et al.* 1999). However, for field populations of *P. australis* the values have reached to 75 pg $cell^{-1}$ (Scholin *et al.* 2000) and 78 pg $cell^{-1}$ (Trainer *et al.* 2000). Levels below regulatory limit (0.75-1.53 µg g^{-1}) found in mussels (Table 2) indicate that *Pseudo-nitzschia* densities were not sufficient to increase DA toxicity even though the cellular DA quota was high.

The presence of curved specimens of *Pseudo-nitzschia* in the field samples (Fig. 2) suggests a low availability of dissolved silicate (DSi) in the

Table 1. DA detected in plankton and shellfish samples from Madre de Dios Island, Magellan Region. (Fig. 1, box map).

	N° St.	Place	Depth	Samples	DA
			(m)		(pg cell^{-1})
Plankton	E13	Soplador Sound	13	(20-200 µm)	17.0
	E14	Eleuterio Channel	4	(20-100 µm)	1.5
				(100-200 µm)	1.3
				(> 200 µm)	1.7
	E15	Escribano Island	3	(20-100 µm)	9.4
				(100-200 µm)	5.4
				(> 200 µm)	-
					(µg g^{-1})
Mussels	E13	Soplador Sound	Beach shore	ribbed	0.75
			Beach shore	blue	1.53
	E15	Escribano Island	Beach shore	blue	-
			Beach shore	clam	-

water although this has not been reported in the literature previously. Past studies indicate a particularly low DSi concentration in the Magellan region (*i.e.* 2.0 ± 0.2 – 7.9 ± 0.8 µM, latitude 47-56° S) in relationship to other coastal areas of Chile (*e.g.* 18.7 ± 1.9 µM, latitude 42-46° S) during the Austral Spring (Torres *et al*. 2014).

It has been pointed out that low concentrations of DSi may induce an increase of cellular DA, which may indicate a difference in physiological state in different stages of *Pseudo-nitzschia* growth (Bates *et al*. 1991; Pan *et al*. 1996 a,b; Pan *et al*. 1998; Fehling *et al*. 2004). Particularly during the 2015-2016 dry warm season, this could result in low DSi input from continental sources into typicaly DSi-depleted Subantarctic oceanic waters (Torres *et al*. 2011), this was particularly true for Madre de Dios Island, since limestone and batholith mineralogy precludes large Si fluxes from the coast to the coastal ocean (Torres *et al*. 2014). However, it is not possible to exclude the role of typically low dissolved iron (Fe) of Subantarctic oceanic waters which can also raise the cellular DA (Wells *et al*. 2005; Trick *et al.* 2010). This hypothesis is consistent with the low frequency of DA events in the Magellan region althought the monitoring frequency is monthly. However the increase in DA may also be spatially localized in waters inside of the fjords. In January 2017, DA at 5-14 µg g^{-1} was detected in the digestive tract of scallops (*Chlamys vitrea*) from two monitoring sites (49° S): Penguin (M08) and Falcon (M09), fjords with glacial influence (Seremi Salud Magallanes, unpublished data). Furthermore, recent evidence has suggested that the presence of copepods may be one of the factors affecting the toxicity of *Pseudo-nitzschia* blooms in the field (Tammilehto *et al.* 2015).In summary, the detection of DA in shellfish and plankton indicates that the diatoms species were also involved in HABs event during 2015.

The detection of DA in areas with little or no human influence such as Subantarctic channels and fjords, lead us to conclude that 2015-2016 period was not only favorable for PSP-causing *Alexandrium catenella* and other dinoflagellates species in the Chilean channels and fjord system.

Such events virtually were undetected in the adjacent coast to the Pacific Ocean of the Magellan region due to restricted access by small vessels to the oceanic areas.

Availability of micro (Fe and Cu) and macronutriens (phosphates and nitrate) in addition to DSi likely play an role in the increase of the cellular DA (Wells *et al*. 2005; Amato *et al*. 2010; Trick *et al.* 2010). However, the understanding of factors regulating DA production requires further research, especiall in Subantarctic oceanic waters and in channels and fjords and with varying degrees of influence between both systems. The biological interactions between microalgae and zooplankton in this region also requires further study.

Our results suggest the importance of macro-scale climatic and oceanographic triggers in regulating the distribution, abundance and composition of

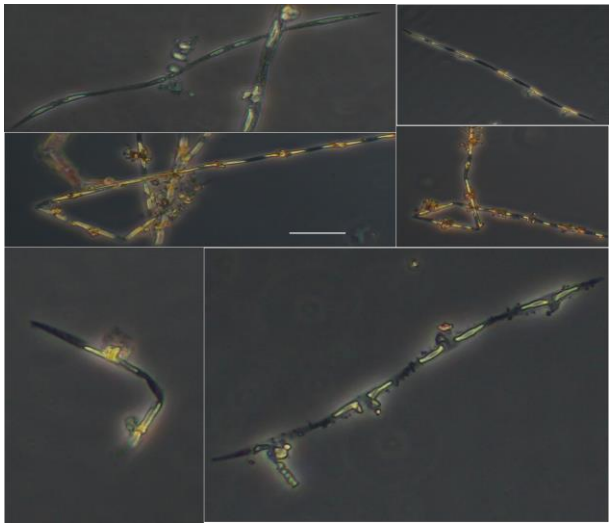

Fig. 2. Photo-micrographs of "curved" specimens of *Pseudo-nitzschia* of the *seriata* (center) and *delicatissim*a (upper and lower) complex. Field samples from Madre de Dios Island. Scale bar = 40 µm.

micro-phytoplankton assemblages, including harmful species.

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Climatic anomalies and harmful flagellate blooms in Southern Chile

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Abstract

The exceptional summer-fall climatic anomalies and "El Niño" of 2016 in southern Chile favored HABs in fjords and oceanic coastal waters and caused significant social and economic impacts. The principal harmful species were *Pseudochattonella* cf. *verruculosa* and *Alexandrium catenella*. Data collected during the bloom included phytoplankton abundance and composition, active fluorescence (Fo, Fm, Fv, Fv/Fm), *in situ* CTD-O profiles, chl-*a*, and backscattering. Large patches of *P.* cf. *verruculosa* were observed, with the highest abundance at ~7700 cells/mL and high chl-*a* and photosynthetic efficiency rates (>38,4 mg/m^3, 55%). The unique features of *P.* cf. *verruculosa* blooms were their occurrence in a remarkable thin layer and exhibiting high ichthyo-toxicity, leading to extensive damage of aquacultured, where more than 40000 ton of fish died within weeks. Profiles showed a sub-surface maximum above the pycnocline of bio-optical parameters consistent with *P.* cf. *verruculosa* cells. The thin layer maximum was coherent with the bloom´s highest temporal peak. Climatic anomalies and thin layer dynamics were key issues in the formation and decay of the *P.* cf. *verruculosa* bloom.

Keywords: P. cf. *verruculosa,* thin layer, climatic anomalies.

Introduction

In 2016, the summer and fall in Southern Chile were characterized by climatic anomalies, due to a strong "El Niño" cycle and probably enhanced by climate change (FAO, 2016). Consequently, more solar radiation, and higher temperatures than normal were observed. A time series analysis showed warmer climatic conditions and less fresh water inputs during the last 12 years, affecting phytoplankton distribution (Clément *et al*. 2016, Buschmann *et al.* 2016). Recently, several HABs have been identified in the inland sea and oceanic coastal waters of the South Eastern Pacific Ocean (Guzmán *et al.* 2010; Clément *et al.* 2016, Mardones *et al.* 2016) with enormous social and economic impacts. These blooms have been the most damaging in South America with losses greater than US$ 500 M. called the Chilean "Godzilla red tide" (G. Hallegraeff, pers. comm).

The coastal ocean circulation around Chile is characterized by a broad eastward West Wind Drift current at approximately 43°S that splits into an equatorward Humboldt current and a poleward Cape Horn Current (Silva and Neshyba, 1977; Strub *et al.* 1998).

Large scale atmospheric physical forcing –like the poleward movement of the South Pacific Anticyclone– produces a southward shift of the West Wind Drift, leading to biogeographic changes (Gatica *et al*. 2009), warmer climatic conditions and variability of freshwater inputs in fjords, disturbing the normal timing of phytoplankton blooms (Iriarte *et al*. 2016). Therefore, climate change will exert a series of complex pressures on phytoplankton distribution and HABs (Wells *et al.* 2015).

Our main objective was to analyse the photoautotrophic flagellates during the summer-fall blooms in Southern Chile, with emphasis on *P.* cf. *verruculosa*. In Chilean waters, *P.* cf. *verruculosa* was observed for the first time in 2004, and frequently in years thereafter. However, these previous observations caused caged salmon mortalities lower than 10% (Mardones *et al*. 2012). Bio-oceanographic studies and HAB monitoring have been a research focus in southern Chile (Guzmán *et al*. 2010; Pantoja *et al*. 2011; Iriarte *et al*. 2014), for at least the past two decades (Clément and Lembeye, 1993; Fuentes *et al*. 2008). For these reasons, we strongly believe that this ichthyotoxic photoautotrophic flagellate

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was absent from southern Chile marine waters prior to 2004.

Pseudochattonella forms recurrent extensive blooms in coastal waters of Japan, New Zealand and North Europe (Eckford-Soper, L. K., & Daugbjerg, N. 2016a). Despite the interesting research done by Scandinavian researchers on *Pseudochattonella* spp., (Andersen *et al.* 2015; Eckford-Soper, L. K., & Daugbjerg, N. 2016a; 2016b; 2017) and others (Chang *et al.* 2014) there are still many gaps on the autecological and practical aspects of this harmful photosynthetic flagellate.

Material and Methods

Climatological data –particularly rainfall– and air temperature time series were obtained from the Chilean Meteorological Service (www. meteochile.cl). The monthly Oceanic Niño Index (ONI) was obtained from the Climate Prediction Center (NOAA). In both cases, the data was processed as time series to detect variability and anomalies.

Phytoplankton composition and abundance data are part of the Monitoring Program of Plancton Andino, performed since 1998 in the marine inland sea (Fig. 1). Phytoplankton were sampled weekly at many fish and/or mussels farms sites located in an extensive area (ca. 700 km). Freshly collected phytoplankton samples (500 mL) were analysed inmediately upon arrival under inverted microscope (Utermöhl, 1958 - modified), either in the Chiloe Archipelago or Puerto Varas laboratories. Results were communicated using a GIS mapping and on-line procedure within 48 hours of collection, by the Plancton Andino SpA digital platform.

Photosynthetic activity expressed as Fo, Fm, Fv, routinely measured using a Fast Repetition Rate Fluorometer (FRRf3) (Oxborough *et al*. 2012). *In situ* variables of T, S (using a CTD-O), chl-*a* and the optical property of Backscattering, Bb (460 & 660 nm), using calibrated sensors at factory (Sea Bird/WetLabs, USA), were measured 15 days in a row in the peak of the HAB.

Time series analysis showes that temperatures were higher than normal, particularly after 2004 (Fig 2). We observed decrease in the monthly rainfall in the Puerto Montt area after 2008 (≈ 42°S, Fig. 2a). The summer-fall of 2016 was dry and warm and the Oceanic Niño Index was the largest observed since the 1997/98 event.

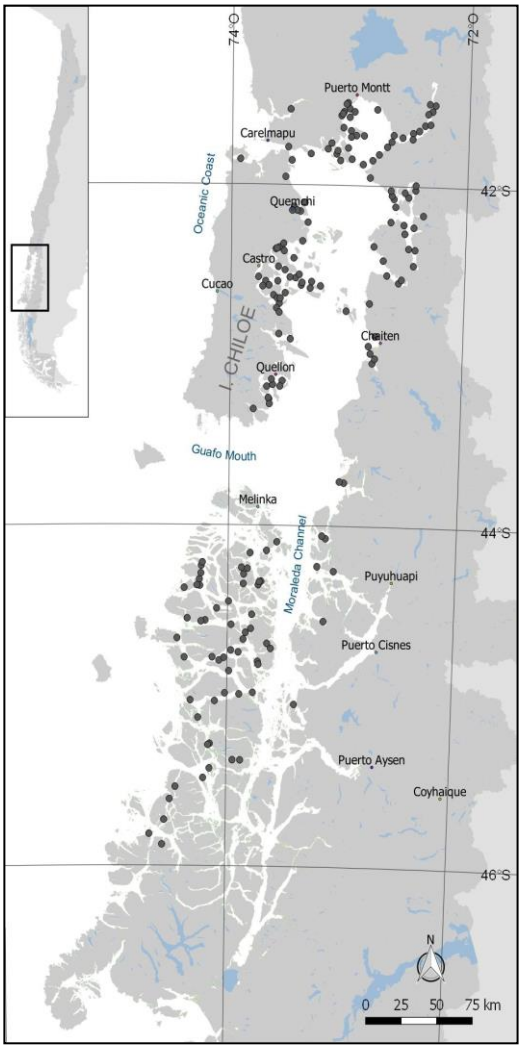

Figure 1. Stations and sites of the Phytoplankton Monitoring Program in the inland sea of southern Chile.

Results and Discussion

The summer of 2016 presented favorable conditions for HABs due to an "El Niño" event, showing its predominant effects as depressed upwelling, and other strong climatic anomalies influencing the ecosystems in the region. We noticed the first outbreak of *P*. cf. *verruculosa* on January 23, at locations at the central Chiloe Archipelago, seriously affecting 3 to 4 salmon farms. However, the greatest impact occurred

during the last week of February (SST max ≈19 °C) and the beginning of March, within the northern inland sea section, (Reloncavi Sound), very close to P. Montt, with >4000 cell/mL (Fig. 3 a, b).

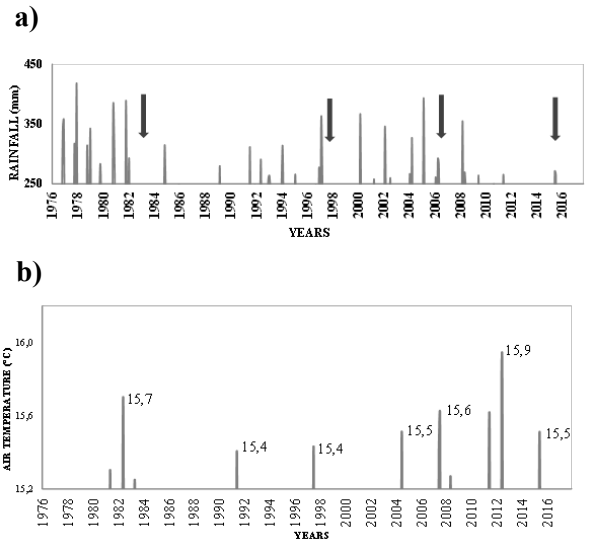

Figure 2. Variation of monthly rainfall > of 250 mm. The arrows show intense "El Niño" events. **(a).** Monthly mean of air temperature > 15.2°C. **(b).** Data from "El Tepual" airport 1976-2016. Puerto Montt, Chile, Source: www.meteochile.gob.cl

In addition to extensive spatial monitoring, we performed daily vertical profiles at a fixed station near Puerto Montt. The main results confirmed a thin layer of *P.* cf. *verruculosa* cells above the pycnocline (Fig. 4). The thin layer distribution corresponded well with the photochemical parameters (PSII). The maximum values of Fo and Fm matched consistently with the maximum *P.* cf. *verruculosa* cell abundance (Fig. 4b and c).

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The thin layer chl-*a* maximum was consistent with actively growing *P.* cf. *verruculosa* cells (photosynthetic efficiency > 50 %, Fm > 14) and coincided with the highest biomass peak of *P.* cf. *verruculosa* cells during the bloom.

Climatic anomalies may be invoked to explain the formation of the bloom, but thin layer dynamics may be related to the decay of the *P.* cf. *verruculosa* bloom. More specifically, water column mixing and pycnocline position (Fig. 4 a) and gradient are thought to have significantly contributed to the HAB decay.

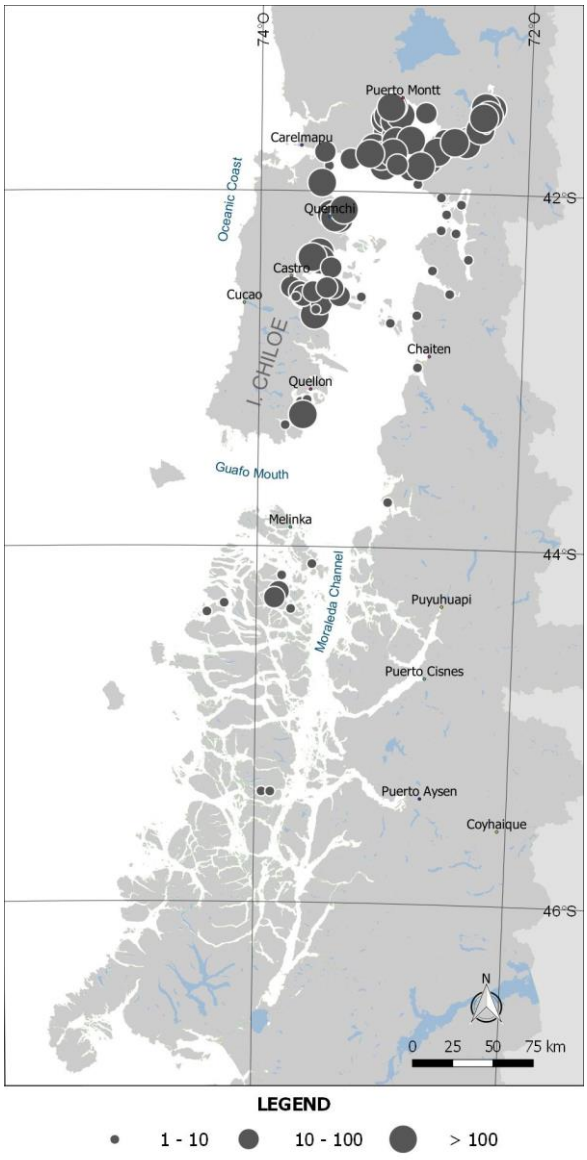

Figure 3. Maximum *P.* cf. *verruculosa* cells/mL abundance and spatial distribution **(a)**. Temporal *P.* cf. *verruculosa* cells abundance (= PS) and chl-a maximum at Reloncaví Sound **(b)**.

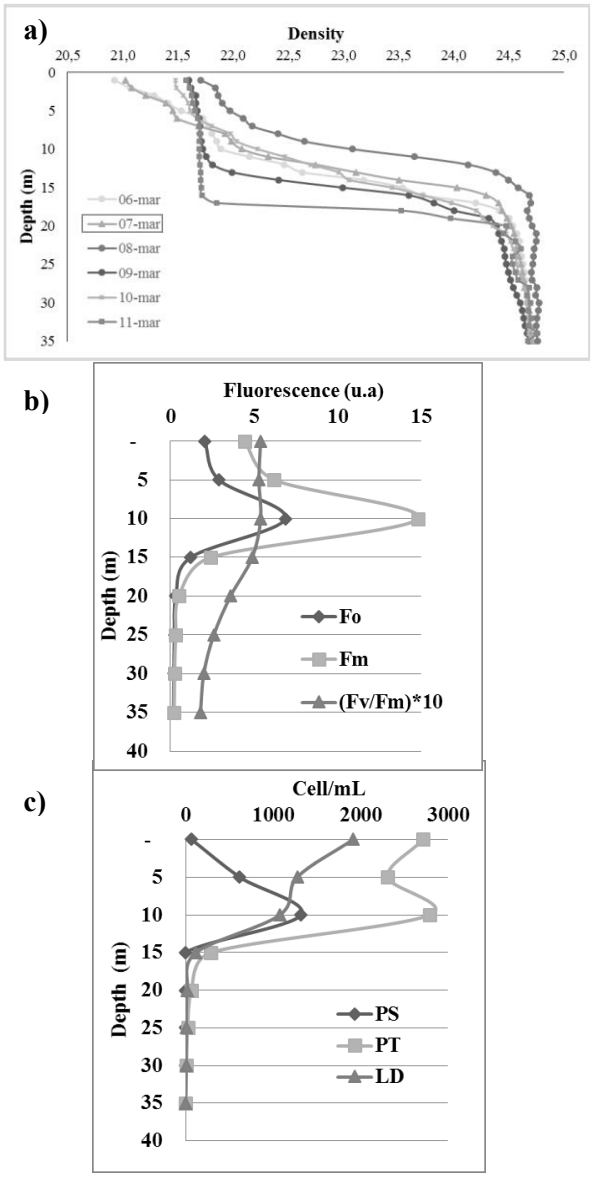

Figure 4. Water column distribution of density as sigma-t **(a),** photosynthetic parameters Fo= Initial Fluorescence, Fm= Max. Fluorescence, (Fv/Fm)*10= Photosynthetic Efficiency. **(b)** and phytoplankton abundance **(c)** during peak of the bloom. PS=*P.* cf. *verruculosa*, PT=Total phytoplankton, LD = *L. danicus*. Reloncaví Sound, March 6, 2016.

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Unprecedented *Alexandrium* blooms in a previously low biotoxin risk area of Tasmania, Australia

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Abstract

During October 2012, a shipment of blue mussels (*Mytilus galloprovincialis*) from the poorly monitored east coast of Tasmania, Australia, was tested by Japanese import authorities and found to be contaminated with unacceptable levels of Paralytic Shellfish Toxins (PSTs; 10 mg/kg). Subsequently local oysters, scallops, clams, the viscera of abalone and rock lobsters were also found to be contaminated. This led to a global product recall and loss to the local economy of AUD 23M. Following low toxicity during 2013 and 2014 and implementation of minimal shellfish farm closures, a more severe bloom event occurred during July-November 2015 and again June-September 2016 (up to 300,000 *Alexandrium* cells/L; 24 mg/kg PST in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing 4 human illnesses resulting in hospitalization after consumption of wild shellfish. While *Alexandrium tamarense* had been detected in low concentrations in southeastern Australia since 1987, all cultured strains belonged to the mostly non-toxic group 5 (now designated *A. australiense;* detected since 1987) and weakly toxic group 4 (*A. pacificum;* detected in 1997). In contrast, the 2012 to 2016 outbreaks were dominated by highly toxic group 1 (*A. fundyense*) never detected previously in the Australian region. Molecular analyses suggest that *A. fundyense* may have been a cryptic ribotype previously present in Tasmania, but newly stimulated by altered water column stratification conditions driven by changing rainfall and temperature patterns. Increased seafood and plankton monitoring of the area now include the implementation of *Alexandrium* qPCR, routine Neogen™ immunological and HPLC PST tests, but ultimately may also drive change in harvesting strategies and aquaculture species selection by the local seafood industry.

Keywords: Alexandrium tamarense complex; PST; unprecedented novel blooms

Introduction

Starting in 1985, the Tasmanian shellfish industry has become used to annually recurrent closures and public warnings of paralytic shellfish poisoning (PSP) risk inflicted by *Gymnodinium catenatum* blooms (reviewed by Hallegraeff *et al.* 2012). This large chain-forming dinoflagellate can be readily recognised by light microscopy, and, in the past, the affected area was primarily confined to the Huon River and d'Entrecasteaux Channel, near the capital city of Hobart. Over time, mussel farms in the most severely affected Huon River all closed business and an economic decision was made to declare the area unsuitable for shellfish farming with no new leases allowed. Early HAB surveys of other Tasmanian locations since 1987, including the east coast, had detected low concentrations of *Alexandrium tamarense* (Hallegraeff *et al.* 1991; Bolch & Hallegraeff 1990; Bolch & de Salas 2007). However, all cultured strains proved to be non-toxic and belonged to what was initially termed the "Tasmanian ribotype" (now designated group 5 or *Alexandrium australiense*; Scholin *et al.* 1995, John *et al.* 2014). A single small bloom event in Spring Bay in 1997 was caused by toxigenic group 4 (or *Alexandrium pacificum*), also widespread along the New South Wales and Victorian coasts of Australia (Hallegraeff *et al.* 1991; Farrell *et al.* 2013). Despite this event, the Tasmanian east coast continued to be classified as a low biotoxin risk and hence was subject to very limited plankton and biotoxin monitoring.

Unexpectedly, in October 2012, a shipment of blue mussels (*Mytilus galloprovincialis*) from the east coast of Tasmania tested by Japanese import authorities was found to be contaminated with unacceptable levels of Paralytic Shellfish Toxins (PSTs; 10 mg/kg). This incident triggered a recall of all Australian shellfish exported to Japan. Subsequent monitoring of the area confirmed PST in mussels, oysters, scallops, clams and rock lobster. A review of this critical incident (Campbell *et al.* 2013) identified: 1. Failure of plankton monitoring to provide timely results and failure to detect *Alexandrium*; 2. Failure of seafood risk assessment by not recognizing the risk of a new mussel farming venture in a poorly monitored area; 3. Failure of PST monitoring by relying only on plankton monitoring as a first screen rather than including shellfish testing. Here we review the results of increased *Alexandrium* plankton and seafood PST monitoring since the 2012 incident with the aim to identify key regions and seafood species at risk as well as environmental variables driving the blooms.

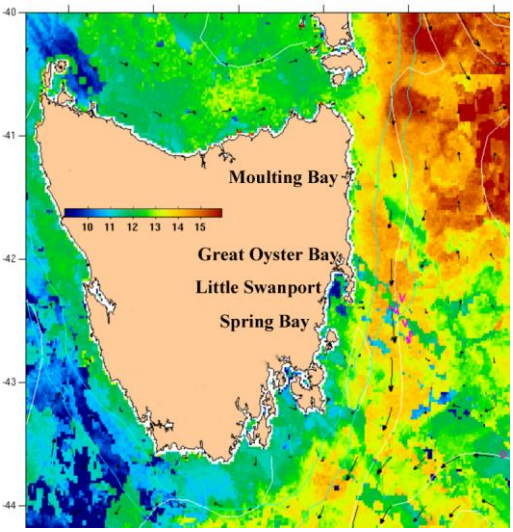

Fig.1. Map of Tasmania, south of the mainland of Australia, showing Sea Surface Temperatures on 27 September 2015 during peak PST, with the East Australian Current (EAC; in red) interacting with the continental shelf. The locations of the main affected shellfish farm areas Moulting Bay, Great Oyster Bay, Little Swanport and Spring Bay are indicated. Source: oceancurrent.imos.org.au.

Material and Methods

Shellfish toxins were monitored at weekly intervals at >20 Tasmanian east coast sites by the Tasmanian Shellfish Quality Assurance Program (TSQAP) using the AOAC approved Liquid Chromatography with fluorescence detection (LC-FLD) method (Lawrence *et al.* 2005). Satellite oceanography of the area was monitored as part of the Integrated Marine Observing System (IMOS; Fig.1). At the height of the August 2016 bloom, additional plankton, toxin and hydrological data were collected along inshore-offshore transects aboard the RV *Southern Cross*. A Seabird SBE 19PlusV2 CTD was used to collect temperature and salinity depth profiles. Plankton counts were obtained by settling 1L Lugol's iodine preserved samples. PST estimates were conducted on 3L of 8µm filtered water using the Neogen™ Reveal 2.0 immunological test kit (modified after Dorantes-Aranda *et al.* 2017). Cyst sediment samples were collected using a Craib corer and processed using primulin staining (Yamaguchi *et al.* 1995).

Results and Discussion

Shellfish toxins

Following low PST detection in 2013 and 2014 (both low rainfall years) with implementation of minimal shellfish farm closures, a more severe bloom event occurred during July-November 2015 (up to 300,000 *Alexandrium* cells/L; 15 mg/kg STX eq. in mussels, 6 mg/kg in *Crassostrea gigas* oysters), also causing 4 human hospitalizations after consumption of wild shellfish. More severe blooms recurred in 2016, following a major flood event in May and blooms lasting until September when up to 24 mg PST/kg was recorded in mussels (Fig. 2). In 2015, the highest PST concentration was measured in the south in Spring Bay, but in 2016 highest PST occurred further north in Little Swanport and Great Oyster Bay. Most shellfish contained high proportions of GTX1&4 (26-88%) and GTX2&3 (8-76%), followed by C1&2 (5-24%) and STX (0-2%) (Dorantes-Aranda *et al.* 2017).

Increased PST flesh testing

The current protocol for sample processing by the Tasmanian Shellfish Quality Assurance Program involves shipping samples to an accredited laboratory in Sydney, leading to frustrating delays (4-12 days) for shellfish growers. The performance of four commercial PST test kits, Abraxis™, Europroxima™, Scotia™ and Neogen™, was compared with the LC-FLD method for contaminated mussels and oysters. Based on their sensitivity, ease of use and performance, the Neogen kit proved the most suitable kit for use with Tasmanian mussels and

oysters. Neogen produced 5% false negatives and 13% false positives when the cut off was altered to 0.5-0.6 mg STX-diHCl eq/kg, whereas the introduction of a hydrolysis conversion step eliminated false negatives. A full single lab and international validation process was conducted (Turnbull *et al*., in press) and once formally approved for regulatory purposes, the Neogen kit will provide shellfish growers with a rapid tool for on-farm harvesting decisions. Rapid screen tests to prevent compliant samples undergoing testing using the expensive LC-FLD method will also result in significant savings (estimated $750k/yr) in analytical costs.

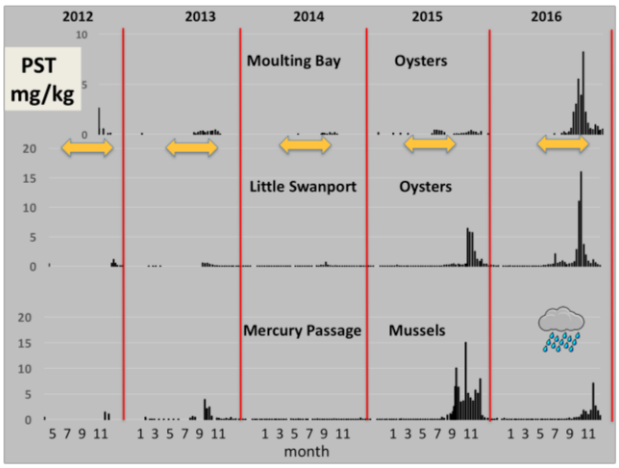

Fig. 2. Shellfish toxicity (mg STX eq./kg) from 2012 to 2016 in Moulting Bay and Little Swanport oysters and Spring Bay mussels. Orange arrows indicate the seasonal 10-15ºC temperature window. The 2016 bloom was preceded by a major rainfall event while anomalously cold water in Great Oyster Bay may explain the 2015 bloom.

Causative dinoflagellates

The causative dinoflagellates morphologically agreed with *Alexandrium fundyense*, possessing a ventral pore in the 1st apical plate (Fig.3a, arrow), and occurring as single cells or division pairs. An unusual feature of field samples was the extreme fragility of cells, ecdysing within 30-60 min after collection (Figs 3 b,c,d). Unexpectedly all cultured strains established during 2012 and 2015 belonged to group 1 never before detected in Australian waters in over 30 years of observations. Unique microsatellite signatures of these cultures (U. John, pers. comm.) suggest an endemic cryptic population being newly stimulated by changing environmental conditions. Paleogenomic research is in progress using dated sediment depth cores from the area to document historic shifts in abundance of *Alexandrium tamarense* ribotypes 1, 4 and 5.

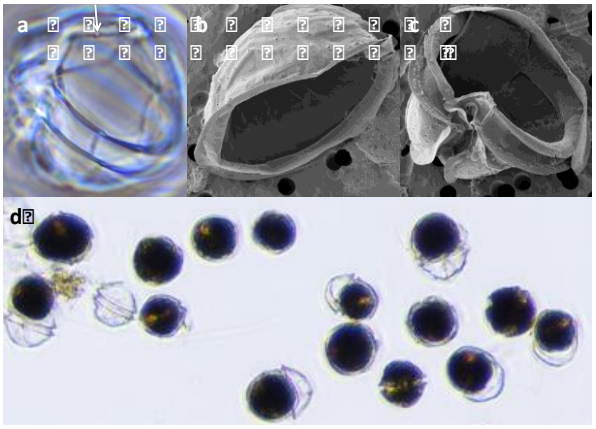

Fig. 3. Light (a,d) and scanning electron micrographs (b,c) of Tasmanian 2015 and 2016 *Alexandrium* field samples. Fig.3a shows ventral pore (arrow) in the first apical plate; Figs.3 b,c,d show the extreme fragility of the cells subject to ecdysis within 30-60 min of collection.

Preliminary views on bloom conditions

The affected Tasmanian coastal region is classified as a climate change "hotspot" resulting from increasing southward movement of the nutrient-poor East Australian Current (Fig.1). These novel *Alexandrium* blooms are not a simple response to increasing water temperatures (2.3ºC increase since the 1940s), as they occur in the cold winter-spring months at water temperatures of 10-15ºC. An observed trend of decreased silica concentrations in these waters would favor dinoflagellates and select against competing diatom blooms (Thompson *et al.* 2009). Preliminary culture growth experiments showed *Alexandrium* growth rates as high as 0.5-0.8 divisions/day, and a preference for low phosphorus and stimulation by humics (R. Quinlan, unpublished). Both culture experiments but notably field estimates using the Neogen test suggest a high cellular toxin content up to 100-500 pg STX eq/cell (Fig. 4, right). In August 2016 *Alexandrium* populations were abundant in inner shelf waters (35-50m deep) (Fig. 4, left) and just inside the sand bars of

the main shellfish growing estuaries of Little Swanport, Great Oyster Bay and Moulting Bay. However *Alexandrium* were virtually absent from the shallow (1-2m) turbid waters of those estuaries, and also were absent from deeper (100m) offshore waters dominated instead by spring bloom diatoms.

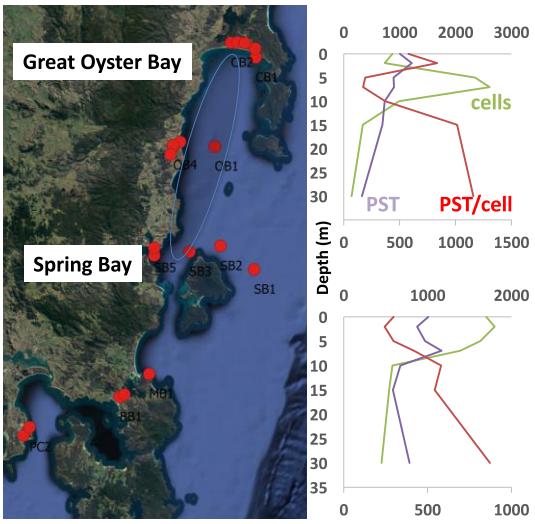

Fig.4. Left: *Alexandrium* bloom patch contained on the inner shelf of east coast Tasmania in August 2016, with no cells detected in offshore deeper waters. Right: Depth profiles of *Alexandrium* cell abundance (top scale), total PST toxins (ng/L) and pg PST eq per cell (bottom scale) in weakly stratified waters of Great Oyster Bay (top) and Spring Bay (bottom).

In 2016 the peak of the *Alexandrium* bloom coincided with a major high rainfall/ flood event that resulted in salinity stratified coastal waters (Fig. 2), while northward flow on the inner shelf was consistent with downwelling favorable conditions along the entire coast. In 2015 the situation was different however with anomalously cold water flowing out of Great Oyster Bay resulting in thermally stratified coastal waters. While both stratified and downwelling conditions are known to favor dinoflagellates over diatoms (Condie & Bormans 1997, Condie & Sherwood 2006), further research is in progress on how these processes control *Alexandrium* blooms off eastern Tasmania.

Alexandrium cyst surveys during August 2016 along the entire east coast of Tasmania found consistently low abundances of cysts (0.1-3 cysts per gram of sediment wet weight), but no dense cyst beds. Most sediments comprised coarse sands reflective of strong current regimes. Preliminary cyst culture experiments indicated a short dormancy period of 1-2 months (compare Hallegraeff *et al.* 1998 for New South Wales *Alexandrium* cysts) suggestive of rapid cycling between plankton and benthos. To protect tourism and human health, the area has now been sign-posted with permanent public PST warnings, which is a first for Australia.

Acknowledgements

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The extraordinary 2016 autumn DSP outbreak in Santa Catarina, Southern Brazil, explained by large-scale oceanographic processes

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Abstract

Diarrhetic shellfish poisoning (DSP) is endemic to the state of Santa Catarina. DSP-related closures of shellfish areas can occur at any time of the year in estuaries and bays, but mostly in autumn and winter months. In May 2016 unusual and sudden high *Dinophysis*. cf. *acuminata* counts (>30,000 cells L^{-1}) were detected by the national monitoring program, followed by positive DSP by mice bioassay in an extensive area. The event was associated with a general salinity drop, leading to a preventive closure along a coastal strip of about 200km. Countings of *D.* cf. *acuminata*, were as high as 580,000 cells L^{-1} in mussel culture areas. Bloom conditions persisted from end of May through June. Further closures occurred northward in the states of Parana and São Paulo. This was the most severe, intense and extensive DSP event ever recorded in Brazil. This outbreak was associated with exceptionally intense southwesterly winds, which induced an inflow of low salinity waters from the La Plata River, located more than 1100km away. Intense winds, combined with high river discharge in the Parana basin induced an extraordinary penetration of buoyant, low-salinity and nutrient-rich waters, leading to such large-scale bloom.

Keywords: Dinophysis acuminata, okadaic acid, La Plata River, Santa Catarina.

Introduction

Diarrhetic shellfish poisoning (DSP) is a worldwide syndrome that causes harmful impacts to human health and aquaculture. The causative poison is okadaic acid found in filter-feeding mussels and its analogues, which are produced by dinoflagellates of *Dinophysis* and *Prorocentrum*. In Brazil, DSP occurrence was described in 1997 (Proença et al. 1998) on the Santa Catarina coast. Anecdotal data suggested that DSP occurred even prior to that, as fishermen used to experience occasional diarrhea and other symptoms of DSP intoxication intrinsic to the act of eating mussels. Since 1997, the mussel production areas in Santa Catarina, the largest mussel producer in the country, have been monitored although extent, varying in time and spatial frequencies. Results have show that the occurrence of *Dinophysis* cf. *acuminata*, associated with levels of okadaic acid above the regulatory limit is relatively frequent and DSP may be considered endemic to the region. Subsequently, observations have shown that DSP occurs along an extensive area, from the Santa Catharina to Rio de Janeiro states (Proenca et al. 2007).

In Santa Catarina, *D.* cf. *acuminata* can occur throughout the entire year over a large range of temperature and salinities in estuaries, bays and other open areas (Proença et al. 2007, Tavares et al. 2009). Although not an exclusive cause, the penetration of the cold and low-salinity La Plata River water from Argentina along the coast has been suggested as a major mechanism which can be related to the appearance of *D.* cf. *acuminata* and consequent DSP in culture areas of Santa Catarina (Proença et al. 1998). The La Plata River water is advected northwards by southerly winds in autumn and winter months (Piola et al. 2005). The presence of the low-salinity, nutrient-rich water from the South is well known to affect the biology and plankton community in the region (Matusura 1996, Ciotti et al. 1997).

In 2016 the penetration of the La Plata River water was exceptional in intensity and extension and was associated with the largest *D.* cf. *acuminata* bloom ever registered in the region. Here we describe the bloom and discuss the major processes associated with this extraordinary event,

which extended over a long section of the southern coast of Brazil and caused human intoxication and economic losses.

Material and Methods

Data of cell counts, salinity and DSP assays were obtained from the official monitoring program of the Santa Catarina state for mussel sanitation control carried out by the State Program for Hygiene and Sanitation Control of Bivalve Mollusks (PECMB), coordinated by the Integrated Agricultural Development Company of Santa Catharina (CIDASC). Mussels and water samples were collected fortnightly and along the mussel production areas. In the laboratory, harmful algae were counted by the sedimentation chamber method (Utermohl, 1958), salinity by laboratory salinometer, and DSP by mouse bioassay (Yasumoto et al. 1978). Sampling was intensified during the bloom.

The evolution of sea surface temperature anomalies was calculated from daily Optimum Interpolation Sea Surface Temperature (OISST) with 0.25ºx0.25º resolution (Reynolds et al. 2007). Sea surface salinity was obtained from Surface Moisture Ocean Salinity products from the Barcelona Expert Center (http://bec.icm.csic.es/). Daily wind analyses were obtained from the Cross-Calibrated Multi-Platform (CCMP) available at Remote Sensing Systems Inc. (http://www.remss.com /measurements/ccmp). Data on the La Plata River discharges from January to April 2016 were obtained from Borús et al. (2016).

Results and Discussion

The largest event ever registered in Santa Catarina was first noticed on May 23th 2016 in Baía Sul, a mussel culture area in the southern part of Florianópolis (Fig 1). From a few cells per litre, counts of *D.* cf. *acuminata* increased and attained 20.3 x 10^3 cells L^{-1} in Baia Norte region with positive results for DSP in mussels. In the following days, high values were also noted in northern coastal areas up to Armação do Itapocoroy, with 16.9 cell L^{-1} x 10^3 on May 25th. This distribution indicated that the bloom occurred along virtually the entire virtually Santa Catarina coast. This scenario led authorities to proceed with a general preventive mussel closure from Baia Sul to São Francisco given the high counts and positive DSP results, where available.

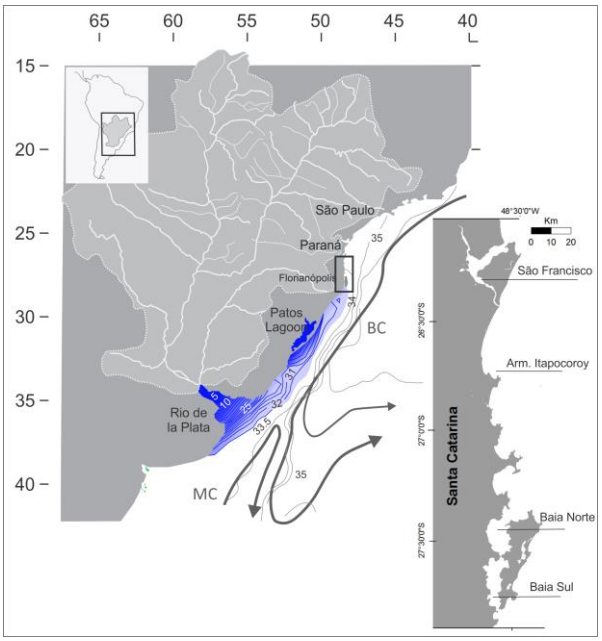

Fig. 1 a) The La Plata basin (light grey) and winter surface salinity distribution in the southwest of the South Atlantic (blue). Salinity distribution was based on historical hydrographic data collected in July-August-September. Contour interval is 1. The thick contour corresponds to the 33.5 isohaline, which marks the offshore extent of the La Plata derived waters. The Brazil Current (BC) and the Malvinas Current (MC) are shown schematically.

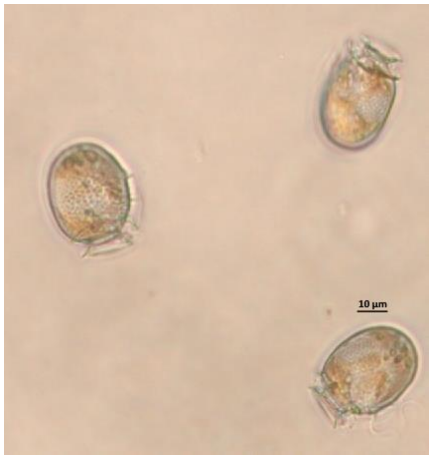

Fig. 2. Live cell of *Dinophysis* cf. *acuminata* from 2016 bloom.

D. cf. *acuminata* (Fig. 2) is endemic to the region and can be found any time of the year in estuaries with salinity as low as 16 to more saline open

marine waters. Although of highly variable occurrence, every year at least one mussel culture area is closed due to DSP levels above the allowed limit (at least 2 out 3 deaths of tested mice in 24h). Most of the time, a gradual influence of the water derived from the Plata River Plume can be tracked along the coast, generally from south to north. In the present case, the low salinity and the associated bloom were noticed along almost 200 km of coastline at the same time. Maximum counts reached up to 580 x 10^3 Cell L^{-1}, but even higher ones were found outside the mussel culture areas. The salinity drop firstly observed in the south was repeated with more or less the same intensity in all regions. Although there are several small to medium size rivers runoff along the region, the general salinity decrease locally observed during the monitoring period could not be explained by local freshwater input. Previous data showed influence of low temperature and salinity waters from the La Plata River Plume (PRP) (Fig. 1) (Piola *et al.* 2005). In mid-June 2016 the continental shelf to the north of the La Plata estuary presented sharp negative anomalies exceeding -4ºC. The analysis of the surface temperature and salinity along the coast of Santa Catarina clearly confirms the large-scale frontal system.

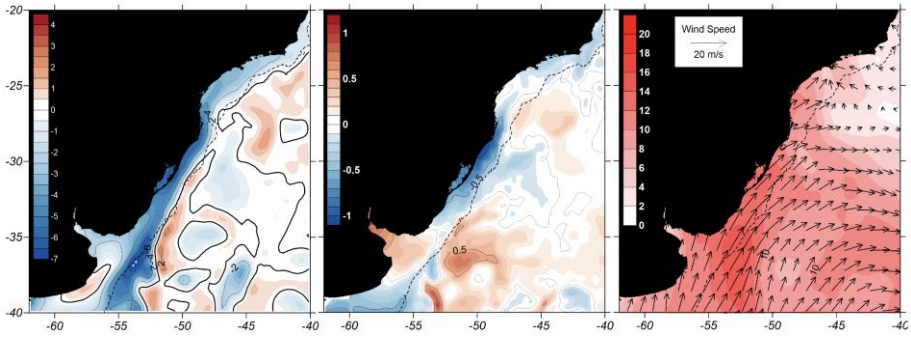

Fig. 3. Mid-April 2016 OISST sea surface temperature anomaly (left), mid-April to mid-May SMOS salinity difference (center) and late April surface winds from CCMP (right).

Interestingly, the region presented warm SST anomalies since January 2016. The cooling initiated in mid-April and rapidly expanded northward (Fig. 3 left). During that period, the southern Brazilian shelf also experienced sharp freshening, with surface salinity decreasing about 0.5 from mid-April to mid-May (Fig. 3, center). Though cooling and freshening are expected in mid-autumn (Piola et al., 2005), the intensity of the 2016 event sharply exceeded the climatology, leading to the switch from warm to cold SST anomalies in mid-April. The surface temperature anomaly and salinity change distributions point to the southern source of cold, fresh shelf waters. As a result of this anomalous event, which redistributed the low salinity waters, the region off the La Plata estuary showed a salinity increase (>0.5) (Fig.3 center).

The process was driven by a sharp change in wind direction and intensity from northeasterly winds in most of April to southwesterly winds in late April and May (Fig. 3, right). It was this wind reversal that triggered the anomalous
northeastward expansion of PRP waters along the shelf of Uruguay and southern Brazil.

This scenario created the mechanism for the extreme *D.* cf *acuminata* bloom to develop for tens of kilometers along the coast, as observed an aerial survey. The severity of the event was rapidly recognized and state authorities instigated a general interruption on mussel extraction and consumption on May 26th. Despite the ban and full media coverage, few cases of human intoxication, not quantified, were reported due to recreational mussel extraction from rocky shores. As cell counts started to decrease, the closed areas were gradually opened after 55 days. Economic losses of R$ 12 million (approx. US$ 4 million) were calculated.

The relationship between the *D.* cf *acuminata* bloom and the PRP, advection can be clearly observed in Figure 4. It shows the driving factors leading to an increase in the chlorophyll *a*: high fresh water input from the La Plata watershed, sudden prevalent wind change, the drop in temperature and salinity. This scenario allowed predicting the DSP occurrence along the Paraná and São Paulo coasts, located to the north of Santa Catarina. In fact, in early June the bloom severely reached the Paraná coast causing a mussel consumption ban and some intoxication cases.

Okadaic acid was detected in mussels, zooplankton and mullets (*Mugil platanus*) (i.e. free- OA in the gizzards and intestines, max.156.5 and 62.1 ng.g-1, L.L. Mafra *pers.com.*). Later, okadaic acid was found in São Paulo, and mussel consumption was also interrupted there.

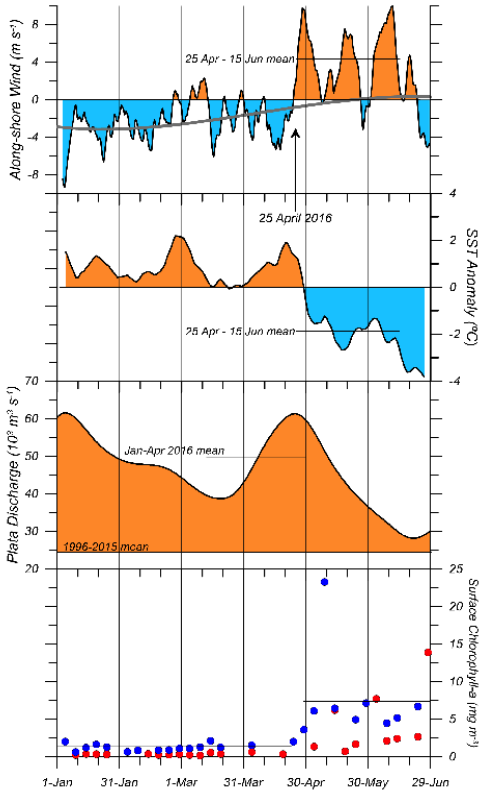

Fig. 4: a) Along-shore wind anomaly (m s-^{1}) over the continental shelf of southern Brazil. The grey line shows the climatological along-shore wind. Also indicated is the mean wind stress anomaly from 25 April-15 June 2016 (+4.3 m s^{-1}). b) Sea surface temperature anomaly (ºC). Also indicated is the mean SST anomaly 25 April-15 June 2016 (-1.88 ºC). c) La Plata discharge (m^3/s). Also shown are the mean discharge from January to April 2016 (49600 m^3/s) and the climatological La Plata discharge from 1996 to 2015 (24300 m^3 s^{-1}). d) MODIS surface chlorophyll concentration (mg m^{-3}) at two sites off southern Brazil. Also indicated are the mean chlorophyll concentrations from 1 Jan-25 April (1.36 mg m^{-3}) and 29 April-29 June 2016 (7.34 mg m^{-3}).

Waters derived from the La Plata River are always present in Santa Catarina during autumn and winter months and cause major changes in the plankton community structure. We do not know why in 2016 *D.* cf. *acuminata* was among the dominant species at the plume front. In the 2015 summer, an exceptional *Dinophysis acuminata* complex bloom occurred at the Uruguayan coast, about 1000 km to the south. This was the longest bloom period (i.e. 5 months) registered in that country, with counts up to 30 x 10^4 cells L-1. The bloom was associated with warmer and saltier waters from the Brazil Current, which contributes to shape up the Uruguayan coast in summer months (Mendez et al. 2017). It is reasonable to consider these two events in South Brazil and Uruguay as directly related. The broad oceanographic conditions during these two events suggest that *D.* cf. *acuminata* is an opportunistic organism with a wide range regional occurrence. Further investigation is necessary to access the factors that enabled *D.* cf. *acuminata* to dominate the plume front and extend the bloom in such a large scale

Acknowledgements

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Origins of Dinophysis blooms which impact Irish aquaculture

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Abstract

The main unresolved issue with *Dinophysis* blooms and their contamination of shellfish with DSP toxins has been the identification of their source. From cruises covering the shelf region south of Ireland over the past years, it was shown that extensive blooms of *D. acuta* develop in summer in the productive region close to the Celtic Sea Front, a tidal front extending from southeast Ireland across to Britain. Of particular note has been the development of *D. acuta* populations with cells ranging from 2,000 cells L^{-1} to 55,000 (2014) and 75,000 (2015) cells L^{-1} located in a layer at the top of the sub-surface chlorophyll fluorescence maximum. Despite the blooms being localized to stratified water adjacent to the tidal front, they extended over a very large area. Over the rest of the Celtic Sea Shelf, cell densities were less than 100 cells L^{-1} at this time. These blooms are transported westwards along the south coast of Ireland towards the economically important shellfish culture region of southwest Ireland where they subsequently do harm. The source of these *Dinophysis* blooms is thus in excess of 300 km from their point of impact.

Keywords: *Dinophysis*, Celtic Sea

Introduction

The contamination of seafood bivalves with Diarrheic Shellfish toxins, nearly always due to species of the genus *Dinophysis*, is the most serious and economically damaging toxic HAB event along the Atlantic seaboard of Europe (Reguera *et al*. 2012). These events are caused by the transport of *Dinophysis* populations into sites of shellfish aquaculture (Raine *et al*. 2010, Whyte *et al*. 2014). The origin of these populations on the coastal shelf, or even further away, remains obscure. This information is a prerequisite for the development of models or early warning systems that are essential to mitigate these blooms. This paper presents results of summer research expeditions which indicate the source of extensive *Dinophysis acuta* blooms which regularly occur around the southwest of Ireland.

Material and Methods

Research cruises were carried out on board the research vessels *Celtic Voyager, Prince Madog* and *Discovery* in the Celtic Sea in the summers of 2014 and 2015. Most sampling stations were located in the northeastern Celtic Sea towards the Celtic Sea tidal front (Fig. 1). At each station, water samples were taken using a CTD rosette (e.g. SBE 9/11+) of multiple 10 L sampling bottles. Targeted depths were in the vicinity of pycnoclines but with up to seven depths sampled per station the entire water column was covered. A relatively high resolution of stations 3 km apart was employed for the most part allowing a certain confidence in sampling *Dinophysis* populations, which can be very heterogeneous in their vertical distribution. Occasionally, a fine scale sampler was deployed capable of collecting 15 samples 20 cm apart (Lunven *et al*. 2005).

Once back on board, two litres of water were taken from each bottle and passed through a 20 µm nylon mesh sieve. Material collected on the sieve mesh was backwashed into 50 ml centrifuge tubes using filtered sea water. The final volume of sample was made up to 30 ml. Enumeration of *Dinophysis* cells was carried out by transferring 3 ml of the concentrated sample into an Utermöhl sedimentation chamber. Either half or the entire base plate was then scanned and *Dinophysis* cells counted, giving a lower limit resolution in the cell density of 5 cells L^{-1}.

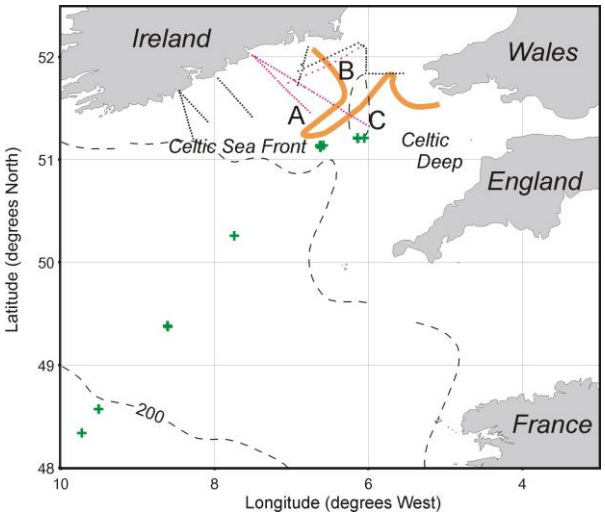

Fig 1. Locations of sampling stations in the Celtic Sea carried out on the *Celtic Voyager* (small filled circles) and *Discovery* (green crosses) in the summers of 2014 and 2015. The markers A, B and C refer to transects of stations, highlighted in red, shown in Figs. 2 & 3. The thick line shows the approximate position of the Celtic Sea Front. Isobaths are in metres.

Results and Discussion

The distribution of *Dinophysis acuta* along two transects of stations sampled in July 2014, one extending southeastwards from the south eastern Irish coast (Transect A) and the other normal to it (Transect B), are shown in Fig. 2. Cell numbers were noticeably higher at either end of transect A where water in the vicinity of the pycnocline was uplifted both at the coast and at the boundary between stratified and tidally mixed water at the seaward end. A more developed *D. acuta* population was observed along an extended transect (Transect C) in July 2015. On this occasion, cell densities were typically 3,000 – 5,000 cells L^{-1} at the top of the seasonal pycnocline found at 25-35 m depth (Fig. 3). Maximum cell densities of *D. acuta* of 55,000 (July, 2014) and 72,000 (July 2015) cells L^{-1} were observed close to the Irish coastline.

Some irregularities in the distribution were observed in 2015 such as the uplift of cells at Km 100-105 along Transect C (Fig. 3). This was related to the local physical oceanography as here the pycnocline was uplifted to near the surface when crossing a tongue of tidally mixed water, which extends south from the Celtic Sea Front (Fig. 1).

The vertical distribution of *Dinophysis* at selected stations is shown in Fig. 4. The subsurface maximum was not directly at the pycnocline, although on occasion this was as a thin layer. It is apparent that the weaker the level of stratification, the more diffuse the sub-surface maximum, as might be expected. However, the sharpness of the pycnocline at station 155 (Fig. 4), which could be elucidated in more detail from a fine scale sampler deployment, suggests that the thin layers are a result of physical aggregation as caused by example the tide, as opposed to biological effects (e.g. migration).

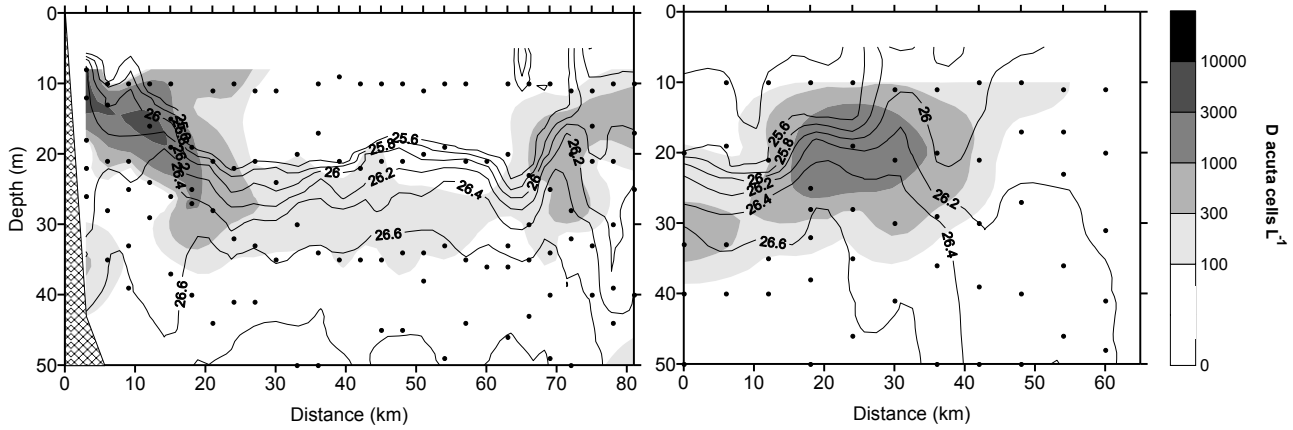

Fig. 2. Distribution of *Dinophysis acuta* along sections in the northeastern Celtic Sea. Left panel: Transect A extending southeastwards from Ireland. Right panel: Transect B across the Celtic Sea Front. Locations of the transects are shown in Fig. 1. Cell densities are superimposed on seawater density (sigma theta, Kg m^{-3}). The hatched area shows the seabed; tick marks along the upper axis are stations; water depths along the main part of the sections were within the range 70-80 m.

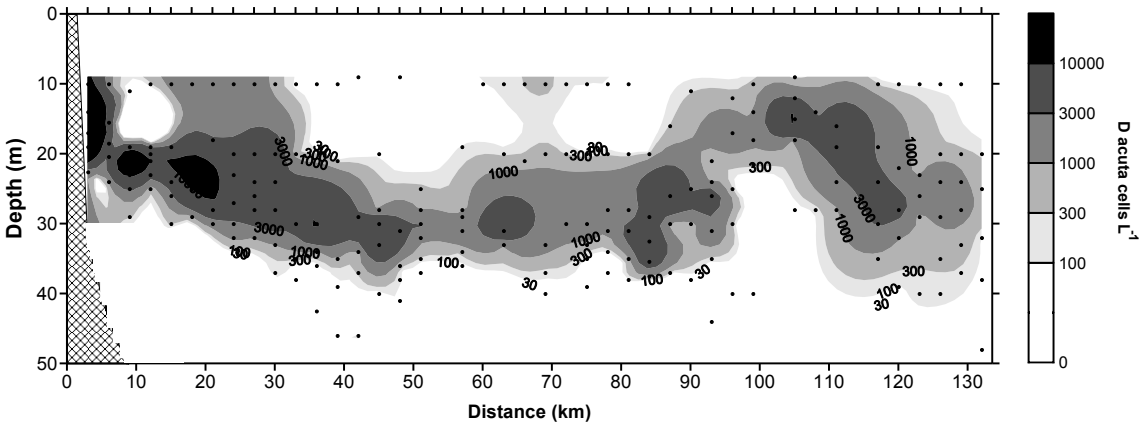

Fig. 3. Distribution of *Dinophysis acuta* along Transect C in the northeastern Celtic Sea extending southeastwards from Ireland and whose location is shown in Fig. 1. The hatched area shows the seabed; water depths along the main part of the sections were within the range 70-80 m increasing to over 100 m on the right of the picture over the Celtic Deep (see Fig. 1).

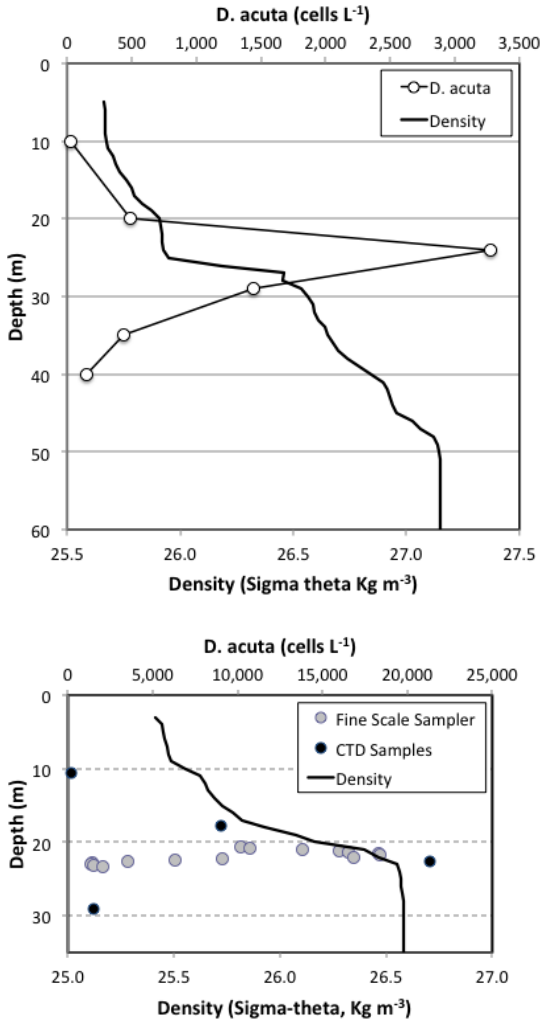

Fig. 4. *Dinophysis* and seawater density distributions at stations 57 (Transect C, km ?; upper panel) and 155 (close to the Irish coast, water depth 47 m) sampled in July 2015 and 2014 respectively.

Central and southwestern parts of the Celtic Sea were sampled in July and August 2015 (See Fig. 1 for station locations). The only water samples bearing cell densities >300 cells L^{-1} were in the vicinity of the Celtic Deep at approximately 51 12'N 6 08'W and 51 08'N 6 37'W where sub-surface maxima of 10,000-14,000 cells L^{-1} were noted at depths of 20-30 m. These results indicate the persistence of *D. acuta* populations in the region.

Dinophysis is a rare species (*sensu* Gaston 1994) in that it seldom dominates the phytoplankton yet it has a very wide, ubiquitous distribution. Most often across continental shelves *D. acuta* is found in cell densities <100 cells L^{-1} (Reguera *et al.* 2014). There is a seasonal aspect to its presence in slightly elevated (ca. 1,000 cells L^{-1}) concentrations as witnessed by monitoring data from the southwest of Ireland (Raine *et al.* 2010) in that it mainly occurs between June and September when shelf waters have become stratified. The cell densities observed in the present study would be considered unusually high.

Tidal fronts are productive systems. They are associated with elevated chlorophyll levels and, historically, those found around the Celtic Sea (the Celtic Sea Front and Ushant Front) have been associated with blooms of *Karenia* (Pingree *et al.* 1975; Holligan 1981). These can be explained and modeled using relatively simple light-nutrient-mixing approaches (e.g. Gentien *et al.* 2007). *Dinophysis* cannot be treated in a similar way as it is an obligate mixotroph. It needs light for growth,

but for nutrition it feeds by the ingestion of particles. Thus successful culture of species of the genus requires feeding with *Mesodinium* (Park *et al.* 2006). There was no obvious visual indication of food source for the *D. acuta* observed under a microscope in this study, but relatively fragile cells of *Mesodinium* would probably have been destroyed with the sampling technique applied. Nevertheless, in 2014 a high proportion (ca. 15%) of the cells were well vacuolated, indicating recent feeding.

So how do these large populations of *D. acuta* develop? With their ubiquitous nature, one can hypothesise that low cell densities (<100 cells L^{-1}) are carried onto the Celtic Sea shelf with water from the Northeastern Atlantic water (see Fig. 30 in McDermott & Raine 2006). When cells meet the productive waters in the vicinity of the tidal front, they grow. Repeat observations in the Celtic Sea (data not shown) suggest that they grow quite quickly and can develop within four weeks. Interestingly, cell densities of *D. acuminata* in water samples were lower by an order of magnitude or more indicating a markedly different ecology for this species.

Developed populations in the vicinity of the tidal front will be transported within the strong, narrow, baroclinic current that flows northwards along the front and then westwards along the southcoast of Ireland (Brown *et al.* 2003; Hill *et al*. 2008). Growth near the tidal front and subsequent transport along the southern Irish coast (Farrell *et al.* 2012) can explain an increasing number of sporadic observations of extremely high (10^5 cells L^{-1}) cell densities of *D. acuta* at locations along the southern Irish coast, whether these have been observed during the national HAB monitoring program or in research surveys.

The cell densities of *D. acuta* observed here are particularly high. They are not as high as are commonly found for diatoms, microflagellates and other taxa of the phytoplankton. To put our observations into context, a population of only 200 cells L^{-1} is enough to contaminate shellfish above the threshold level allowable under current EU and other legislation of 0.16 µg gm^{-1} whole tissue in okadaic acid equivalents (Van Egmond *et al.* 1993). It is quite possible that small, undeveloped populations will advect into bays used for shellfish culture along the Atlantic seaboard of Ireland and render the shellfish unsuitable for human consumption for a short period. Nevertheless, we have shown how significantly larger blooms arise and can be transported to aquaculture sites causing extensive toxic events. It is worth pointing out that the origin of these blooms would be some 300 km or more away from the site of impact. This would have substantial consequences on our ability to predict them.

Acknowledgements

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Fine scale physical-biological interactions in a *Dinophysis acuminata* population during an upwelling-relaxation transition

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Abstract

Increased input of energy, shoaling of isoclines and shear stress inflicted to planktonic cells may lead to important changes in the rate of physiological processes. The cruise "ASIMUTH-Rías" 2013 was carried out on 17-21 June 2013 in the Galician Rías and adjacent shelf to study small-scale physical processes associated with spring blooms of *Dinophysis acuminata*. The cruise coincided with the initiation of an upwelling pulse following relaxation. A 36-h cell cycle study carried out on 18-20 June showed a brusque decline (0.51 to 0.25 d^{-1}) in division rates (μ), associated with increased vertical diffusivity (Kz). Negative effects in division rates (μ = 0.5 d^{-1}) were not observed at a mid shelf station used as reference. Here we describe microscale changes in water column structure and in the distribution and physiological status of the *D. acuminata* population and co-occurring ciliates as a result of increased mixing. Upwelling pulses, in addition to a direct physical effect of advective dispersion, appeared associated with a physiological disturbance to the cells probably through shear stress. The short-term impact of upwelling pulses in this population and the role of mid shelf populations as a relatively undisturbed reservoir of *Dinophysis* cells for subsequent blooms inoculation is discussed.

Keywords: Dinophysis acuminata; Physical-biological interactions; Fine scale structure; Division rates; Circadian variability; Galician Rías.

Introduction

Low density (10^2-10^4 cells L^{-1}) populations of diarrhetic shellfish poisoning (DSP) toxin-producing *Dinophysis acuminata* are the main cause of endemic shellfish harvesting bans in the Galician Rías Baixas. The growth season of *D. acuminata* is tightly coupled to the local spring-summer upwelling season (Díaz *et al*. 2013; Velo-Suárez *et al*. 2014). Therefore, interactions between biological processes in *Dinophysis* populations—including effects of ciliate *Mesodinium* prey availability—and quick changes of water-column structure associated with upwelling-downwelling cycles need to be scrutinized for the development of realistic prediction models (Ruiz-Villarreal *et al*. 2016).

The *in situ* specific growth rate, μ, is an important intrinsic parameter in the growth equation that needs to be determined. Models tend to use constant division rates, derived from laboratory experiments, for short (week) time-scale forecasts, but fast day-scale shifts in coastal wind-driven circulation, modulated by tides, may have dramatic effects on the division rate of a given species. The model of Carpenter & Chang (1988), based on the mitotic index approach (McDuff & Chisholm 1982), has been successfully used to estimate *in situ* division rates of *Dinophysis* populations in different coastal environments (Chang & Carpenter 1991; Reguera *et al*. 2003). In many cases, an integrated value of μ for the whole water column, estimated from vertical haul samples, was obtained. But in two cases when division rates at different depths (μ_z) were estimated, important vertical heterogeneities were observed (Velo-Suárez *et al*. 2009; Farrell *et al*. 2014).

Here we examine the short-term response of a *D. acuminata* population to a shift from upwelling relaxation conditions to a new upwelling pulse in the Galician Rías Baixas to study small-scale physical processes associated with late spring blooms of *D. acuminata*.

Material and Methods

Field work was part of the cruise "*ASIMUTH-Rías 2013*", carried out on board R.V. *Ramón Margalef* from 17 to 21 June 2013 in the rías of Vigo and Pontevedra and their adjacent shelf (Fig. 1).

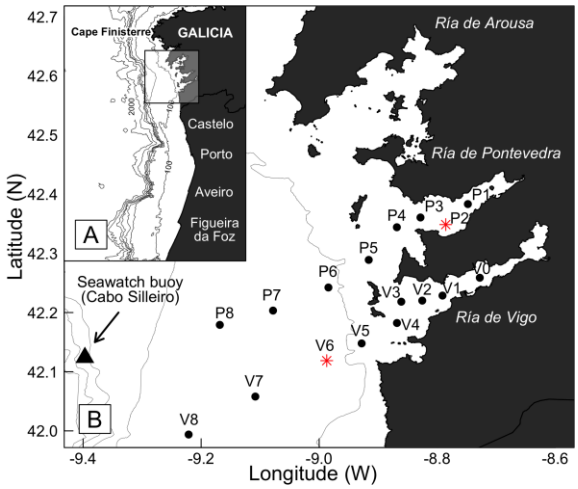

Fig. 1. A) Northwest coast of Spain and the northern half of Portugal; B) Survey's sampling stations in the rías of Vigo (V0-V8) and Pontevedra (P1-P8).

Sampling for the 36 h study, from 18 to 20 June, was carried out at a fixed station (P2, Bueu) from Ría de Pontevedra that has won the reputation of being one of the hot spots for DSP occurrence in SW Europe. High-frequency physical measurements were carried out using a microstructure profiler (MSS)—equipped with sensors to measure shear stress and horizontal acceleration at a sampling rate of 1000 samples for second—and temperature and salinity with a CTD probe (SBE25) attached to a rosette of 12 Niskin bottles (2.5 L). Bottle water samples were collected at 5-6 discrete depths between 30 and 3 m for quantitative analyses of phytoplankton to describe circadian tidal variability of *D. acuminata* and *Mesodinium*. Sampling for division rate estimates was carried out every 2 hours from 10.00 to 04.00 next day, and every hour from 04.00 to 10.00, which is the time window before and after dawn when phased division of *Dinophysis* spp. is observed and proportion of dividing and recently-divided cells changes very rapidly (Reguera *et al*. 2003). *In situ* division rates were estimated, with a "post-mitotic index approach", following the model of Carpenter & Chang (1988).

Results and Discussion

In June 2013, a succession of 1-2 week-scale periods with alternation of positive and negative Ekman's transport estimates were observed, within the average values (mean from last 38 years) during upwelling-downwelling cycles in the late-spring in the region (Fig. 2).

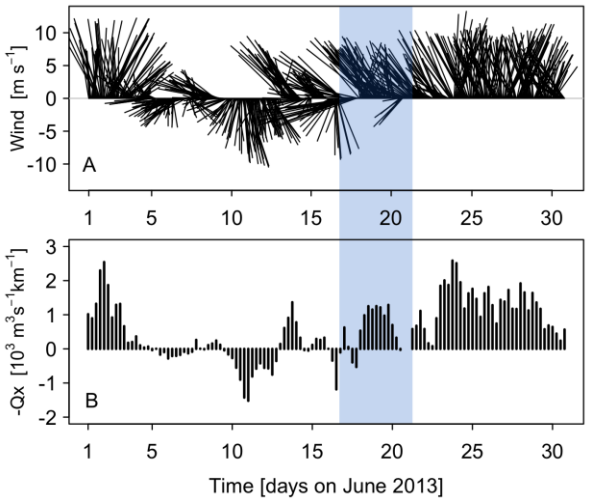

Fig. 2. Time series of A) Hourly records of vector diagrams of wind direction and velocity (m s^{-1}) at Cabo Silleiro buoy (positive values correspond to northerly winds), B) Estimates of daily upwelling indices, Q_x (m^3 s^{-1} km^{-1}; positive values correspond to upwelling). Shaded area indicates the days of the cruise.

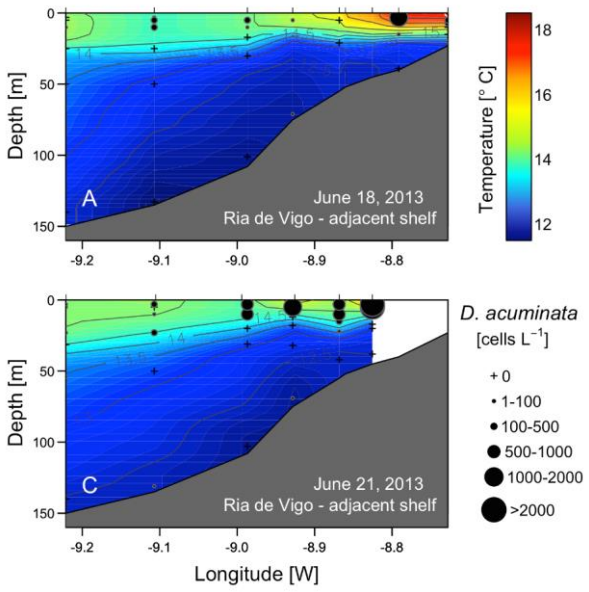

Fig. 3. Vertical distribution of temperature (ºC) and *D. acuminata* (cells L^{-1}) in a Ría de Vigo-shelf transect on 18 and 21 June.

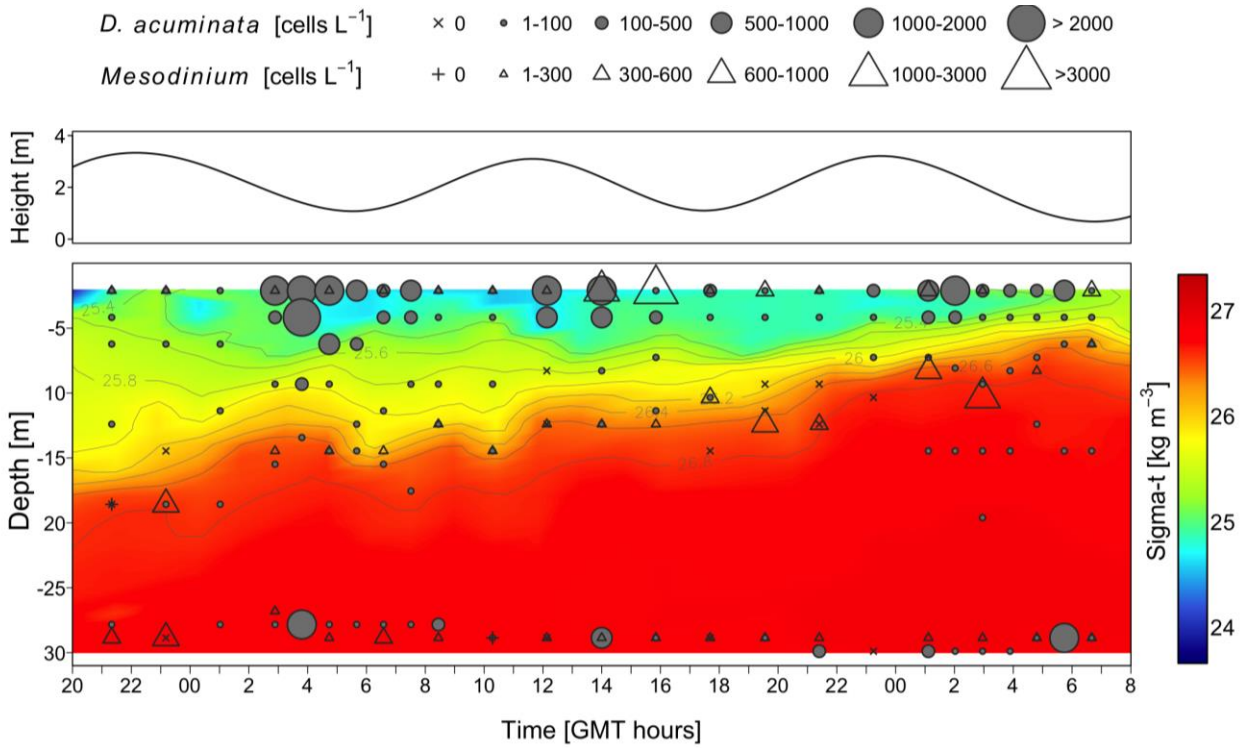

Fig. 4. Vertical distribution of seawater density (Sigma-t), *Dinophysis acuminata* (grey circles) and *Mesodimum* cells (empty triangles) at a fixed station (P2) during a 36-h cell-cycle study, 18-20 June 2013. Hourly recorded of sea level (m), are shown in the top panel.

During relaxation, ría-shelf transects perpendicular to the coast showed that maximal densities of *Dinophysis* (1×10^3 cells L^{-1}) were located near the surface (3-5m), in the middle reaches of the rías, in the warm (>16ºC) water that had been advected from the shelf the previous days (Fig. 3A). After the upwelling pulse, mixed surface waters cooled down (15ºC) and *Dinophysis* maxima (2.2×10^3 cells L^{-1}), located in the top layer (5 m), had moved off to the outer reaches of the rías and shelf waters (Fig. 3B).

During the 36h study, there was no evidence of daily vertical migration of *D. acuminata*, and the cell maxima remained in the warmer (>16º) and more brackish (< 35) surface (3-5m) layer, above the 25.5 σ_t isopycnal (Fig. 4). Cell densities increased during the tide outward flow (ebb tide), and the cell maximum, 2.4×10^3 cell L^{-1}, was observed at 3 m, 05.00 on 19 June, coinciding—as in the haul samples—with low tide. Likewise, cells maximum of *Mesodinium*, 5×10^3 cell L^{-1}, was observed at the same depth, 18:00 on 19 June, during low tide (Fig. 4). Nevertheless, maxima of both species did not co-occur. High turbulent diffusion rates (Kz) were observed at the surface layer (0-10 m; 3.8×10^{-2} m s^{-1}) and close to the bottom (>25 m depth; 1.1×10^{-3} m s^{-1}) throughout the 36-h cycle, due to wind stress forcing and bottom friction, respectively.

Maximal frequencies of mitotic cells were observed at 3-5 m—above the 25.5 σ_t isopycnal—where the cell maxima were found throughout the two cycles on 19 June (day 1) and 20 June (day 2). Distribution of frequencies of dividing (*fc*) and recently divided (*fr*) cells of *D. acuminata* showed a clear-cut phased cell division on day 1 and day 2, but maximum frequency of dividing cells (*fc+fr*) on the first day (0.32) was more than double that on the second (0.15) (Fig. 5). Slight differences were also found in the timing of maximal frequencies of dividing (05.00 and 04.00 GMT) and recently divided cells (06.00 and 05.00 GMT), i.e. maximum *fc* was just at dawn (05.00) on day 1 and one hour earlier on day 2. Thus, estimates of μ_{avg} and μ_{min} were 0.51 d^{-1} and 0.28 d^{-1} on day 1 and 0.25 d^{-1} and 0.14 d^{-1} on day 2, respectively. Nevertheless, the time lag between the peaks of cytokinesis and sulcal-list regeneration was the same (1h) the two days, although the distribution of recently divided cells (*fr*) showed a sharper curve the first day (Fig. 5).

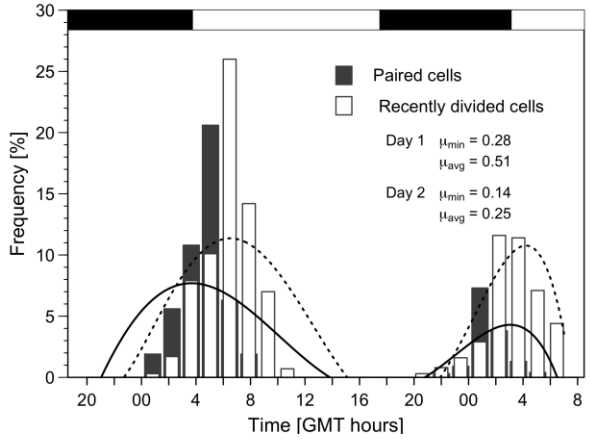

Fig. 5. Distribution of frequencies of paired (dividing, black bars) and recently divided (grey bars) cells of *D. acuminata* during the 36-h cell cycle, fitted to a 5th degree polynomial curve. Black shading in top bar indicates the period between sunset and sunrise.

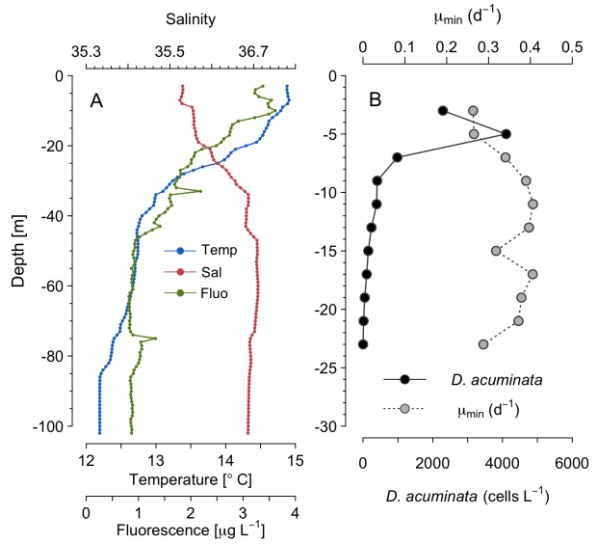

Fig. 6. A) Vertical distribution of temperature, salinity and *in vivo* fluorescence and B) Vertical distribution of *D. acuminata* (cells L^{-1}) and μ_{min} on 21 June 2013 at shelf station V6.

Vertical distribution of physical properties on a shelf station (100 m deep) off Ría de Vigo on 21 June showed that changes in water column structure there were not as drastic as inside the rías (Fig. 6A). Vertical distribution of *Dinophysis* cell densities showed the cell maximum (4.1×10^3 cell L^{-1}) was at 5m (Fig. 6B). Distribution of μ_{min} at each depth $(\mu_{min})_z$ at peak-division time (06.00 GMT) on 21 June showed that the maximum μ_{min} value (0.5 d^{-1}) was not found in the layer of the cell maximum (5m) but between 11 and 17 m, where cell densities were much lower (Fig. 6B). Still the value of μ_{min} (0.3 d^{-1}) at the cell maximum was slightly higher than the estimate inside the rías during the 36-h cell-cycle study before the intrusion of cold water.

Our results suggest that downwelling-upwelling transitions during the upwelling season have a double effect, both contributing to decreased cell densities in established populations of *D. acuminata*: an indirect physical effect of advective dispersion and a direct effect of physiological disturbance by increased shear stress (horizontal velocities) inflicted on the cells. Thus, when *Dinophysis* and other dinoflagellate species are transported off the rías to mid-shelf waters during upwelling pulses, this region will act as the pelagic seed bank (*sensu* Smayda 2002) where cells will remain and keep in good condition (high division rates) until the next relaxation period promotes their re-introduction in the rías.

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Effect of different taxonomic groups on the growth and toxin content in *Gymnodinium catenatum* cultures from the Pacific coast of Mexico

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Abstract

The effect of cells and cell-filtrates of microalgae belonging to different taxonomic groups were tested on the growth and toxin content of *Gymnodinium catenatum* cultures. Cultures of a raphidophyte (*Chattonella marina* var. *marina*), a dinoflagellate (*Margalefidinium polykrikoides*), and a bacterial community isolated from *G. catenatum* were used. All these were cultivated in GSe media, maintained at 24 ± 1°C, salinity 34, and a 12:12 light:dark cycle. Samples for cell counts and microscopic observations were taken. *Chattonella* caused a strong and fast inhibition of *G. catenatum* growth; mortality of *G. catenatum* occurred with and without direct cell contact, indicating that toxic metabolites were released to the culture medium. *Margalefidinium polykrikoides* also caused mortality of *G. catenatum* but required a longer time. The growth response of *G. catenatum* towards the bacterial community was isolate-specific. Changes in of *G. catenatum* morphology, i.e. loss of flagella, swelling, evident nucleus and lysis, were observed in all cases, and cells resembling pellicle cysts in some. The results suggest that biotic factors affect *G. catenatum* growth and life history transitions, providing new insights on the interactions between *G. catenatum* and other co-occuring planktonic species.

Keywords: biotic interactions, growth, *Gymnodinium catenatum,* morphology, paralytic shellfish toxins

Introduction

The dinoflagellates *Gymnodinium catenatum* and *Margalefidinium polykrikoides,* and the raphidophyte *Chattonella marina* var. *marina* form harmful algal blooms (HABs) (Band-Schmidt *et al*. 2010; López-Cortés *et al*. 2011), and co-occur in Mexican waters (Gárate-Lizárraga *et al*. 2009; López-Cortés *et al*. 2011). The three species are toxic. *Chattonella* and *Margalefidinium* species produce haemolytic and haemo-agglutinating compounds, as well as superoxide and hydroxyl radicals (Kim *et al*. 2007; Kim and Oda, 2010; Kuroda *et al*. 2005), free polyunsaturated fatty acids (Dorantes-Aranda *et al*., 2009) and brevetoxin like substances (Band-Schmidt *et al*. 2012). *G. catenatum* produces paralytic shellfish toxins, including benzoil analogues (Bustillos-Guzmán *et al*., 2015).

In laboratory conditions *G. catenatum* tolerates wide ranges of salinity, temperature and N:P ratio (Band-Schmidt *et al*. 2010, 2014; Bustillos-Guzmán *et al*. 2012), a fact suggesting that this species may be present in the water column all year round.

Few studies have been done to understand biological interactions (e.g. allelopathy, parasitism, competence) in this species. Grazing studies showed that *Noctiluca scintillans* and the copepod *Acartia clausi* are relevant predators of *G. catenatum*, and may have an important role in the regulation of its blooms (Palomares-García *et al*. 2006; Bustillos-Guzmán *et al*. 2013). Bacteria also affects dinoflagellates growth, toxicity and nutrient assimilation (Amin *et al.* 2007; Green *et al*. 2010; Albinsson, 2011; Bolch *et al.* 2011).

Studies on species interaction are important for understanding bloom dynamics of co-occurring species. Little is known about interactions among *G. catenatum* and other planktonic species. In this study, we studied the effect of *C. marina* var.

marina, *M. polykrikoides* and a bacterial community on *G. catenatum* growth under experimental conditions to gain new insights onto this issue.

Material and Methods

Strains and culture conditions.
G. catenatum (BAPAZ-5, GCCV-7, GCMV-7, G7 and 62L), *C. marina* var. *marina* (CMPV-1), and *M. polykrikoides* (MPPAZ-11) were isolated from the Gulf of California and the Mexican Pacific coast. The bacterial community was isolated from *G. catenatum* strain G7. Strains were inoculated in 20 mL of GSe enrichment medium (Blackburn *et al*. 1989) with addition of earth worm soil extract, prepared with seawater from Bahía de La Paz, all components filter sterilized (0.2 µm). Cultures were maintained at a salinity of 34, temperature of 24 °C, a photon flux of 150 µmol m^{-2} s^{-1} and a 12:12 light:dark cycle of 12:12, and progressively scaled up to 150 mL. From this final volume, 400 cells mL^{-1} were inoculated in 150 mL of GSe medium in 250 mL Erlenmeyer flasks. In all cases cells were harvested at late exponential phase.

Bi-algal culture experiments with cell contact. Cells of each species were harvested during exponential phase and mixed at three different ratios: 1:1 (20 x 10^3 cells L^{-1} of each species), 2:1 (20 x 10^3 cells L^{-1} of *G. catenatum* and 10 x 10^3 cells L^{-1} of *C. marina* var. *marina* or *M. polykrikoides*), and 1:2 (10 x 10^3 cells L^{-1} of *G. catenatum* and 20 x 10^3 cells L^{-1} of *C. marina* var. *marina* or *M. polykrikoides*). Mixed cultures of each ratio, were inoculated into 150 mL GSe culture medium in 250 mL Erlenmeyer flasks, as well as monoalgal controls for both experimental cultures, with the same cell densities described above.
Bi-algal culture experiments with no cell contact. Triplicate 250 mL Erlenmeyer flasks, containing 150 mL of culture medium, were inoculated with *G. catenatum* at a density of 20 x 10^3 cells L^{-1}. Immediately, a 15 mL Falcon tube was introduced, the bottom was removed previously and adapted to fit a 10 µm Millipore nylon membrane. Within the tube, 15 mL of *C. marina* var. *marina* or *M. polykrikoides* where inoculated in proportions of 1:1, 1:2, and 2:1.
Filtrates of *C. marina* var. *marina* and *M. polikrikoides*. *Chattonella marina* var. *marina* and *M. polykrikoides* were cultivated until the late exponential phase was reached; cells were then gently removed by filtration, using GF/F (0.7 µm) glass fiber filters (Whatman International). Fifty mL of the filtered culture of *C. marina* var. *marina* or *M. polykrikoides* were inoculated in 250 mL Erlenmeyer flasks containing 150 mL of *G. catenatum* culture. Two controls were used, containing unialgal cultures; in 150 mL of *G. catenatum*, and 150 mL *C. marina* var. *marina* or *M. polykrikoides*, 50 mL of filtrate were added of *G. catenatum*, *C. marina* var. *marina*, and *M. polikrikoides*.

Bacterial community. Cells of *G. catenatum* and a bacterial community were harvested during late exponential phase and mixed at an initial cell abundance of 500 cells mL^{-1} of *G. catenatum* and 9000 cell mL^{-1} of the bacteria. Mixed cultures were inoculated into 150 mL GSe culture medium in 250 mL Erlenmeyer flasks, as well as monoclonal controls of each strain, with the same cell densities described above.

Morphological changes. Changes in morphology of *G. catenatum* cells were recorded with a 3.0 ScopePhoto camera mounted on a Zeiss Axioskop light microscope at 10x and 20x. **Cell density**. All trials and controls were performed by triplicate. A 2 mL aliquot of *G. catenatum* was fixed with Lugol's iodine solution at different incubation times, while cell abundance of *C. marina* var. *marina* and *M. polykrikoides* was determined with live cultures due to the fragility of the cells. Cell density was determined using a 1 mL Sedgewick-Rafter counter chamber under an inverted microscope. For recording bacteria cell density a 500 µL sample was taken and diluted 1:2 with PBS 1%, fixed with formaldehyde at 4% and stored at 4°C in darkness. Bacteria samples were analyzed by epifluorescence microscopy. Cultures were non-axenic and pH was adjusted to 8.

PSP toxins. Toxin analysis and toxicity was calculated as described in Bustillos-Guzmán *et al*. 2013.
Statistical analysis. Tests for normality of the data on the number of individual cells and chains length did not meet the parametric criteria. A non-parametric Kruskal-Wallis test was applied to determine differences between treatments, with significance set at $p<0.05$. Analyses were performed with Statistica 8.0 software (Statsoft, Tulsa, OK).

Results and Discussion

Maximal inhibition of *G. catenatum* growth was observed after 72 h exposure to *C. marina* var. *marina* cultures (Table 1). Mortality was lower (0-33%) but was also observed when cell contact was prevented, indicating that toxic metabolites are liberated to the culture medium. When exposed to culture filtrates a mortality from 16 to 48% occured in 24 h.

Table 1. Average mortality(-)/growth(+) (%) and standard deviation of *G. catenatum* exposed to *C. marina* var. *marina* (CM) and *M. polykrikoides* (MP) in mixed cultures with cells contact, without cell contact and exposed to culture filtrates at different cell density proportions

	Bi-algal cultures. Cell contact			Bi-algal cultures. No cell contact			Culture filtrates		
	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1
CM	-18 ± 3[a*]	-100 ± 8[a*]	-100 ± 4[a*]	-6 ± 1[a]	-0 ± 1[a]	-33 ± 2[a*]	-16±2[b*]	-48 ± 5[b*]	-47 ± 3[b*]
MP	-98 ± 1[c*]	-99 ±0.1[c*]	-98 ±0.8[c*]	+34 ± 2[c*]	+97 ± 6[c*]	+79 ± 8[c*]	-99±1[b*]	+37 ± 9[b*]	+33 ± 9[b*]

[a]72 h, [b]24 h, [c]5 days, *significant difference with control

When *G. catenatum* was exposed to *M. polykrikodes* a high decline (98-99% of the population) was also noted in cultures with cell contact in a longer time span (5 days) (Table 1). Bi-algal cultures without cell contact favoured the growth of *G. catenatum* (34-97% density increase) and when exposed to cultures filtrate, only in the proportion 1:1, a mortality of 99% of the population was observed.

The effect of the bacteria community in general reduced the growth of *G. catenatum*, however big differences among strains was observed, from no significant differences with the control to a growth inhibition of 88% (Table 2). Toxin content per cell also had a wide variation among strains, in two strains it increased (from 6 to 30 %) and in two strains it decreased (from 29 to 37%).

Table 2. Average mortality (-)/growth (+) (%) and cell toxin content in strains of *G. catenatum* exposed to a bacterial community.*significant difference with control

Strain	Mortality/growth (%)	ng STXeq/cel (%)
GCCV-7	+3.5 ± 1.4	+29.95 ± 4.1*
GCMV-7	-38.08 ± 8.3*	+5.87 ± 1.2*
62L	-87.62 ± 6.4*	-36.73 ± 7.3*
G7	-17.28 ± 1.7*	-29.12 ± 2.1*

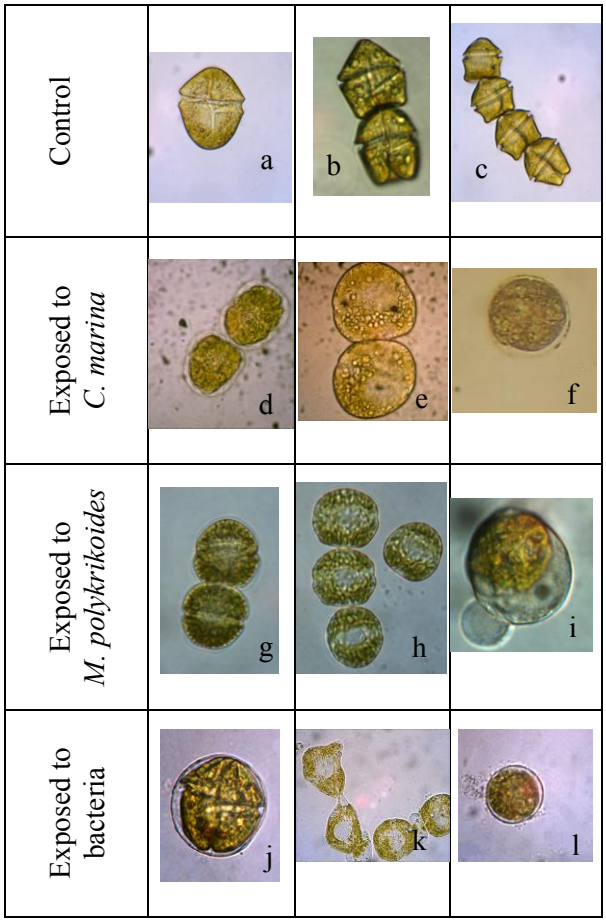

Figure 1. *G. catenatum* exposed to *C. marina*, *M. polykrikoides* and bacteria. (a-c) normal morphology, (d), 2-celled chain with detached amphiesma, (e, g, h, k) swollen cells with evident nucleus, (i, j) single cell surrounded by a membrane, (f, l) cells resembling pellicle cyst.

Changes in the morphology of *G. catenatum* occurred in the presence of *C. marina*, *M. polykrikoides*, and the bacteria community. Observed changes were loss of flagella, swelling, loss of girdle and sulci, evident nucleus and cell lysis. When exposed to cell filtrates of *C. marina* and to bacteria, cells resembling pellicle cysts were observed (Fig. 1).

These results suggest that when there is cell contact, both *C. marina* and *M. polykrikoides* inhibit *G. catenatum* growth. *C. marina* had a stronger effect since inhibition ocurred in a few hours, compared with *M. polykrikoides* where the inhibition ocurred after several days. *C. marina* also liberated toxic metabolites to the medium, thus reducing *G. catenatum* growth. In the case of *M. polykrikoides* the metabolites liberated favoured *G. catenatum* growth. Also *Chattonella marina*, *M. polykrikoides* and the bacteria caused similar changes in the morphology of *G. catenatum* providing new insights of interactions between these species.

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Distribution and abundance of vegetative cells and cysts of harmful dinoflagellates in Quellón Bay, southeast of Chiloé Island

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Abstract

Quellón Bay is an important area for mussel farming and a relevant port for shellfish landings. Five harmful dinoflagellates were studied from February 2012 to April 2013 in relation to the abundances of vegetative cells and cysts, and toxicity in shellfish. *Dinophysis acuminata* was the most abundant species, although the number of positive samples for lipophilic toxins by mouse bioassay was low. These results were attributed to false positives for YTXs and/or PTXs because they have already been associated with *Protoceratium reticulatum* and *D. acuminata*, respectively, in this region. *Alexandrium catenella* and *A. ostenfeldii* were frequently recorded in plankton showing maximum abundances in different periods, while *A.* cf. *tamarense* was occasionally observed and scarce. Cysts of *A. catenella, A. ostenfeldii, Protoceratium reticulatum* and *Lingulodinium polyedrum* were identified in traps and surface sediments, but no *L. polyedrum* vegetative cells were detected in plankton samples. *L. polyedrum* reached higher cyst estimates, but estimations for all species were low, suggesting that hamrful species could only be significantly detected in sediments after intense blooms. Regarding to toxicity, no PSTs were detected using mouse bioassays; while HPLC analysis detected GTX-2, GTX-3 and GTX-4. The PST producer is uncertain, but could be associated with *A. ostenfeldii*.

Keywords: dinoflagellate, cyst, toxin, *Alexandrium*, *Dinophysis*, Chile.

Introduction

The inner waters of Chiloé Island have been affected by a variety of harmful blooms since 1970, being notorious those caused by *Dinophysis acuta* (Lembeye *et al.* 1993), *Heterosigma akashiwo* (Parra *et al.* 1991), *Alexandrium catenella* (Mardones *et al.* 2010) and *Pseudochattonella verruculosa* (Clément *et al.* 2016); with the exception of *A. catenella*, none of them have presented a recurrent temporal and spatial pattern.

The study was conducted in Quellón Bay, an important area for mussel farming and a relevant port for shellfish landings. The aim of this study was to record spatial and temporal distribution of *A. catenella*, as well as other harmful taxa, both in its vegetative form and as resting cyst, and also the detection of paralytic shellfish toxins (PSTs) and lipophilic toxins.

Material and Methods

Sampling was carried out on 9 locations (Fig. 1), from February 2012 to April 2013 in a total of 41 cruises. Vertical net (23 µm) samples, and quantitative phytoplankton samples by a hose were collected. Surface sediments and sediment traps were used for collecting dinoflagellate cysts following Garcés *et al*. (2004), and clams or mussels were collected from six sites for toxin analysis.

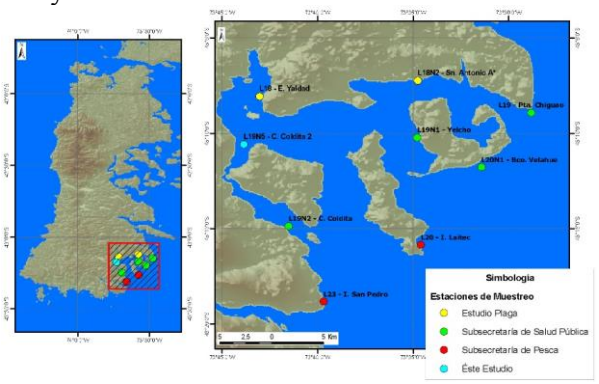

Fig. 1. Study area and sampling sites for southeast Chiloé area.

Relative abundance (RA) of 5 harmful species previously known for the study area (*A. catenella, A. ostenfeldii, D. acuminata, Dinophysis. acuta,*

P. reticulatum) was estimated from net samples using a RA scale (Guzmán *et al*. 2012). Cell density was estimated by the Utermöhl method (Hasle 1978). Cysts from surface sediment and traps were analysed following Matsuoka and Fukuyo (2000). Shellfish (*Venus antiqua* and *Mytilus chilensis*) samples were analyzed for PSTs and diarrhetic shellfish toxins (DSTs) using the mouse bioassay following A.O.A.C. (1990) and Yasumoto *et al*. (1984) methods, respectively, while HPLC was used for saxitoxins (STXs) (Franco & Fernández-Vila 1993). Data analysis included cluster analysis for RA and cyst vertical flux calculations according to formula proposed by Joyce and Pitcher (2004).

Results and Discussion

Phytoplankton analysis

Dinophysis acuminata was the most numerically important harmful species, accounting for the 58% of the total counts of harmful species for RA, being followed by *A. ostenfeldii* (16%), *P. reticulatum* (15%), *A. catenella* (10%), *D. acuta* (2%).

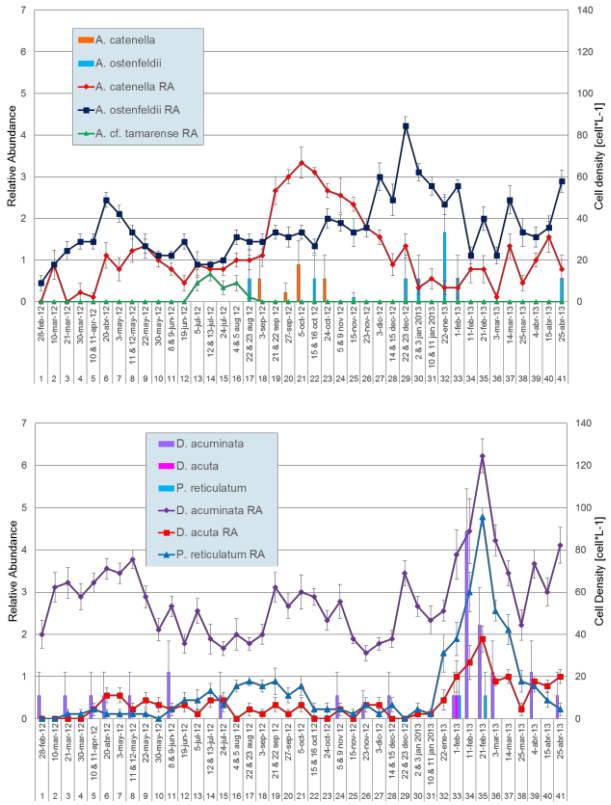

Fig. 2. Relative abundance (RA, lines) and cell density (bars) results for harmful dinoflagellates.

The morphospecies *A.* cf. *tamarense* was also detected (<1% of total count). This pattern was also reflected in quantitative samples, but their numbers were substantially lower and the species were detected on fewer occasions (Fig. 2), confirming that RA estimates is a better and more sensitive measure of harmful microalgae species for monitoring purposes (Guzmán *et al.* 2012). *A. catenella* was more abundant but still in low numbers from September to December 2012, while *A. ostenfeldii* reached its higher numbers between November 2012 to February 2013. *A. catenella* is typically absent during July and August (Guzmán *et al*. 2010), months with lower temperatures and solar radiation, and it is usually more abundant during summer in this area (Guzmán *et al.* 2016). *A. ostenfeldii* has shown to be more regularly distributed during all the year in this area, reaching higher numbers from late winter to summer (Guzmán *et al.* 2016). *A. ostenfeldii* has been considered to have an artic-boreal distribution (Okolodkov 2005), although it is also found in warmer waters such as the Mediterranean Sea (Bravo *et al*. 2006) and in the Peruvian coast (Sánchez *et al*. 2006).

The recent record of *Alexandrium* cf. *tamarense* morphospecies, defined by the presence of a ventral pore on the 1' plate border (Fig. 3a) was detected only in net samples at least once in every sampling station between July to August 2012, but in very low numbers. John *et al*. (2014a) has shown recently using a molecular approach that the complex *tamarense-catenella-fundyense* constitutes a unique species, suggesting to keep the complex under *Alexandrium fundyense*. However, Fraga *et al*. (2015) based on the zoological nomenclatural code sustained that the valid name should be *A. catenella*, which was later confirmed by Prud'homme van reine (2017). The species detected in this study showed morphological characters similar to *A. tamarense* (Balech 1995). However, morphological variability in the presence/absence of the ventral pore in *A. catenella* (formerly *A. tamarense*, John *et al.* 2014b) populations have been reported (Gayoso & Fulco 2006). This could be happening with the *Alexandrium* cells recorded from the studied area but genetic studies are needed to define their identity.

On the other hand, a notorious increase in February 2013 for *Dinophysis* species and *P. reticulatum* was observed.

Cluster analysis using RA data generated 2 groups of sampling stations; I. Laitec (L20) and I. San

Pedro (L23), segregated independently, mainly because of their general higher RA counts for *A. catenella*, *P. reticulatum* and *D. acuminata*. Both sampling stations are located in a more exposed, area of southern Chiloé.

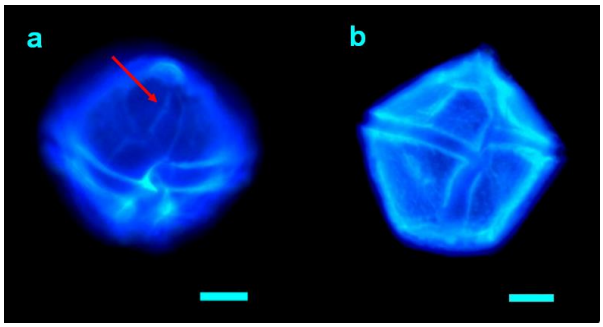

Fig. 3. a. *Alexandrium* cf. *tamarense* morpho-species (with ventral pore); b. *Lingulodinium polyedrum* cell.(scale bar 10 µm)

Cyst analysis

In general, abundance of harmful dinoflagellate cysts in surface sediments and traps was low (Fig. 4). For the study period, the total cyst counts for surface sediment were 421 cysts·mL^{-1} wet sediment and 394 cysts·L^{-1} for sediment traps. Taxa identified in these samples were *A. catenella*, *A. ostenfeldii*, *P. reticulatum*, and *Lingulodinium polyedrum*. Cyst record per season of the year showed that *L. polyedrum* was the most abundant species in surface sediments (Fig. 4a) and sediment traps (Fig. 4b), especially during Spring 2012 (164 cysts·mL^{-1}) and Summer 2013 (124 cysts·L^{-1}), respectively. Cyst vertical flux was also higher for this species (max. value 8,679 cyst·m^{-2}·day^{-1} during Autumn 2013). Cysts of *A. catenella*, *P. reticulatum*, and *L. polyedrum* have been previously reported in the area (Alves-de-Souza *et al*. 2008, Salgado *et al*. 2011), but not those of *A. ostenfeldii* being this the first record. The low abundance of *A. catenella* and *P. reticulatum* cysts for southeast of Chiloé agrees with that reported by Seguel *et al*. (2010).

As in the study by Salgado *et al*. (2011) -the first record of *L. polyedrum* cyst in this area-, vegetative cells of the species were not identified here. However, in a posterior study carried out in 2015 (Guzmán *et al.* unpublished data), phytoplankton samples presented *L. polyedrum* vegetative cells (Fig 3b), for which further studies and excystment experiments are needed. This record would extend its latitudinal southern distribution, being its previous record 32º S on Lambert's Bay , South Africa (Joyce *et al.* 2005). *L. polyedrum* is considered a cosmopolitan, eurihalyne and eurithermic species (Mudie *et al.* 2017), distributed in temperate and equatorial regions for the Northen Hemisphere, but only for subtropical regions for the Southern Hemisphere. Nevertheless, the temperature and salinity range where it has been detected (Zonneveld *et al.* 2013) includes those recorded for this area.

The low abundance of harmful dinoflagellate cysts recorded in the studied area could be related to the absence of intense blooms during this period, but for *L. polyedrum* it is a different situation; there also could be no density-dependent relationship between the abundance of planktonic cyst-forming dinoflagellates and the number of cysts recovered (Godhe *et al*. 2011).

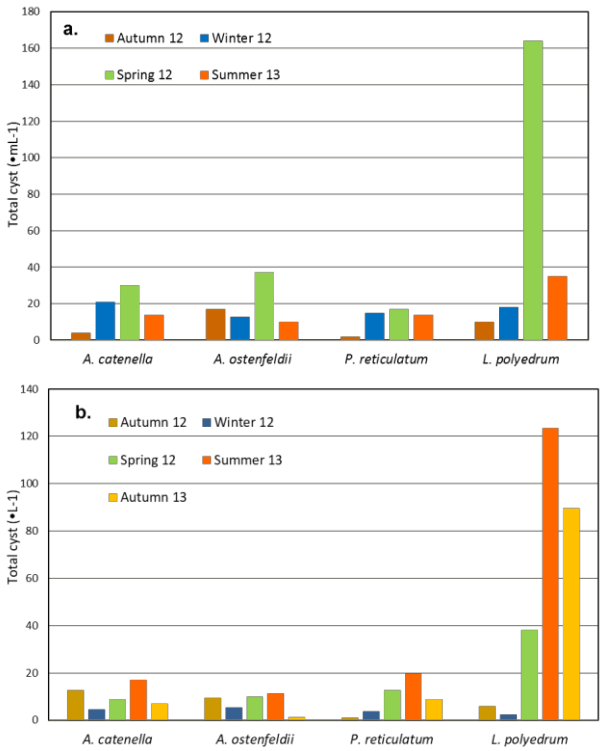

Fig. 4. Cyst abundance in (a) surface sediment and (b) sediment traps.

Toxin analysis

All mouse bioassays for PSTs were negative, and HPLC analysis was negative for STX and Neo-STX. Only trace levels of GTX-2, GTX-3 and subtoxic levels of GTX-4 were mainly recorded during May to September 2012 and reappeared in April 2013 (Fig. 5). These toxin profiles are more similar to what has been associated to *A. ostenfeldii* from another area of Southern Chile which showed GTX-2, GTX-3 and STX (Pizarro *et al*. 2012, Salgado *et al*. 2015).

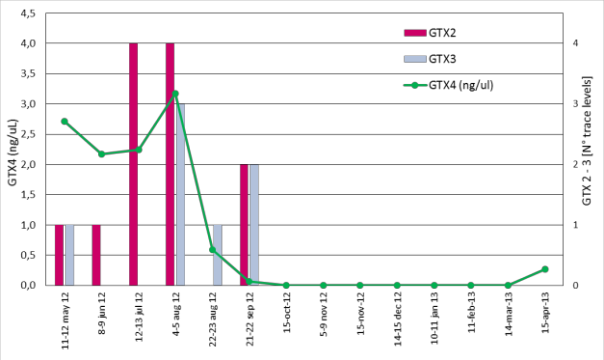

Fig. 5. Toxin profile for HPLC analysis.

Only two positive samples were detected for lipophilic toxins by mouse bioassays. These results could represent false positives, considering previous evidence showing YTXs linked to *P. reticulatum* (Vivanco *et al*. 2012), and that the toxin profile for *D. acuminata* in this area -the most abundant harmful species during the sampling period- was associated with PTX-2 but not okadaic acid and congeners (Fux *et al*. 2011), similar to the toxin profile found by Blanco *et al.* (2007) in northern areas of the Chilean coast.

Acknowledgements

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Changes in phytoplankton species composition during various algal blooms in bays of Manzanillo and Santiago, Colima, Mexico (April-May, 2015)

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Abstract

Algal blooms in the bays of Manzanillo and Santiasgo, Colima, in the central Mexican Pacific, are unpredictable recurrent events. Environmental data and phytoplankton samples were obtained from 13 April to 11 May, 2015, in order to study the algal blooms that appeared in the bays of Manzanillo and Santiago. Phytoplankton showed changes in its composition, where the athecate dinoflagellates *Gymnodinium catenatum* and *Akashiwo sanguinea* were dominant from 13 to 20 April, and from 24 April the dinoflagellates *Alexandrium monilatum* and *Gymnodinium catenatum* became dominant. Along the study period, the dinoflagellate *Dinophysis caudata* was a constant species, and less abundantly other dinoflagellates such as *Alexandrium minutum*, *Prorocentrum micans, Tripos dens* and *T. furca*. These algal blooms decreased from 4 May and the dinoflagellates *Alexandrium minutum*, *Gyrodinium* sp., *Lingulodinium polyedra* and *Scripsiella trochoidea* appeared in low densities, together with several species of the diatom genera *Dactyliosolen*, *Pseudosolenia* and *Rhizosolenia*. The chain-forming diatoms *Pseudo-nitzschia multistriata*, a potentially toxic species, and *Skeletonema pseudocostatum* were also present. This is the first record of the species *Alexandrium monilatum* in Bahia Manzanillo. The variation in temperature, associated to a sudden turbulence of the water column caused by strong winds, and unexpected rain episodes, apparently affected the temporal structure of the phytoplankton communities.

Keywords: Algal blooms, Diatoms, Dinoflagellates, Mexican Pacific, Phytoplankton, Species succession.

Introduction

In coasts of the Mexican Pacific there have been many reports on harmful phytoplankton and red tides, mainly produced by dinoflagellates, as well as other harmful and non-toxic events caused by species of diatoms, ciliates and cyanobacteria (Hernández-Becerril *et al*., 2007). Some regions along these coasts have historical records of these phenomena (Cortés-Altamirano and Núñez-Pastén, 1992). The bays of Manzanillo and Santiago, Colima, located in the central Mexican Pacific are important places for tourism, fisheries and oceanic transportation, as the Manzanillo port might be considered the busiest one in the Mexican Pacific. The continuous rise of human populations and the accelerated industrial and commercial activities have led to an increased pollution in these areas (Ortiz and Jiménez, 2006). Algal blooms in Bahia Manzanillo and adjacent areas have been known for a number of years, as they are recurrent, although absolutely unpredictable events (Ortiz and Jiménez, 2006).
In this paper we report the monitoring of algal blooms during the period of 13 April to 11 May, 2015, where various potentially toxic species formed red tides and showed a succession of species in Bahía Manzanillo.

Material and methods

Phytoplankton samplings (obtained every 3 or 4 days, in non-fixed spots, with bottle and net at surface) and environmental data acquisitions were carried out in various points where red tides appeared in the bays of Manzanillo and Santiago (Fig. 1). Water temperature, salinity, dissolved oxygen and pH were measured and the light penetration in the water column was estimated with a Secchi disc during the period of 13 April to 11 May, 2015. The composition, abundance and distribution of the phytoplankton were studied, analyzing the bottle samples in a compound LM,

identifying and counting phytoplanktonic species using Sedgewick-Rafter chambers. Net samples were also analyzed to confirm species identifications. Some samples or isolated organisms were studied by SEM.

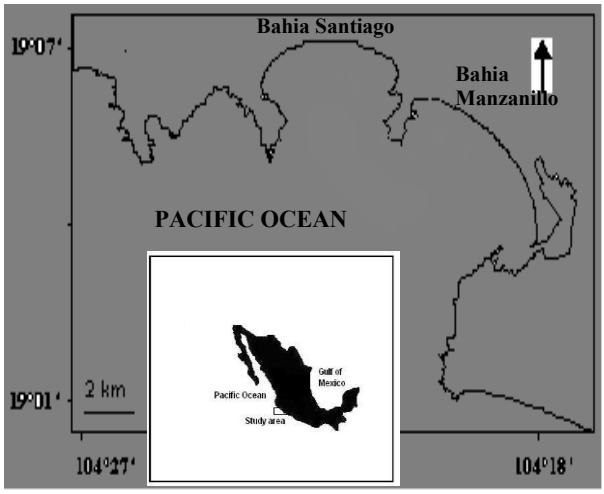

Figure 1. Map showing the study area, with the bays of Manzanillo and Santiago, in the central Mexican Pacific.

Results and discussion

The first indication of the algal blooms occurring by 13 April, 2015, was the pale olive-green color of the water in the bays of Manzanillo and Santiago, which prevailed throughout the study period. The blooms ceased by 4 May, 2015, but some extra sampling and environmental data were taken until 11 May, 2015.

During the period studied, the water temperature was less than 25° C, until 7 May, 2015, and then it increased to 27° C, whereas salinity was fairly constant (around 33), pH varied from 7.5 to 8.1, dissolved oxygen had an average of 8.9 mg L^{-1}, and the Secchi readings gave less than 2 m depth. No toxins were determined (e.g. Saxitoxins, Domoic acid, etc.) during the period of the phytoplankton blooms.

The phytoplankton community showed changes in its composition, where the athecate dinoflagellates *Gymnodinium catenatum* and *Akashiwo sanguinea* were dominants from 13 to 20 April, with maximum densities of the former reaching 3 X 10^4 cells L^{-1}. From 24 April, the thecate dinoflagellate *Alexandrium monilatum* (with a maximum density of 2.6 X 10^4 cells L^{-1}) became dominant, and on the 2 May, both dinoflagellates *Alexandrium monilatum* and *Gymnodinium catenatum* were dominants in numbers (up to 1 X 10^4 cells L^{-1}) (Table 1). The algal blooms decreased from 4 May and the dinoflagellates *Alexandrium minutum*, *Gyrodinium* sp., *Lingulodinium polyedra* and *Scrippsiella trochoidea* appeared in low densities, together with several species of the diatom genera *Dactyliosolen*, *Pseudosolenia* and *Rhizosolenia*.

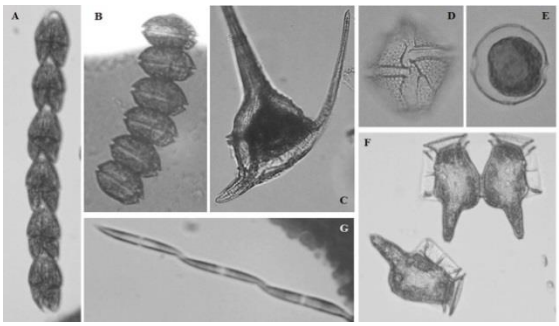

Figure 2. Some phytoplankton species recorded during the period of study. A- *Gymnodinium catenatum*. B- *Alexandrium monilatum*. C- *Tripos dens*. D- *Lingulodinium polyedra*. E. *Alexandrium minutum*. F. *Dinophysis caudata*. G. *Pseudonitzschia multistriata*.

Along the whole study period, the dinoflagellate *Dinophysis caudata* was a constant species, reaching to a maximum density of 0.8 X 10^4, and less abundantly other dinoflagellates such as *Alexandrium minutum*, *Prorocentrum micans*, *Protoperidinium conicum*, *Tripos dens* and *T. furca*. The chain-forming diatoms *Pseudo-nitzschia multistriata*, a potential producer of the toxin domoic acid, and *Skeletonema pseudocostatum* were also present, in discrete densities.

Most phytoplankton species found in this study are commonly recorded in the subtropical waters of the Mexican Pacific and in Bahias Manzanillo and Santiago (Ortiz and Jiménez, 2006), including those potentially toxic, such as the dinoflagellates *Akashiwo sanguinea* and *Lingulodinium polyedra*, and the diatom *Pseudo-nitzschia multistriata*. However, this is the first record of the species *Alexandrium monilatum* in Bahia Manzanillo. Fig.

2 shows some of the species found in this study, including dinoflagellates and a diatom.

We postulate that the unstable climatic conditions, where the variation in temperature, associated to a sudden turbulence of the water column caused by strong winds was evident, and unexpected rain episodes, apparently affected the temporal structure of the phytoplankton communities and favored the succession of species forming blooms in the bays of Manzanillo and Santiago. The wind stress and changes in temperature are related to the dissipation of red tides of another athecate dinoflagellate, *Karenia brevis*, in neritic waters of the northern Gulf of Mexico (Tester and Steidinger, 1997).

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Tester, P.A. & Steidinger, K. (1997). *Gymnodinium breve* red tide blooms: Initiation, transport, and consequences of surface circulation. Limnol. Oceanogr. 42: 1039-105

Date	Dominant microalgae	Maximum density (cells L^{-1})
13-Apr-15 to	*Gymnodinium catenatum*	3 X 10^4
16-Apr-15	*Dinophysis caudata*	0.8 X 10^4
20-Apr-15	*Dinophysis caudata*	0.6 X 10^4
24-Apr-15	*Alexandrium monilatum*	2.6 X 10^4
	Dinophysis caudata	0.3 X 10^4
27-Apr-15	*Alexandrium monilatum*	1 X 10^4
	Dinophysis caudata	0.5 X 10^4
02-May-15	*Alexandrium monilatum*	1 X 10^4
	Gymnodinium catenatum	1 X 10^4
	Dinophysis caudata	0.2 X 10^4

Table 1. Phytoplankton species identified during the study period in the bays of Manzanillo and Santiago, with their density.

Relationship between viable cell transport of the diatom *Didymosphenia geminata* and other invasive species in Tierra del Fuego Island, Chile

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Abstract

The Magellan region (southern Chile) has been aware of the introduction and spread of Invasive Alien Species (IAS). For decades, it has been exposed to the impact by species from Argentina, which arrived in 1946 with 25 pairs of beavers that were released in the north of Lake Fagnano, and in 1948 75 males and 155 females of muskrat were released in lentic systems of Tierra del Fuego island. The presence of IAS on the island has become even more complex with the detection in 2013 of the invasive microalga *Didymosphenia geminata* (Didymo) in two connected freshwaters sources, the Grande and Blanco rivers (in Chile and Argentina respectively). This study aims to explain the possible relationship between wildlife mobility and Didymo cell transport within the Grande river watershed, proposing beavers as a target species. A total of 10 individuals of the beaver *Castor canadensis* were collected and analysed. The presence of viable cells of *Didymosphenia geminata* was identified on different parts of the body of the individuals obtained. This finding suggests that IAS such as beavers can act as vectors for Didymo in Tierra del Fuego and wildlife should be considered in management strategies to limit the spreading of *D. geminata* from one watershed to another.

Keywords: Didymosphenia geminata, invasive alien species, freshwater system, river

Introduction

The Magellan region (southern Chile) has been aware of the risk from introduction and spread of invasive alien species (IAS) (Simberloff, 2011). For decades, it has been exposed to the impact caused by several species from Argentina, which began in 1946 with 25 pairs of beavers, that were released in the north of Fagnano lake, and in 1948 a total of 75 males and 155 females of muskrat were released in various lentic systems of Tierra del Fuego Island (Jaksic *et al.,* 2002). Both species, as well as mink, crossed the boundary that divides the two countries and spread throughout the entire Chilean Patagonian territory, causing negative impacts on the biodiversity of the Tierra del Fuego Archipelago (Sielfeld y Venegas, 1980; Rojel, 2009). The presence of IAS in the island has become even more complex with the detection in 2013 of the invasive microalga *Didymosphenia geminata* (Lyngbye) Schmidt 1899 (Didymo) in two connected freshwaters sources, the Grande and Blanco rivers (in Chile and Argentina respectively) (Figure 1). Didymo is a very aggressive benthic diatom, capable of producing massive algal blooms, which can completely cover the benthic substrate of the watercourses that it affects, because of excessive production of extracellular stalks, which ends up causing severe physical, chemical and biological impacts on ecosystem processes (Kilroy, 2004; Blanco & Ector 2009; Whitton *et al.,* 2009; Bishop & Spaulding, 2017)

Both the Grande and Blanco rivers, have special hydric and limnological characteristics (Niemeyer, 1982), which allow to maintain stable ecosystems of Tierra del Fuego Island. They sustain a great diversity of habitats, and are the favorite locality for numerous native and invasive wildlife, which use water as a source for drink,

shelter, breeding and feeding at several times per year (Schiavini & Raya-Rey, 2001).
This study aims to explain the possible relationship between wildlife mobility and Didymo cell transport within the Grande river basin, using beavers as a target species that act as a vector of viable Didymo cells from one freshwater source to another. Simultaneously we seek to explain the ecological relationship between Didymo transport and propagation upstream of the Grande river basin.

Material and Methods

Sampling

A total of 10 individuals of the beaver *Castor canadensis* were collected between the Grande river and Blanco lake in Tierra del Fuego, using a Conibear 330 trap, with a high degree of efficiency (Lizarralde et al., 1996).
One of its main advantages is that it captures animals without changing the natural state of the sample site, minimizing loss and favoring the recolonization of fur. Trapping was performed under international standards of hunting, which avoids unnecessary suffering of animals and protects the safety of people (Lizarralde & Elisetch, 2002).

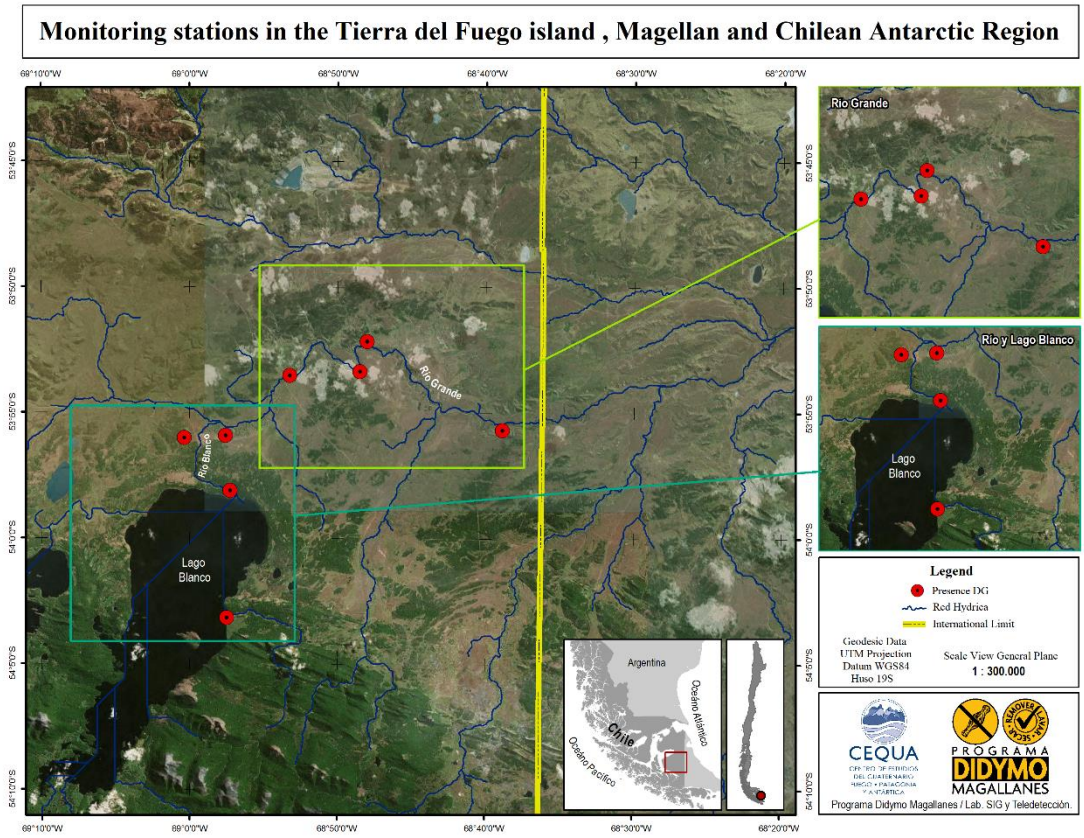

Fig. 1. Monitoring stations at the Tierra del Fuego Island areas with Didymo and beavers presence in the Grande and Blanco rivers and Blanco lake. The square shows the sampling stations.

Following this procedure, several body sections of each trapped animal were brushed (head, tail, and front and rear trunk limbs). The samples obtained were placed into 60 ml flask and then preserved using formalin 5%. Finally, each animal was weighted and their total length and length of each sampled body sections was measured. In the laboratory, we analysed each sample by counting and identifying the phytoplankton groups using an inverted microscope.

Results and Discussion

The presence of *Didymosphenia geminata* viable cells was detected on different parts of the body of the beavers, showing a zoochoric transport relationship by the beavers. Bus-Leone *et al.,* (2014) found a similar result for minks (*Neovison vison*) from the Aysen region, where the presence of Didymo cells on different sections of the animal body was detected. Early information about zoochoric information in wildlife can be found in Atkinson (1972), which pointed out that both birds and mammals are potential vectors of invasive and colonizing species.

Regarding the distribution of Didymo cells on different parts of the beaver's body, the head part showed the presence of viable cells in almost 60% of the sampled specimens; for the abdomen and trunk section a total of 100% presence was obtained for all specimens. On the tail zone, 40% presence was observed, and for the front and back legs between 40 and 70% were found, respectively. Along with Didymo, many other species of benthic diatoms were observed on the beaver fur, particularly the genera *Cocconeis, Encyonema, Fragilaria*, *Gomphonema*, *Navicula*, *Pinnularia and Tabellaria*. According the descriptions associated with the proliferation of *D. geminata* in different water courses, these benthic diatoms correspond to well-known groups of species. It should be noted that in benthic sampling of rivers, the same patterns were observed in areas with Didymo. In the case of a beaver sampled from an area without Didymo (one specimen), only some diatom genera were detected, such as *Gomphonema*, *Nitzschia*, *Fragilaria* and *Pinnularia*. (Figure 2).

Native animal species as well as invasive ones can be transporters of Didymo cells through the freshwater systems in Tierra del Fuego Island, but the differences in the hydrological characteristics (physical, chemical and biological) of this system can be a limiting factor for the establishment and development of the microalgae. An example is that the Grande river contains a zone with abundance of *Sphagnum* moss (peat), which creates an anoxic (low concentration of nitrogen) and acid (pH values of 4-5) environment, with high level of sedimentation (Van Breemen, 1995) allowing patchy Didymo distribution through the river systems. Not all cells that are being transported have the same possibility to generate extensive epilithic mats in freshwater systems.

This study is only the second work after Bus Leone et al., (2014) demonstrating that wildlife can act as vectors of Didymo propagation in the watersheds from southern Chile. Future work on biosecurity and control plans for the spreading of Didymo in Patagonia should therefore consider integrated management strategies for multiple invasive species. Beavers are only a vector for transport of the algae, and its effectiveness as a dispersing agent depends on the environmental conditions where Didymo proliferates massively in the basins that the beaver inhabits.

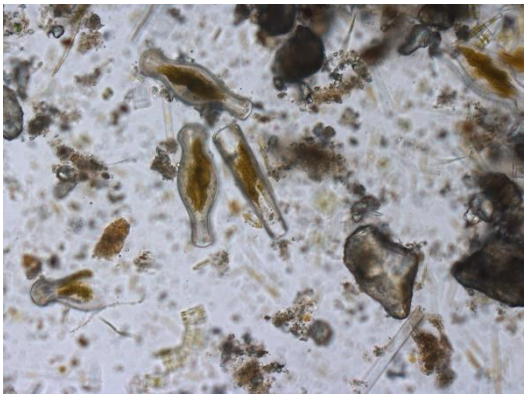

Fig. 2 Presence of *Didymosphenia geminata* and other diatoms genera obtained the beaver's fur from the freshwater systems of Tierra del Fuego Island (Grande and Blanco rivers).

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Using a matrix of scales to understand better the effects of toxic components produced by harmful algae

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Abstract

Compiling a 3dimensional matrix of the scales of length, time and stress (defined as force/area) focuses attention on the ecologically relevant scales at which to measure physical quantities of processes in HAB toxicity and in other aquatic systems. Examples of these scales relevant to mechanisms of toxicity and harm from four selected HAB target organisms, *Karenia, Alexandrium, Ostreopsis* and *Gambierdiscus,* are discussed. A downloadable template matrix, acro.pagespro-orange.fr/RheFFO/matrix.docx, is provided for researchers to try this approach to other HAB species of interest. Data obtained are given, but these are primarily an illustration of new thinking that this approach can stimulate, whether related to HABs or not.

Keywords: Harmful algae, physical scales, toxicity, novel thinking.

Introduction

Harmful algae produce different types of toxic or otherwise harmful actions. Each action acts at a point or in a cloud situated at the intersection (co-ordinate) of three scales, length [m], time [s] and stress (= force/area) [Pa = kg m^{-1} s^{-1}]. Instead of mass [kg], stress is used here because it physically better describes fluidics in the medium surrounding plankton (Jenkinson & Sun, 2010). Stress is a direct quantitative descriptor of the fluidics of swimming and feeding by nano- (Kiørboe, 2016) and picoplankton, whereas mass is considered to be a less intuitive quantitative descriptor. Much of the fluidics close to plankters is non-Newtonian, because of the presence of polymers (tightly and loosely bound exopolymeric substances, including glycocalyxes), and because even in Newtonian fluids close to surfaces the classical no-slip-no-sticking assumption is frequently violated (Rothstein, 2010; Jenkinson, 2014).

The importance of length and time scales in science is well known, but only in the last two decades has the availability of suitable instruments allowed acquisition of force data at the scales of molecules and atoms. I here argue that verbal descriptors of processes may be more precisely expressed as length-time-stress co-ordinates.

Some examples of these scales relevant to mechanisms of toxicity and harm for 4 different HAB target organisms, will be discussed.

A matrix is presented of: harmful algae (*i*); target organisms (*j*); and the three scales of harm as described (*k*) (Table 1A). Because of space limitation, the matrix is limited to four selected HABs (Table 1B; *Karenia mikimotoi, Alexandrium catenella/ pacificum/ tamarense/ fundyense complex, Ostreopsis ovata, Gambierdiscus toxicus*) (Table 1C), the target species being harmed, and a verbal description plus the three quantitative descriptors of scale (Table 1D). Examples of the magnitudes of some processes are then compiled in the corresponding Tables 2A-D.

Material and Methods

Incidences of toxicity and harm from HABs on target organisms were retrieved from the literature. Verbal descriptions of this harm have been assessed for rough values of quantitative descriptions in terms of 3D co-ordinates of length, time and stress. The work was greatly facilitated by the recent compendium by Lassus et al. (2016) but also other sources as noted in the results.

Results

Tables 2A-C show, respectively, verbal descriptions of likely harmful processes and their length, time and stress scales. In Table 2D, however, corresponding data on stress were found only for flow in teleost fish gills, and therefore examples of stresses at scales of molecules and cells have been cited from cell-science literature. All scales

are tentative and are intended to inspire more extensive future compilations. A template matrix, acro.pagespro-orange.fr/RheFFO/matrix.docx, may be downloaded and completed as required for each research project, or pair of species, *i,j*. These results need to be verified and completed, as the real aim of this little paper is the technique, not the results.

Discussion

Measurement and compilation of forces at scales of molecules and cells is progressing rapidly in cell-science in order to understand processes inside cells, near their surface and cross-membrane (Brenner et al., 2011; Sukharev & Sachs, 2012). To understand processes in HAB cells similar techniques are required. Compiling a matrix of length, time and stress scales, as illustrated here, may be a useful tool to guide a programme for research on the physical quantities involved in HAB biodynamics and on their toxic and other harmful effects. Each particular scale involved will dictate the technique(s) required. Scales of length (itself 3D) and deformation in addition require verbal description of geometry, particularly when far from compact, as in convoluted flow-ways.

Table 1A. Dimensions, *i, j, k,* of the matrix

Designation	***Description***
i	Toxic/harmful alga.
j	Target species potentially harmed.
k	Quantitative description: 1) length; 2) time; 3) stress.

Table 1B. Examples of toxic/harmful algae

i	***Toxic/harmful alga***
1	*Karenia mikimotoi*
2	*Alexandrium catenella/pacificum/tamarense*
3	*Ostreopsis* cf. *ovata*
4	*Gambierdiscus toxicus*-complex

Table 1C. Examples of target organisms

j	***Target organisms potentially harmed***
1	Protists
2	Teleost fishes
3	Molluscs
4	Mammals, including humans

Table 1D. Description and scales of harm

k	***Description and scales of harm***
0	Verbal description of harm
1	Length scale(s) of harmful processes
2	Time scale(s) of harmful processes
3	Stress scale(s) of harmful processes

Techniques for measurement should first consider atomic force microscopy, because it can both measure forces and image structures at the same molecular- and cellular scales. For other techniques, see Brenner et al. (2011).

This matrix may be useful in research not only on HABs but also on other bioaquatic systems that involve capillaries and filtration, including lab-on-a-chip, bioreactors, desalination plants and water-purification systems. I have found trying to complete Table 2 forced me to think both deeply and laterally, giving me new ideas. I hope it can benefit other researchers similarly, helping them develop new insights in the science and management of harmful algae as well as of many other fields.

Acknowledgements

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Table 2A. k = 0, Verbal description of harm. Letters in brackets show references after Table 2D, in addition to Lassus et al. (2016)

Alga i	*Target j*
Karenia mikimotoi	**Protists. 1.** Allelopathic to flagellates and diatoms, partly using PUFA, particularly octodecapentoic acid (a). **2.** Allelopathic growth-rate reduction of *Dunaliella salina* (b). **Teleosts. 1.** ROS damages fish gills. **2.** Mucous thickening in gill passages, increases viscosity and reduces flow, modelled (c) and found experimentally (d) sufficient to kill fish by suffocation. **Molluscs. 1.** Mortality of bivalves, gastropods. **2.** Toxic effect on growth of king scallops. **3.** Produces deformations in bivalve larvae. **3.** Mortality of squid in Hokkaido, Japan (e). **Mammals incl. humans.** No health effect known.
Alexandrium catenella	**Protists, bacteria.** Lytic and non-lytic strains showed both negative and positive allelopathic effects on other phytoplankton, but mainly beneficial effects on bacteria (f). **Teleosts. 1.** Ichthyotoxicity (haemolytic activity) in axenic culture media (g) related to gill lesions preceding mortality in Chilean farmed salmon (h). Damage to gill epithelia considered not to be by PSTs, but by a combination of ROS and/or PUFAs (i). Larval teleosts having ingested *A. fundyenense* PSTs vectored by copepods suffer behavioural anomalies (j). **Molluscs.** Clearance rate and gape in bivalves reduced associated with toxic strains of *Alexandrium monilatum* (j)**;** behavioural impairment, and increased susceptibility to viral disease in bivalves (k). **Mammals incl. humans. 1.** PSTs cause PSP in mammals. **2.** Toxic to mammals that eat PST-contaminated organisms.
Ostreopsis ovata	**Protists, Teleosts.** No harm noted **Molluscs. 1.** Mortality of limpets, *Patella*, mussels. "Stranded" octopuses. Mortality of sea urchins. **2.** All these organisms contaminated by palytoxin; ovatoxin-a dominant. **3.** Mucus abundant (l). **Mammals incl. humans. 1.** Respiratory problems, apparently resulting from water-soluble toxins and cell debris released by the algae, then incorporated into wind-borne aerosols. **2.** Marine organisms fed *O. ovata* experimentally can be toxic to mammals.
Gambierdiscus toxicus	**Protists, Molluscs.** No harm noted. **Teleosts**. Produces CTX, which is ichthyotoxic (m). **Mammals incl. humans.** Effects, often lethal, of nerve toxins CTX and MTX.

Table 2B. k = 1, Length scales of harm.

Alga i	*Target j*
Karenia mikimotoi	**Protists. 1.** Toxicity to plankton due to unstable PUFA leading to a toxic sphere of radius **O(175µm)** around each cell (a). **2.** Radius of PUFA molecules, octadecapentoic acid, **O(10nm)** (n, m). **3.** Length of action of PUFA on target cell **O(10-100nm)**, but larger length scales associated with glycocalyxes. **Teleosts. 1.** ROS, free O radicals, radius **O(5-10nm)** (n). ROS terminations on polymers may have larger functional length scale. **2.** Secondary lamellar gill-pore width **O(17-53µm)**, length **O(180-1600µm)** (o). **3.** Quasi-fractal rheological aggregate-lumpiness effect may also occur. **Molluscs.** More study required of toxicity mechanisms on molluscs, both bivalves and squid. **Mammals incl. humans.** No effect known.
Alexandrium catenella	**Protists.** Length scales could be comparable to those in effects by *K. mikimotoi.* **Teleosts. 1.** For damage to gill epithelia by haemolytic activity, length scales are similar to haemolytic activity by *K. mikimotoi*. **2.** Radius of toxin molecules, **O(10nm)**. **3.** No evidence of harm by algal mucus. **Molluscs.** Radius of toxin molecules, **O(10nm)**, Shell gape **O(1mm)**.

	Mammals incl. humans. 1. Diameter of PST molecules, **O(5-10nm)**. **2.** Scales in human intoxication related to ingestion of contaminated seafood, absorption of toxins, and neurotoxicity, with possible scales **O(1mm-1m)**. **3.** Scales of contamination by PST of shellfish important, but not considered.
Ostreopsis ovata	**Protists, Teleosts.** No harm noted. **Molluscs. 1.** A priori, length scales for intoxication of adult bivalves, as well as for octopus, through their gills similar to those for gills of teleosts by *K. mikimotoi*. **2.** More investigation needed on scales related to the absorption and effects of algal toxins in octopus. **Mammals incl. humans.** Respiratory effects: diameter of hydrated toxin molecules, **O(5-10nm)**, secretion of toxin **O(1µm)**; cells and cell debris **O(5-50µm)**; diffusion in the sea, **O(0.1mm-1m)**, air-sea flux, **O(0.1-1mm)**; wind transport, **O(1m- 1km)**; diffusion in human body, **O(1dm)**; diffusion across lung epithelia, **O(10µm-1mm)**.
Gambierdiscus toxicus	**Protists, Molluscs.** No harm noted. **Teleosts.** Diameter of GTX toxin molecules, **O(5-10nm)**; abnormal fish behaviour **O(mm-m)** **Mammals incl. humans.** Wide diversity of obligate and facultative processes is involved, from GTX and MTX production by the alga to feeding by herbivorous fishes, to absorption of the toxin molecules, followed by predation by carnivorous fishes, fishing, and transfer to humans, including trade and international tourism. Length scales range from molecule diameter, **O(5-10nm)** to trade and tourist routes **O(1-10,000km)**.

Table 2C. k = 2, Time scales of harm.

Alga i	*Target j.*
Karenia mikimotoi	**Protists.** Half life of allelopathic principle **O(50min)** (a). **Teleosts. 1.** ? **2.** Flow time through secondary gill pores (Length/flow speed) **O(0.023-0.028s)**; **3.** For damage by O radicals, see under *A. catenella*.
Alexandrium catenella	**Protists.** Half life of molecules toxic to protists might be similar to those toxic to teleost gill epithelia **Teleosts. 1.** For damage to gill epithelia by haemolytic activity, time scales of gill flow are *a priori* similar to haemolytic activity by *K. mikimotoi*. **2.** Half life of molecules toxic to gill epithelia may be **O{~µs}** for hydroxyl radicals and **O(3-300s)** for superoxide (k Bricelj 2011). **Protists, molluscs.** No harm noted. **Mammals, incl. humans. 1.** Time scales of toxic action by PST molecules on target structures (Na^+ channels): To be determined; **3.** See length scale.
Ostreopsis ovata	**Protists, Teleosts.** No harm noted. **Molluscs.** See remarks for length scales. **Mammals incl. humans.** Respiratory effects: action of toxin molecules, **TBD**; secretion of toxin **O(1s?)**; diffusion in the sea, **O(1s-1h)**, air-sea flux, **O(0.1-1s)**; wind transport, **O(1s-1h)**; diffusion in human body, **O(1s-1d?)**; diffusion across lung epithelia, **O(1ms-1min?)**; evolution of human pathology **O(1h-10y?)**.
Gambierdiscus toxicus	**Mammals incl. humans.** See remarks for length scales. Times might range from molecular **O(µs)** to trade and tourist routes **O(1h-1wk)** to evolution of human pathology **O(1h-10y)**.

Table 2D. k = 3, Stress scales of harm and some other effects.

Alga *i*	**Target *j***
Karenia mikimotoi	**Teleosts. 2.** Hydrostatic pressure generated across gills P = **O(5Pa)**, and stress at the surfaces of the gill pores, $\tau \sim P.r/2.L$, where r is pore "radius" and L is pore length, giving τ=**O(0.1-0.5Pa)**, perhaps less in teleost larvae.
Diverse harmful algae	**Diverse target systems.** Taking human arteries, for comparison with some target systems, their endothelial glycocalyx (GC), composed of **O(6nm-dia.)**, **O(2µm long)** fibres, cushions the endothelial cell membranes from flow-induced shear stress τ. At the tip of the GC fibres τ=**O(1.5 Pa)**, but it is modelled that τ falls to **O(6x10^{-7}Pa)** at the fibre roots on the cell membrane (p). Comparable GCs occur on respiratory surfaces of harmful algae and their target organisms.

References: a – Gentien *et al.* (2007); b – He *et al.* (2016); c - Jenkinson (1989); d – Jenkinson & Arzul (1998); e – Shimada *et al.* (2016); f – Weissbach *et al.* (2010); g - Ogata & Kodama (1986); h – Fuentes *et al.* (2008); i – Mardones *et al.* (2015); j Samson *et al.* (2010); k – Bricelj *et al* (2011); l – Giussani *et al.* (2011); m – Lewis (1992); n – Conlisk (2013); m – Jenkinson (2014); o – Hughes (1966); p – Liu *et al.* (2011).

Imaging FlowCytobot provides novel insights on phytoplankton community dynamics

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Abstract
The Imaging FlowCytobot (IFCB) combines flow cytometry and video technology to capture images of individual cells which, together with machine-learning technology, enables near real-time reporting of individual phytoplankton species abundance and community composition. The IFCB is designed for automated submersible operation. Over a decade of sustained high temporal resolution observations (hourly to daily) at the Martha's Vineyard Coastal Observatory (MVCO) and the Texas Observatory for Algal Succession Time series (TOAST) are providing new insights into dynamics of phytoplankton community structure. In the Gulf of Mexico at TOAST, the IFCB has provided successful early warning and observation of eight harmful algal bloom (HAB) events. Initial stages of HABs are detected with sufficient time to close shellfish harvesting and prevent human illness. On the east coast of the US at MVCO, multi-year trends in blooms have been linked to regional climate-related variables. Time series data can also identify novel interactions, life cycle stages, and quantify processes that might otherwise be missed. As the network of IFCBs expands globally, an unprecedented level of information will be available on the variability and diversity of the phytoplankton community. Of particular interest are those species that are harmful or early indicators of ecosystem response to environmental change.

Keywords: Karenia brevis, Dinophysis ovum, early warning, life history stages, parasites, predation

Introduction

Early warning is essential for successful mitigation of harmful algal blooms (HABs). Although light microscopy is the traditional method for monitoring, it is labor intensive and often does not provide sufficient temporal resolution to identify initial stages of a bloom. A new approach using imaging-in-flow cytometry provides automated continuous sampling and processing for measuring phytoplankton abundance (Sosik and Olson 2007). The resulting time series deliver not only data at a frequency sufficient for early warning, but also novel observations that furnish insights on interactions among the plankton. The IFCB is now commercially available (McLane Research Laboratories, Inc., East Falmouth, MA, USA) and there are currently ~25 instruments in operation worldwide.

Material and Methods

The Imaging FlowCytobot (IFCB) combines flow cytometry and video technology to obtain high resolution images (1μm/pixel; Olson and Sosik 2007). Samples (5 ml) are collected approximately every 20 minutes and images are recorded for cells that are within the size range of ~10 - 150 μm (Fig. 1). Images are processed and classified with automated software-based analysis tools (Sosik et al. 2016; https://github.com/hsosik/ifcb-analysis/wiki).
Over 200 features, including biovolume (Moberg and Sosik 2012), are computed for each image. A supervised random forest algorithm is used to classify images (often to genus or species). Classification results are then used to provide near real time estimates of taxon-specific cell abundance and biomass.

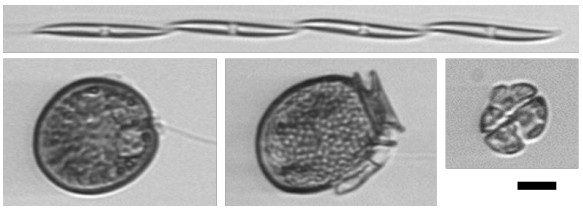

Fig. 1. Examples of HAB images at TOAST from top right: *Pseudo-nitzschia multistriata*, *Karenia brevis*, *Dinophysis ovum*, *Prorocentrum texanum.* scale bar = 10 μm

We have established two phytoplankton

observatories utilizing the IFCBs: (1) at MVCO off the east coast of the US in 2006; and (2) at TOAST in the Gulf of Mexico at Port Aransas, TX in 2007. At both of these decade-long time series, data downloading, processing, and classification are automated. For selected taxa, once cell abundance exceeds a threshold (currently set at 2 cells mL^{-1} to avoid excessive false positive notifications), automated emails are sent to state managers. Messages include information on cell abundance for the previous 10 h and a link to the IFCB Dashboard (Fig. 2), where all images are displayed and identifications can be confirmed (Campbell et al. 2013). The IFCB Dashboard and associated web services provide open access to all IFCB images and data products (Sosik and Futrelle 2012) at http://ifcb-data.whoi.edu/mvco for MVCO and http://toast.tamu.edu/ for TOAST.

Fig. 2. IFCB Dashboard, example during a 2014 *Dinophysis* bloom on the Texas coast.

Results and Discussion

Early warning of harmful algal blooms

Identification of novel species, including HAB species, has been one of the major successes at TOAST. In 2008, *Dinophysis ovum* was detected at bloom levels in Texas and resulted in the first shellfish harvesting closure and recall due to diarrhetic shellfish poisoning (DSP) in the US (Campbell et al. 2010). Since then, IFCB has contributed to the detection of seven additional events, including five *D. ovum* events (2010, 2011, 2012, 2014, 2015) and two *Karenia brevis* events (2009 and 2011). Despite dangerous levels of toxicity, no human illnesses have been reported due to these HAB events, in part because of the effective early warning provided by IFCB data. The cooperation between researchers operating the time series and local managers has also been critical.

Continuous operation of the IFCB at the TOAST site has shown Port Aransas to be an effective "hot spot" for HAB detection on the Texas coast. Cell concentrations can be extremely patchy, however. For example, during a 2009 *K. brevis* bloom, the peak in average daily abundance was ~90 cells ml^{-1}; however, at 2-h resolution, it was clear that patchiness is extreme and cell concentration can vary greatly over just a few hours (Fig. 3). Additionally, standard discrete sampling at weekly intervals could have missed entirely the peaks in this event entering the estuary (Fig. 3).

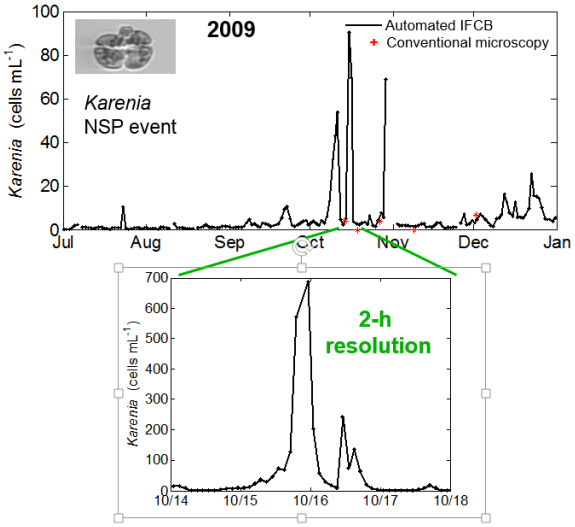

Fig. 3. Time series of *Karenia brevis* abundance during the 2009 bloom at TOAST. upper: daily average abundance; weekly manual sample counts (+); lower: IFCB time series at 2-h resolution. Daily resolution provided reliable early warning in September when cell abundances were very low, while the 2-h resolution emphasized the extreme patchiness of the main bloom, which was easily missed by intermittent manual sampling.

Identification of a new species, *Prorocentrum texanum* was also possible from IFCB observations (Henrichs et al. 2013). This new species has two distinct morphotypes (var. *texanum* and var. *cuspidatum*) that are not distinguishable genetically based on the 5 genes examined. One isolate of *P. texanum* var. *texanum* produced okadaic acid.

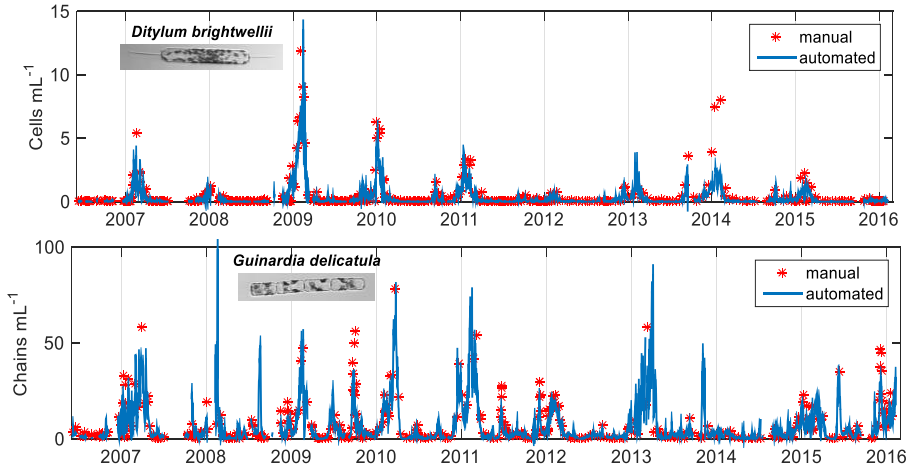

Fig. 4. Time series of two important diatom species at MVCO, *Ditylum brightwellii* and *Guinardia delicatula*. Blue lines indicate daily resolved abundance estimates from automated classification and red symbols correspond to estimates from images that have been manually verified for ~1 hour of data collection approximately every 2 weeks.

Bloom dynamics

The decade-long time series at MVCO has revealed marked patterns in seasonality, as well as considerable interannual variability. At MVCO, diatoms dominate microplankton biomass and many species bloom most dramatically in winter, though IFCB records emphasize that there are species-specific differences in bloom timing and amplitude (Fig. 4).

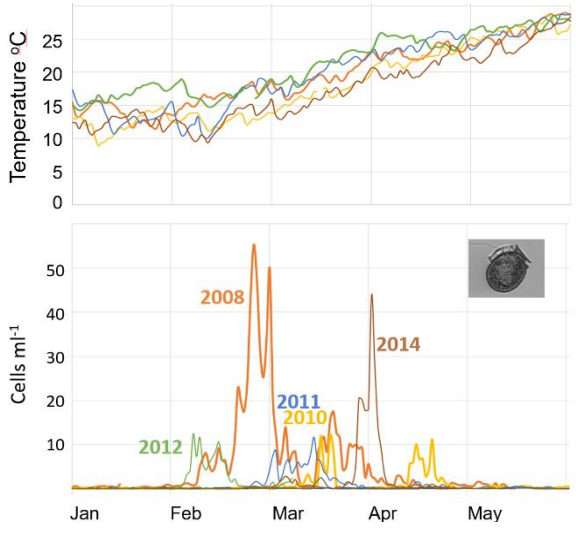

Fig. 5. Annual blooms of *Dinophysis ovum* and water temperature at TOAST

Long-term observations can also inform on the phenology of phytoplankton blooms. For example, at the TOAST site, *Dinophysis ovum* blooms occur each spring, however the timing of initiation has ranged from early February to early April. Interannual variability appears to be related to temperature; earlier onset of *D. ovum* in 2008 and 2012 coincided with >15ºC water temperature (Fig. 5).

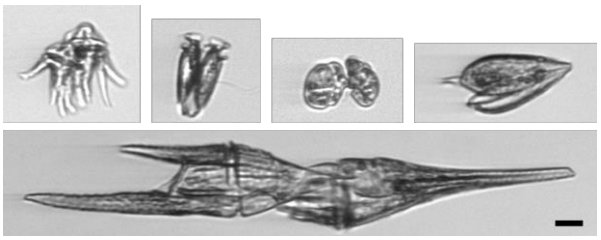

Fig. 6. Examples of dividing dinoflagellates (from top left: *Brachidinium capitatum, Dinophysis ovum, Karenia brevis, Prorocentrum micans, Tripos furca*). Phased diel patterns of cell division have been used to estimate *in situ* growth rates. scale bar = 10 µm

Cell division and life history stages

The high frequency sampling of IFCB time series has also enabled novel observations of cell cycle stages and interactions among different types of plankton. For many microplankton species, dividing cells can be distinguished and quantified (Fig. 6). In dinoflagellates, diel patterns in cell division are typical, and this phasing allows growth rate estimation with the frequency of dividing cells approach (Carpenter and Chang 1988). Specific growth rates have been calculated for *D. ovum* (Campbell et al. 2010), *Brachidinium*

capitatum (Henrichs et al. 2011), and *Alexandrium fundyense* (Brosnahan et al. 2015). In addition to providing essential information about growth under *in situ* conditions, this approach is advantageous for species that are difficult to culture, or are not available in culture.

Life cycle stages, which may be short-lived, can be difficult to document in field samples. Sustained imaging from IFCB continuous sampling increases detection of rare events. For *Alexandrium* species, for example, the formation of resting cysts is an essential step in recurrence of blooms, yet the factors influencing the onset of gamete production and subsequent cyst formation are not well defined. With the IFCB, Brosnahan et al. (2015) were able to capture a continuous record of an *A. fundyense* bloom cycle, including the transition from vegetative growth to the sexual cycle, and to measure division rates. Importantly, growth rates measured *in situ* exceeded laboratory measurements at the same temperature, highlighting the importance of being able to determine rates for *in situ* populations under natural conditions.

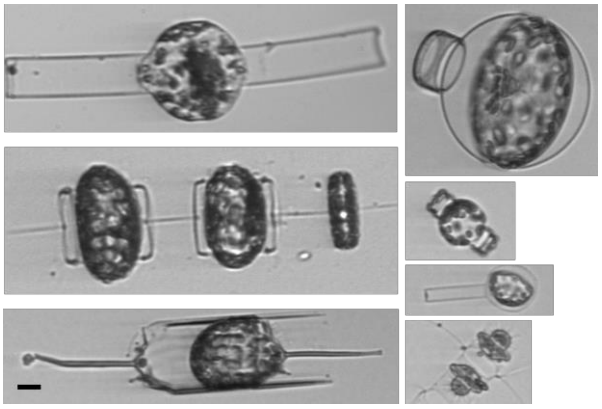

Fig. 7. Diatom life cycle stages. From top rows: auxospores, bottom row: resting spores. scale bar = 10 µm

Diatom life cycle stages have also been recorded at both MVCO and TOAST (Fig. 7). Although the vegetative life cycle stage of diatoms is dominant and may last for years, sexual reproduction is an obligate life cycle stage for most species (Chepurnov et al. 2004). Gametogenesis and auxosporulation are induced by cell size reduction, but environmental factors may also play a role (e.g., Godhe et al. 2014). These stages may last for only hours to days (Chepurnov et al. 2004), so are known from cultures and are not well documented in the field. The IFCB time series may provide more detailed information on timing of life history stages in nature, which should provide new insights into the role of resting stages in seeding blooms

Symbiotic, epiphytic and parasitic interactions

The heterocystous diazotroph *Richelia intracellularis* is well known as a symbiont in diatoms of the genera *Rhizosolenia, Hemiaulus* and *Guinardia* (Mague et al. 1974; Zeev et al. 2008). Most previous diatom-diazotroph associations have been reported from the tropical oligotrophic ocean, so observations documented at TOAST (Fig. 8) are unexpected, possibly having an offshore origin.

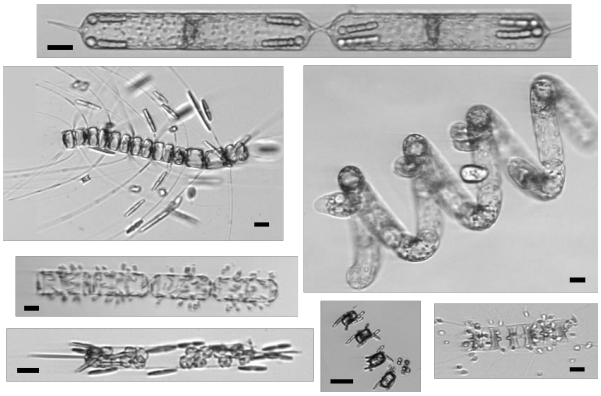

Fig. 8. Examples of symbiotic (*Guinardia cylindrus* with *Richelia*) and epiphytic flagellates, pennates or chytrids on the diatoms *Chaetoceros, Cerataulina, Ditylum, Guinardia, and Thalassiosira*. scale bars = 10 µm

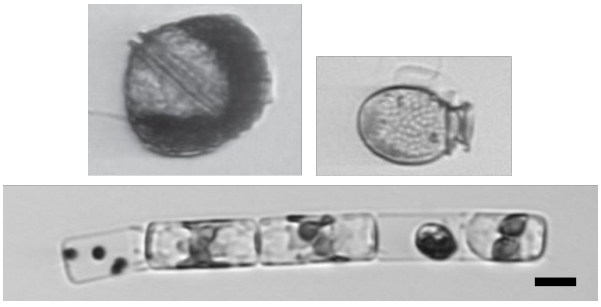

Fig. 9. Examples of *Alexandrium, Dinophysis ovum*, and *Guinardia delicatula* with developing parasites. scale bar = 10 µm

Epiphytic and parasitic interactions are also documented in IFCB images (Fig. 8 and 9). In a notable case at MVCO, Peacock et al. (2014)

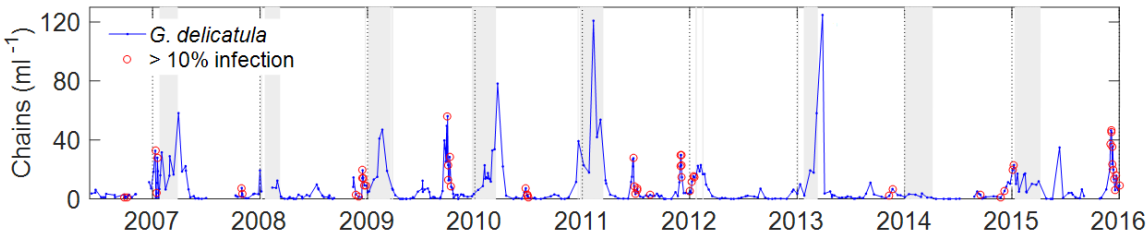

Fig. 10. Time series of *Guinardia delicatul*a abundance at MVCO, with occurrence of high rates (>10% of host population) of parasite infection indicated. Parasite infection occurs every year, but is apparently inhibited when water temperatures fall below 4°C (denoted by gray bars). After Peacock et al. (2014), but updated through 2015.

showed that these kind of interactions can be very important ecologically. The time series of high-resolution images revealed that the dominant chain-forming diatom (*Guinardia delicatula*) is subject to recurrent high mortality from a nanoflagellate parasitoid (Fig. 10). Overall, the level of parasitoid infection evident in the population was a strong predictor of bloom magnitude, and warm winters were linked to enhanced infection and bloom collapse (Peacock et al. 2014). In essence, cold waters provide the diatom a refuge from parasitoid mortality. These kinds of quantitative assessments of natural interactions over multiyear periods are now leading to intriguing climate-related hypotheses. For example, in the case of *G. delicatula*, as warming trends continue in the waters of New England, periods of cold winter waters may be reduced in duration and intensity and dramatic winter blooms of this diatom may not persist.

Protist feeding mechanisms

Predation by microzooplankton is a major source of phytoplankton mortality (Calbet et al. 2008). A variety of feeding mechanisms by protists are recognized, such as pallium and peduncle feeding by heterotrophic dinoflagellates (Jacobsen 1999), but these are not well documented in natural communities. Although the IFCB was intended to detect phytoplankton, well-fed (chlorophyll-containing) herbivorous and mixotrophic protists can also be imaged (Fig. 11) (Brownlee et al. 2016). Many micrograzers are quite fragile cells and may not be preserved well for identification. By imaging live cells, the IFCB provides novel observations and improved potential for quantifying these interactions. To improve detection of micrograzers, the Sosik Laboratory now has a prototype staining IFCB (IFCB-S) with automated live cell fluorescent staining. Initial results with this IFCB-S have shown that more organisms, not just those that have fed, are imaged with IFCB-S and the time series revealed different seasonal patterns for different microzooplankton grazers (Brownlee et al. 2016).

A unique feature for the successful culture of the mixotrophic dinoflagellate *Dinophysis* is the requirement for a specific ciliate prey item, *Mesodinium rubrum* (Park et al. 2006). Thus, *Mesodinium* distributions may be useful in predicting blooms of *Dinophysis* in the Gulf of Mexico (e.g., Harred and Campbell 2014). Long-term time series were used to identify seasonal patterns and correlations between *Mesodinium* and *Dinophysis*. Environmental conditions, as well as *Mesodinium* abundance and species composition were found to affect bloom initiation (Harred and Campbell 2014).

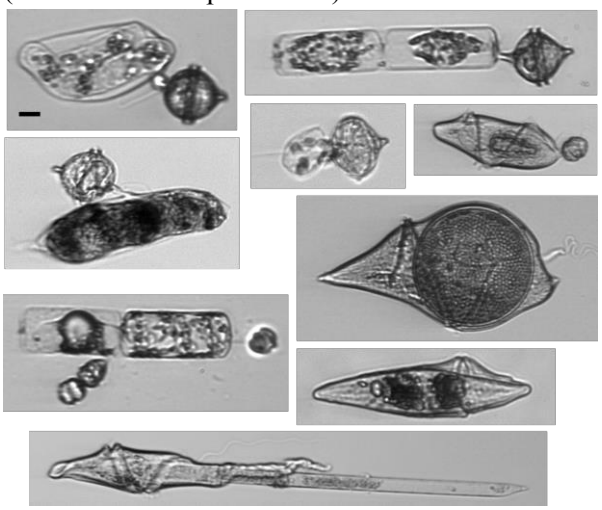

Fig. 11. Examples of heterotrophic feeding by dinoflagellates preying on microplankton. scale bar = 10 µm

New developments for the IFCB

In addition to the IFCB-S mentioned above, advances for the IFCB include new instrument capabilities and new deployment platform options. A high-throughput version of the IFCB that uses acoustic focusing to gently pre-concentrate cells above the flow cell is in development to allow for better counting statistics of rarer cells. A version of the IFCB that operates horizontally for integration with autonomous surface vehicles is also being tested now as a means for responding to or following bloom events.

Conclusions

The high temporal resolution IFCB time series provides a unique view of microplankton communities. Species-specific phytoplankton time series are a powerful tool to mitigate HABs and human health risks through early warning and bloom forecasting. In Texas, the IFCB has already provided early warning and monitoring for six *Dinophysis* and two *Karenia* HAB events since 2007.

Continuous and automated analysis also permits the opportunity to observe novel species, variations in community composition in response to environmental forcing, trends with climate change, phenology, and interactions among species (symbiotic, epiphytic, parasitic, and grazing). Continuous observation is advantageous in that it allows quantification of processes within the plankton that might otherwise be missed.

Acknowledgements

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First report of the epiphytic genera *Gambierdiscus* and *Ostreopsis* from the coast of El Salvador, Eastern Tropical Pacific

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Abstract

Toxin-producing algal blooms are recurrent on the coast of El Salvador; however, there are no reports of ciguatera poisoning due to benthic algal blooms. The analysis of epiphytic dinoflagellates in the intertidal rocky platform of Los Cóbanos reef system revealed the presence of *Gambierdiscus* and *Ostreopsis* for the first time on the El Salvador coast. These species have been previously identified as potentially toxic and involved in ciguatera outbreaks around the world. During April to May 2013, macroalgae samples were collected in the intertidal zone of Los Cóbanos by free diving and using plastic bags; samples were then vigorously shaken to remove dinoflagellates, the water was filtered through several sieves and the retained fraction in the 20µm sieve was analyzed using an inverted microscope. Both *Gambierdiscus* and *Ostreopsis* were found in low density (3 ± 2 cells g^{-1} wet weight macroalgae); with *Gambierdiscus* species showing a preference for Rhodophytes. In addition, four species of *Prorocentrum* were identified: *P. lima, P. micans, P. mexicanum,* and *P. minimum*. *Procentrum lima* showed the highest abundance with 80 cell g^{-1} macroalgae. Though *Gambierdiscus* and *Ostreopsis* species are still being confirmed, this is the first report of the genera in El Salvador and one of the few reports from the Eastern Tropical Pacific.

Keywords: epiphytic dinoflagellates, ciguatera, El Salvador, Eastern Tropical Pacific

Introduction

Toxin-producing algal blooms are recurrent in El Salvador and are dominated by paralytic shellfish poisoning events (Espinoza et al. 2012, Espinoza et al. 2013a, Espinoza et al. 2013b, Amaya et al. 2014). To date, there are no reports of Ciguatera Fish Poisoning (CFP) due to benthic algal blooms. Ciguatera poisoning is known to be caused by dinoflagellates in the genera *Gambierdiscus* that produce ciguatoxins (CTXs) and other related toxins, such as maitotoxins (MTXs); these toxins can be accumulated in fish from tropical and subtropical regions and cause CFP in humans (Parsons et al. 2012). It is assumed that toxins flow from herbivorous fish, which feed from macroalgae, to carnivorous fish (Litaker et al. 2010).

Potentially toxic epiphytic dinoflagellates are known to occur in the Atlantic, Pacific, and Indian Oceans (Parsons et al. 2012). Intestinally, few benthic dinoflagellate species have been reported from the Eastern Tropical Pacific with the exception of those occurring along the Costa Rica (Vargas-Montero et al. 2012) and México coasts (Okolodkov and Gárate-Lizárraga, 2006). Central America Pacific has not been identified as CFP endemic area, despite the presence of *Gambierdiscus* and a higher CFP incidence rate in the Pacific than the Atlantic (Tester et al. 2010).

The goal of this study was to identify potentially toxic epiphytic dinoflagellates in Los Cóbanos, one of the most important marine ecosystems and the only coral reef in the coast of El Salvador.

Material and Methods

Study area

Los Cóbanos is a marine protected area with 15 km^2 and located in the western coast of El Salvador in the Eastern Tropical Pacific (figure 1). This area contains the only coral reef in the country and is characterized by a rocky intertidal zone with extensive macroalgae covering.

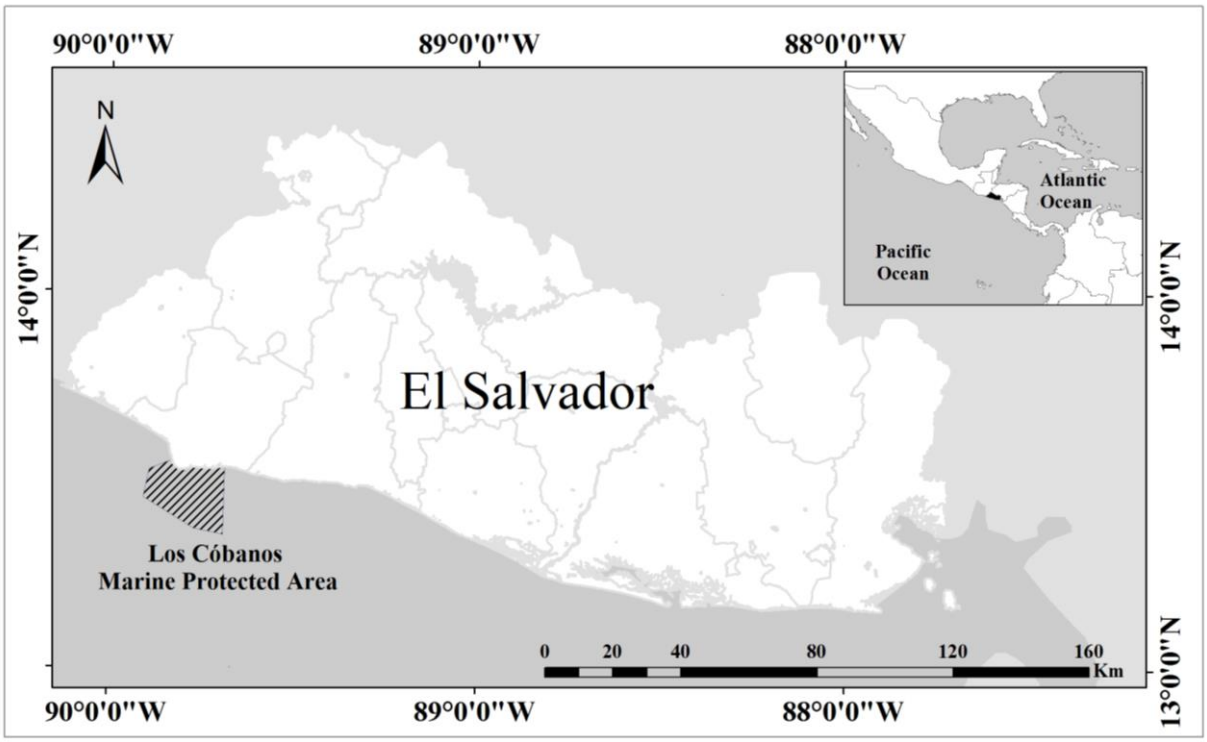

Figure 1. Location of Los Cóbanos Marine Protected Area.

Methodology

During April to May 2013, macroalgae samples were collected in the intertidal zone of Los Cóbanos. Macroalgae were collected by free diving and using plastic bags; samples were then taken to laboratory for processing.
Dinoflagellates were removed from macroalgae by manual shaking during three minutes, and then water retained in plastic bags was sieved. The 20µm retained fraction was analyzed using an inverted microscope and Sedgewick-Rafter chamber. Each macroalgae was weighted so that dinoflagellates were quantified as cells per gram of wet weight of macroalgae.

Results and Discussion

Gambierdiscus Adachi & Fukuyo 1979 and *Ostreopsis* Johs.Schmidt 1901 were found as epiphytes on macroalgae and are reported for the first time on the coast of El Salvador (Fig. 2).
Gambierdiscus species were mostly found on Rhodophytes, particularly *Hypnea sp*. and the invasive species *Acantophora spicifera.*

Along with these genera, four species of *Prorocentrum* were identified: *P. lima*, *P. micans*, *P. mexicanum*, and *P. minimum*; *P. lima* showed the highest abundance with 80 cells g^{-1} macroalgae.
Mean densities of each species per group of macroalgae are shown in Fig. 3.

Gambierdiscus and *Ostreopsis* abundance was considerably low, with maximum abundances of 4 cell g^{-1} and 9 cell g^{-1} wet macroalgae, respectively.

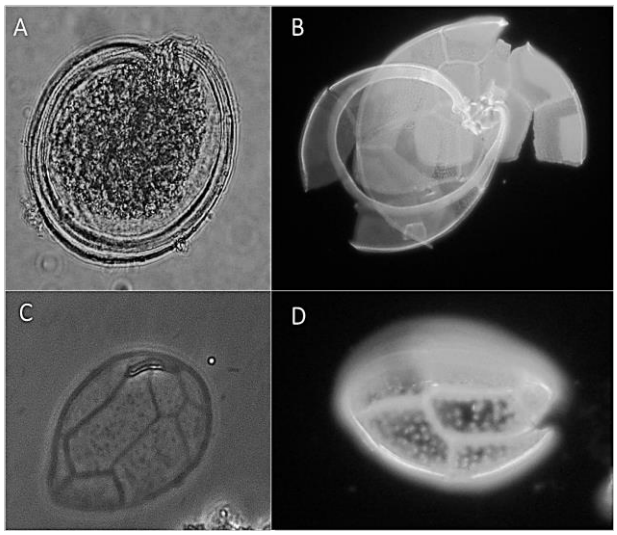

Figure 2. A and B: *Gambierdiscus* sp. C and D: *Ostreopsis* sp.

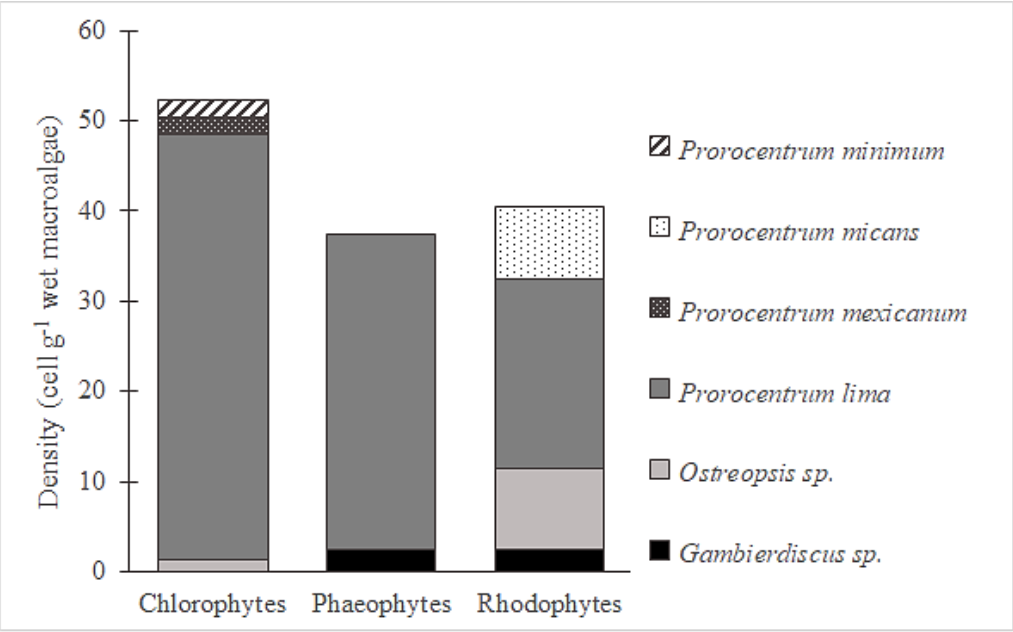

Figure 3. Mean densities of each dinoflagellate species per group of macroalgae.

The abundance found in this study are below those detected in the only abundance report from the Eastern Tropical Pacific region, in Isla de Coco in
Costa Rica (Vargas-Montero et al. 2012), where *Gambierdiscus* was found with a mean abundance of 15 cells g^{-1} wet macroalgae.
However, *Gambierdiscus* abundance found in Los Cóbanos, are within the range of population levels from the tropical Pacific region (Litaker et al. 2010), where around 20% of the average abundance estimates were 1 to 10 cell g^{-1} wet weight macroalgae.

This preliminary result suggests a low risk of CFP in Los Cóbanos, since studies have shown that CFP events are preceded by an increase in *Gambierdiscus* cell densities (Gillespie et al. 1985). Additionally, cell abundance below 1000 cell g^{-1} wet weight macroalgae are considered to represent background levels of *Gambierdiscus* that
pose little or no risk of CFP (Litaker et al. 2010); however, these assumptions need to be widely tested in the Central American Pacific where *Gambierdiscus* population studies are scarce.

Moreover, considering that prolonged elevated water temperature and environmental disturbances are optimum for increased *Gambierdiscus* abundance (Chateu-Degat et al. 2005, Tester et al. 2010), climate change scenarios of increasing temperature might entail a favorable environment for the increase of *Gambierdiscus* populations in

Central America. Thus its presence in Los Cóbanos reef should be monitored and further research is needed in order to address *Gambierdiscus* and *Ostreopsis* abundance through time.

Though *Gambierdiscus* and *Ostreopsis* species taxonomy are still being confirmed, this is the first report of the genera in El Salvador and one of the few reports from the Eastern Tropical Pacific, along with Costa Rica and México.

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Systematics and diversity of genus *Ostreopsis* in the East Australian Current region

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Abstract
Specific *Ostreopsis* species/strains produce complex toxic polyketide molecules like palytoxin (PLTX, $C_{129}H_{223}N_3O_{54}$) and its analogues, which are associated with human illnesses through consumption of contaminated seafood and direct contact through aerosolic exposure during blooms. In this study, we analyzed the diversity, distribution and transcriptomic comparisons of *Ostreopsis* species from the East Australian current region. In sites along a ~2000 km coastline, we identified 3 species, including *O.* cf. *siamensis, O.* cf. *ovata* and a recently described species, *O. rhodesae*. LC-MS/MS was used to determine the PLTX-like analogues from the cellular extracts and the transcriptomes of representative strains of the three *Ostreopsis* species were analysed using high throughput sequencing technology. The results from this study present several candidate genes that can aid in bridging the gap between biodiversity and functional genetics through multigene phylogenies in marine phytoplankton, and are an important step forward in understanding the genetic basis of PLTX production.

Keywords: Ostreopsis, palytoxin, East Australian Current, phylogenetics, RNA-Seq

Introduction

Species of the genus *Ostreopsis* Schmidt (1902) are known to occur in tropical and temperate waters binding epiphytically to macroalgae, seagrass, rocks, coral rubble and sand (Rhodes, 2011). Certain *Ostreopsis* species produce highly toxic palytoxin (PLTX, $C_{129}H_{223}N_3O_{54}$) and/or its analogues, such as Ostreocin-D, ovatoxins a-g and isobaric palytoxin (Dell'Aversano et al., 2014; Usami et al., 1995). PLTX and/or its analogues have been associated with human poisonings through the consumption of contaminated fish and shellfish and have been linked to clupeotoxism incidents (Amzil et al., 2012; Tubaro et al., 2011). *O.* cf. *ovata* blooms in the Mediterranean Basin have been associated with skin and eye irritations as well as respiratory illnesses from exposure to toxic aerosols (Ciminiello et al., 2006; Ciminiello et al., 2014). Blooms of *O.* cf. *siamensis* in New Zealand have been linked with large scale sea urchin mortalities (Shears and Ross, 2009), thereby highlighting the global ecological and human health impact caused by these species and toxins.

Distinguishing *Ostreopsis* species from one another based upon morphological differences is difficult, due to their similarity in size, shape and thecal plate patterns, and also their co-existence in the environment (Hoppenrath et al., 2014; Penna et al., 2005). Along the East Australian coast, the increase of up to 2.0 °C in ocean temperature over the past few decades has been linked to the southern range extension of the East Australian Current (EAC) with an increase in harmful algal taxa reported along the south-eastern coastline (Ajani et al., 2013; Thompson et al., 2009). Little is known about the distribution and diversity of *Ostreopsis* species along the EAC despite recurrent occurrences in shellfish producing estuaries from the sub-tropical and temperate regions of the coast. To investigate the diversity of *Ostreopsis* species in the EAC region and to elucidate the unique biology of these PLTX producing dinoflagellates, we sampled various locations along the East Australian coast to establish *Ostreopsis* cultures and investigate the diversity using ribosomal rDNA genes, toxin profiles using LC-MS/MS and transcriptomes through high-throughput sequencing technology.

Material and Methods

Macroalgal and seagrass samples (*Padina*, *Saragassum, Hormosira* and *Zostera* spp.) were collected from nine sites as listed in Table 1 during April-June 2014. Eighty clonal strains were established in f/10 media as described in Verma et al. (2016b). DNA was extracted using modified CTAB- phenol-chloroform method and 5.8S-ITS region were sequences using previously described primers (Verma et al., 2016a). Maximum Likelihood tree was produced in MEGA v6 using Tamura 3+G+I with 1,000 bootstrap replications. Bayesian analysis was performed using MrBayes v3.2.2 using general time reversible + G model as described in Verma et al. (2016b).

Table 1: Geographic location of sampling sites in Australia, temperature and salinity measurements and number of *Ostreopsis* clones isolated during this study

S.no	Location	Latitude; Longitude	Temp. (^{0}C); Salinity (psu)	No. of clones
1	Heron Island	23°27′S, 151°55′E	20; 34	14
2	Minnie Water	29°77′S; 153°29′E	NA	10
3	Bonny Hills	31°58′S; 152°82′E	NA	10
4	Wallis Lake	32°23′S; 152°48′E	19; 34	10
5	Lake Macquarie	33°09′S; 151°88′ E	17; 34	10
6	Patonga Creek	33°51′S; 151°28′ E	18; 35	8
7	Gordons Bay	33°91′S; 151°26′ E	18;34	7
8	Kiama	34°67′S; 150°85′E	18; 34	7
9	Merimbula Lake inlet	36°53′S; 149°54′E	19; 28	4

All *Ostreopsis* cultures were harvested in late stationary phase by centrifugation (50 mL; 2,300 *g*; 10 mins; room temperature) and the cell pellets were freeze dried for PLTX screening using a quantitative LC-MS/MS method at the Cawthron Institute, New Zealand as described in Selwood et al., (2012). This approach monitors sub-structures generated by the oxidative cleavage of vicinal diol groups present in the intact toxins using periodic acid. It yields an amino-aldehyde, used for quantification, common to known palytoxins, ovatoxins and ostreocins, and an amide-aldehyde that varies depending on the toxin type (Selwood et al., 2012)

Triplicate 1 L cultures of representative isolates of each species; *O*. cf. *ovata* (HER27), *O*. cf. *siamensis* (BH1) and *O*. *rhodesae* (HER26) were sampled at mid-late exponential growth phase (days 11-14) for RNA extraction. RNA extraction was performed using Tri-reagent and purified using RNeasy Mini Kit. The libraries were prepared using TruSeq RNA Sample and sequencing was performed using NextSeq500 generating 75 base pair (bp) paired end reads. Raw reads were quality filtered and assembled as described in Kohli et al. (2015).

Results and Discussion

Light microscopy analyses on the isolated *Ostreopsis* strains did not reveal any significant differences between the strains/species. Phylogenetic analysis using ITS1-5.8S-ITS2 regions reported *Ostreopsis* cf. *siamensis* (HER24), *O.* cf. *ovata* (HER27) and the pseudo-cryptic *O. rhodesae* (12 strains) from Heron Island (Fig. 1). *O. rhodesae* diverged from other previously described *Ostreopsis* clades with full nodal support and was found to be sister group to *O.* cf. *siamensis* (Fig. 1) (Sato et al., 2011; Tawong et al., 2014). . This is the first molecular characterization of the *O. ovata* ribotype from Australian waters (Heimann et al., 2009). The remaining 66 isolates (identical ribotype to CAWD203) were all identified as *O*. cf. *siamensis* thereby highlighting a large cosmopolitan spatial distribution of this species along the EAC region (Fig. 1). Previously, *O. siamensis* blooms have been reported in New Zealand and its seasonal occurrence has been linked to the transportation from tropical/sub-tropical waters via the East Australian current and the Tasman front (Murray et al., 2014; Rhodes, 2011). This may highlight a potential source population that can possibly migrate to the southern temperate regions via ocean currents during warmer seasons.

LC-MS/MS analysis of the oxidized cell extract from *O.* cf. *ovata* showed the presence of PLTX-like analogues in the extract resulting in an estimate of 1.8 pg cell^{-1}. No PLTX-like analogues were detected from cellular isolates of *O. rhodesae* strains (Verma et al., 2016a). Variable amounts of PLTX-like analogues, ranging from 0-

1.5 pg cell^{-1}, were determined from *O.* cf. *siamensis* extracts, highlighting a high amount of inter-strain variability in toxin production within this species (Verma et al., 2016b).

A total of 87.5, 87.6 and 75.8 million reads were extracted from *Ostreopsis* cf. *ovata*, *O. rhodesae* and *O.* cf. *siamensis* RNA-Seq libraries respectively. After removing redundancies, 87,382, 91,824 and 88,929 contigs were assembled of which, 68,759 (Mean length: 1002 bp), 71,151 (Mean length: 955 bp) and 67,786 (Mean length: 854 bp) contigs were greater than 300 bps amongst *O.* cf. *ovata*, *O. rhodesae* and *O.* cf. *siamensis* transcriptomes respectively.

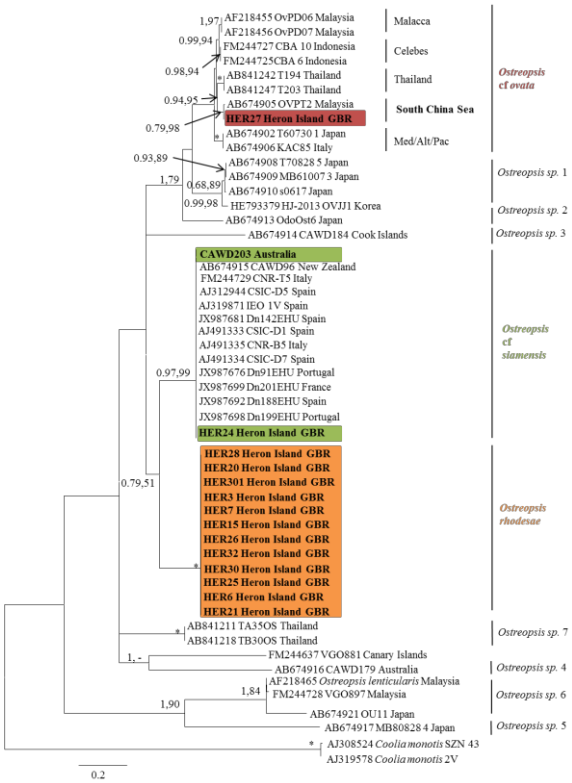

Fig. 1: Maximum Likelihood and Bayesian Inference phylogenetic tree of various *Ostreopsis* species using 5.8S-ITS primer set. Numbers at nodes represent posterior probabilities from Bayesian Inferences and bootstrap support values from Maximum Likelihood based on 1000 bootstraps.

458 highly conserved proteins were identified in the transcriptomic data using the Core Eukaryotic Genes Mapping Approach (CEGMA). The transcriptomes of *O.* cf. *ovata*, *O. rhodesae* and *O.* cf. *siamensis* contained 392, 371 and 379 of these proteins, respectively. This core of conserved annotated proteins is comparable to other dinoflagellate transcriptomes investigated to date (Kohli et al., 2017; Kohli et al., 2015; Meyer et al., 2015; Ryan et al., 2014).BLASTx analysis using BLAST2GO (e-value cut-off 10^{-3}) yielded 26.6% annotated and 25.3% non-annotated matches for *Ostreopsis* cf. *ovata*, and 25.4% annotated and 25.3% non-annotated matches for *O. rhodesae*. 25.2% and 25.1% annotated and non-annotated matches were found in *O.* cf. *siamensis* respectively. The remaining 48.1% (*O.* cf. *ovata*), 49.3% (*O. rhodesae*) and 49.5% (*O.* cf. *siamensis*) of the contigs lacked similarity to any sequence in the GenBank *nr* database. Such percentages have been observed in other protist studies, particularly amongst dinoflagellates, due to their complex and unknown genomic features (Keeling et al., 2014; Kohli et al., 2016; Murray et al., 2016). Of the transcripts with positive BLAST hits and annotations with Gene Ontology terms, similar distribution of transcripts involved in keystone molecular and metabolic functions were observed for the three *Ostreopsis* species (Fig. 2).

'Cryptic' species also appear to be common amongst dinoflagellate species and further complicate their identification when using techniques such as light and scanning microscopy. *Ostreopsis siamensis* was initially described from the Gulf of Thailand (Schmidt, 1902), however, no culture material from the type location is available to date. Since then, numerous microscopy based studies have reported conflicting descriptions of pore sizes and cingulum undulation for *Ostreopsis* species and have generally lacked genetic data (Hoppenrath et al., 2014). In order to compare between published findings and to establish accurate and more recent historical phylogenetic reconstruction of this genus, reporting sequences from different genes and the use of different molecular techniques is essential. In this study, we report three *Ostreopsis* species from the EAC region using ribosomal molecular markers and used a *de novo* transcriptomic approach to unravel several conserved genes that can be potential candidates for multigene phylogenies that can improve in deciphering the functional differences between these species and understand the genes involved in toxin biosynthesis.

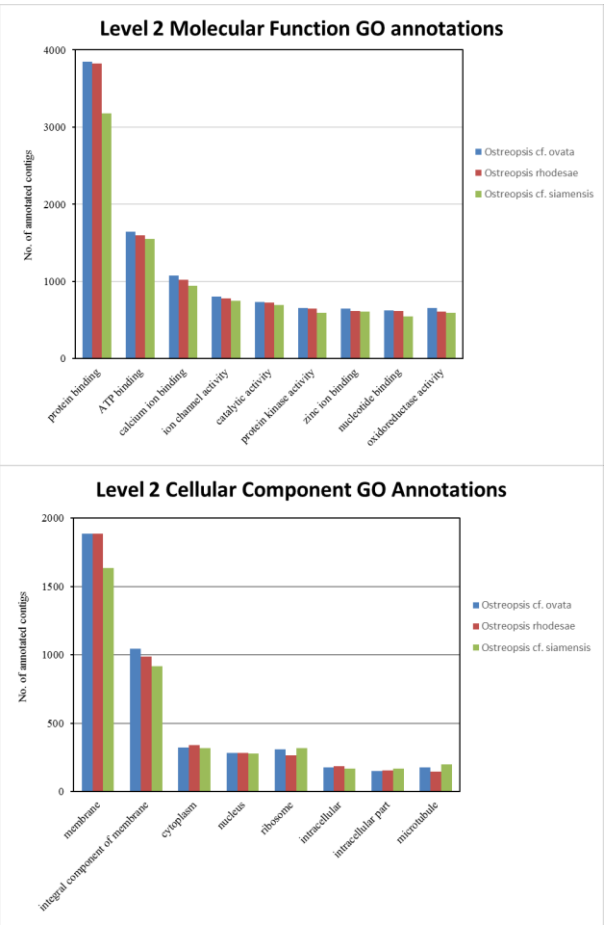

Fig. 2: Distribution of second-level cellular component and molecular function GO annotations in annotated *O.* cf. *ovata*, *O. rhodesae* and *O.* cf. *siamensis* transcripts.

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The authors would like to thank Risa Fujise, Michaela Larsson, Jennifer Clark Lewis and David Hughes for collecting the macroalgal samples and the Australian Research Council for Funding. Analyses performed at the Cawthron Institute were supported through the Safe New Zealand Seafood programme (CAWX1317).

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Notes on morphology, phylogeny and toxicity of a dominant community of toxic benthic dinoflagellates from southern-central coast of Cuba

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Abstract

In the island nations of the Caribbean, most toxin outbreaks come from benthic dinoflagellates associated with ciguatera. The composition, abundance, morphology, phylogeny and toxicity of some of the most abundant toxic benthic dinoflagellates was investigated between May 2011 and March 2012, in a coral reef lagoon from the southern-central coast of Cuba. Preliminary cultures of *Prorocentrum hoffmannianum* were established. The distinctive toxic benthic dinoflagellate assemblage on macroalgae comprised *Ostreopsis* cf. *lenticularis*, *Prorocentrum hoffmannianum* and *Gambierdiscus caribaeus*. *Ostreopsis* cf. *lenticularis* was the dominant species and always registered the highest densities, with a maximum of 5.31 x 10^5 cells g^{-1} FW during February, dry period. Results indicated the presence of palytoxin-like molecules in a benthic mat extract at concentration of 0.12 pg/cell. Preliminary samples analyses by LC-MS/MS displayed masses which matched with others described in the bibliography for CTXs toxins, predominantly CTX 3C. Okadaic acid (43.41 pg.$cell^{-1}$) but not DTXs, were determined from preliminary cultures of *Prorocentrum hoffmannianum*. The presence of abundant and diverse toxic benthic dinoflagellates constitute a potential threat for human health in the southern-central region of Cuba since its related toxins like CTXs toxins, palytoxins and okadaic acid have been associated to poisonings worldwide.

Keywords: Cuba, ciguatera, toxic benthic dinoflagellates, *Gambierdiscus caribaeus*, *Ostreopsis* cf. *lenticularis*, *Prorocentrum hoffmannianum*

Introduction

Ciguatera is the most prevalent form of phycotoxin-related seafood poisoning affecting human health in the Caribbean. The causative agent of this neurodigestive intoxication are the ciguatoxins from the dinoflagellate genus *Gambierdiscus*, being *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri* and *G. ruetzleri* the most representative species in the Caribbean region (Litaker et al. 2010, Parsons et al. 2012). However, there is the potential for other toxins such as palytoxin from the genus *Ostreopsis*, and okadaic acid and dinophysistoxins from the genus *Prorocentrum*, to contaminate seafood (Tosteson et al. 1989, Skinner et al. 2013). The objective of this study was to characterize the temporal variability of the epiphytic dinoflagellates community and provide a preliminary investigation on morphology, genetic and toxicity in a distinctive toxic benthic dinoflagellates assemblage from southern-central coast of Cuba, Caribbean region.

Material and Methods

The study area was an oligotrophic shallow-sheltered lagoon reef rich in brown and red macroalgae, located 30 km offshore from Cienfuegos Bay (21°55´ N y 80°18´ W) on the

southern-central coast of Cuba (Fig. 1). Weather in the study area is divided in two seasons: dry (November-April) and rainy (May-October) season. Sea Sampling was conducted from May 2011-March 2012, with samples taken once monthly. Two independent samples of macroalgae with surrounding seawater were collected and placed in plastic bags. These were shaken vigorously and contents passed through a 250 µm, then 150 µm and finally 20 µm. This last fraction, retaining the dinoflagellates, was suspended in a known volume of filtered seawater and fixed with Lugol's iodine solution. Cell enumeration was performed with an inverted microscope according to Utermöhl's sedimentation method, and recorded as the number of cells per gram of fresh weight of macroalgae (cells g^{-1} FW).

For observation with light microscope (LM), living and preserved cells were observed at 20×, 40× objective and 100× oil objective using an OLYMPUS BX-41. A Jeol-JSM 6360LV scanning microscope (LSM) was employed for observing the valve surface of *Gambierdiscus* species. For examination with scanning electron microscopy, the Lugol's-fixed material was rinsed with distilled water dehydrated in an ethanol series, critical point dried, and finally coated with gold. From a single cell of *Ostreopsis* and *Gambierdiscus*, primers of the D1-D2 regions of the LSUrDNA were utilized, and conditions of PCR amplifications were according to Litaker *et al*. (2003). LSUrDNA sequences were inspected and aligned using CLUSTALW multiple alignment in Bioedit. The phylogenetic relationships were determined using a General Time Reversible model (GTR) in MrBayes v3.1. For *Gambierdiscus* species, samples were screened using species-specific polymerase chain reaction (PCR).

From a natural microalgal extract, palytoxin was analysed by mouse bioassay, haemolytic assay and the presence of palytoxin was confirmed by LC-MS/MS (Riobó *et al*. 2006). Concentrations of okadaic acid and dinophysistoxins were determined by LC-MS/MS from preliminary cultures of *Prorocentrum hoffmannianum*. Qualitative analyses for ciguatoxins molecules were determined by LC-MS/MS for natural extracts of *Gambierdiscus*.

Fig. 1. Map of Cuba and location of the study area, the arrow indicates the littoral of Cienfuegos Province in the southern-central coast of the island.

Results and Discussion

The distinctive toxic benthic dinoflagellate assemblage on macroalgae comprised *O.s* cf. *lenticularis*, *P. hoffmannianum* and *G. caribaeus* (Fig. 2). Based on the morphological and phylogenetic results of *Ostreopsis* we might suggest that *O. lenticularis* was the species present (Fig. 3). For *Gambierdiscus*, the morphology and phylogenetic analysis indicated the presence of *G. caribaeus* (Litaker *et al*. 2009). *G. caribaeus* occurs in both the Pacific and Caribbean regions indicating a circumtropical distribution (Litaker et al. 2010, Parsons et al. 2012). In the Caribbean, high temperatures (~25–30 ºC) are optimal for the growth of *Gambierdiscus* species (Morton et al. 1992; Tester et al. 2010; Kibler et al. 2012). The morphological characteristics of *P. hoffmannianum* are in agreement with the description for this species (Faust 1990, Herrera-Sepúlveda et al. 2015).

Fig. 2. LSM (A) and LM (B-C) photomicrographs of the distinctive toxic benthic dinoflagellate assemblage. A. *Gambierdiscus caribaeus*. B. *Ostreopsis* cf. *lenticularis*, C. *Prorocentrum hoffmannianum*.

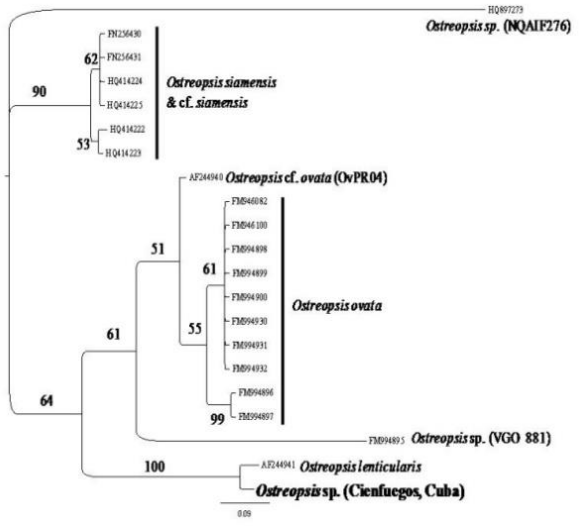

Fig. 3. LSU phylogeny (D1-D2 region) showing the relationship between the *Ostreopsis* sequence obtained from single cell isolates in Cienfuegos Province, Cuba, and other *Ostreopsis* species.

Ostreopsis cf. *lenticularis* was the dominant species and registered the highest densities during all months of the sampling period (Fig 4). Its relative abundance was always higher than 85 % and the maximum concentration was registered during the dry period (bloom of 5.31 x 10^5 cells g^{-1} FW in February). These results were similar to those reported by Ballantine *et al*. (1985) for coral reef environments in southern Puerto Rico, where *O. lenticularis* has been implicated as the major vector in *ciguatera (Tosteson et al. 1986, 1998; Mercado et al. 1995)*. The increase of toxicity in *O. lenticularis* has been associated with high temperatures (Ashton 2003). *Prorocentrum hoffmannianum* and *G. caribaeus* showed similar densities although peak abundances of *P. hoffmannianum* (max 5.53 x 10^4 cells g^{-1} FW) were higher than *G. caribaeus* densities (max 2.18 x 10^3 cells g^{-1} FW). Although a clear seasonality was not observed, the highest densities for all benthic dinoflagellates were recorded in dry season (February-March) while the lowest occurred in October, coinciding with the end of the rainy season (Fig. 4). October is temporally unstable (strong rains and storms) which could affect the benthic algal communities that serve as substrate for the toxic epiphytic dinoflagellates (Tindall and Morton 1998).

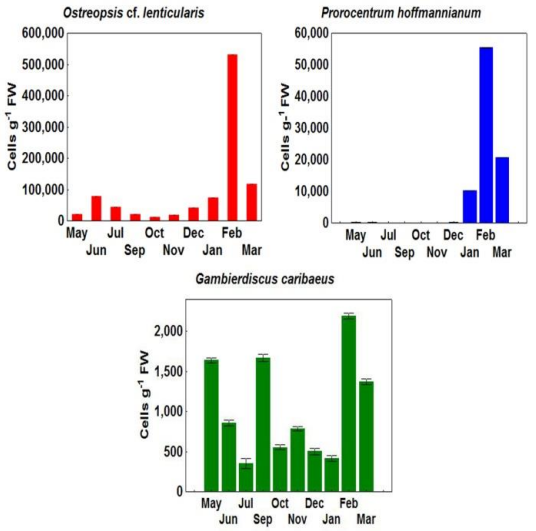

Fig. 4. Cell densities of the distinctive toxic benthic dinoflagellate assemblage during the study period, May/2011 to March/2012.

Another diverse community of potentially toxic benthic dinoflagellates were recorded in lower abundance and frequency, including *Amphidinium* spp., and several *Prorocentrum* species: *P. concavum*, *P. lima* and *P. mexicanum* (Fig. 5).

The *most important* marine phycotoxins produced by benthic dinoflagellates were detected in southern-central Cuba. Toxin analyses performed in this study showed the presence of palytoxin like compounds (0.12 pg/cell) in the natural extracts of *O.* cf. *lenticularis*. Okadaic acid (43.41 $pg.cell^{-1}$) but not DTXs, were determined from preliminary cultures of *P. hoffmannianum.* Preliminary toxins studies were performed with natural extracts of *G. caribaeus*. Samples showed hemolytic activity and also masses which match with others described in the bibliography for CTXs toxins (predominantly CTX-3C) were recorded by LC-MS analyses (Tsumuraya et al. 2006; Chinain et al. 2010).

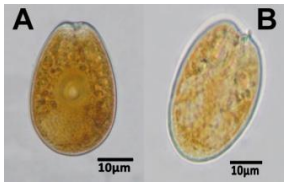

Fig. 5. Other potentially toxic benthic dinoflagellates recorded in lower abundance. A. *Prorocentrum lima*. B. *P. mexicanum*.

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Ecophysiological responses of the toxic dinoflagellate *Ostreopsis* cf. *ovata* under different water motion conditions

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Abstract

Hydrodynamic conditions affect marine microalgae. In the case of some harmful epiphytic species, field observations suggest that water motion and wave action play a selective role in determining their spatial distribution and ecology and regulating cell physiology. In order to obtain new insights on this topic, laboratory experiments were performed with Mediterranean strains of *Ostreopsis* cf. *ovata*. Monospecific cultures were exposed during 3 weeks to the turbulent motion generated by an orbital shaker at 50 rpm in order to simulate the wave movements in their natural habitat. The growth curve and toxin concentrations in the shaken cultures were compared to those maintained under still, control conditions. Shaken *O.* cf. *ovata* populations entered stationary phase earlier, reached lower cell yield and had 30% lower ovatoxin-a intracellular content compared to control ones. In the two treatments, the cell toxin content in the exponential phase was lower than in the stationary phase. These results contribute to understand the dynamics of benthic HABs and their impacts to the ecosystem and human health.

Keywords: Ostreopsis, BHAB, Mediterranean, toxicity, small-scale turbulence

Introduction

Since the late 1990s, the toxic benthic dinoflagellate *Ostreopsis* has caused recurrent blooms in temperate areas, including the Mediterranean coasts. Some of these bloom have been associated with human respiratory disorders (Fig. 1 in Ciminiello *et al.* 2014 and references therein). Interestingly, these adverse effects only occurred during certain phases of the bloom (Vila *et al.* 2016). Some of the blooms have also been associated with massive macrofauna mortalities (e.g. Shears and Ross 2009). These negative impacts on the marine habitat and human health have motivated research on *Ostreopsis* bloom dynamics. Field observations suggest that water motion and wave action, among other environmental factors, could play a selective role on the spatial distribution and ecology of this harmful epiphytic species although no clear relatrionship has been defined so far. For instance in the Mediterranean, *O.* cf. *ovata* was reported from shaken and slightly shaken habitats by Vila *et al.* (2001) while it was found in sheltered areas by Accoroni *et al.* (2012). These apparent contradictory observations arise in part from the lack of quantification of water turbulence in the field, and from the difficulty to discriminate the specific role of this factor from its interaction with other drivers (e.g. depth, light intensity, macroalgal substrates). However, small-scale turbulence has been described to exert negative species-specific effects in some planktonic dinoflagellates (e.g. Berdalet et al., 2011 and references there in). In this study, we present the preliminary results of our investigation of physiological responses of an *Ostreopsis* cf. *ovata* strain exposed to still and turbulent conditions.

Material and Methods

Ostreopsis cf. *ovata* strain "Ostreo BCN1_2014" was isolated from Llavaneres beach, a hot spot where the species bloomed annually since, at least, 2004 but probably since 1998. Aliquots of an exponential monospecific culture grown in f/2 medium were used as inoculum for the 12 experimental (250 ml sterile plastic flasks) cultures with 200 cells·ml^{-1} as initial concentration. The 12 flasks were incubated at

23ºC under a 12-12 hours light-dark cycle, and an irradiance of 150 µmol photon·m^{-2}·s^{-1}. After 24h, the initial cell concentration in each flask was determined on 1-ml Sedgewick Rafter chamber (in duplicate). Six experimental flasks, so called "Control", were maintained under still conditions. The other six flasks, referred to as "Turbulence" were continuously agitated on an orbital shaker at 50 rpm with a 0 – 10º angle inclination variability range in order to simulate the wave movement in their natural habitat. Shaking started on day 1, i.e. 24 hours following inoculation. The experiment lasted for three weeks.

Population growth was characterized by sampling each flask every 2 days. Cell counts on Lugol fixed samples were performed in duplicate as described above. Growth rate was calculated following Guillard (1973) where the growth rate is the slope of the Ln of the cell counts over time during the exponential phase.

At the beginning of the stationary phase (day 11), when the differences between the growth curves in Control and Turbulence treatments were evident, cell size analyses were conducted on around 100 randomly chosen cells from each treatment. Four cell measures were done: dorsoventral diameter including the theca (DVt), dorso-ventral diameter of the inner cytoplasm (DVc), trans-diameter including the theca (Wt) and trans-diameter of the inner cell (Wc). Cell size parameters at each treatment were compared by one-way analyses of variance (one-way ANOVA; STATISTICA). The shape of each measured cell was noted and several microphotographs were also taken a using Leica-Leitz DMIRB inverted microscope (Leica Microsystems, Wetzlar, Germany) and ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik Systeme).

Samples for toxin determination were collected twice, during the exponential phase (day 7) and at the end of the experiment (day 23). Each sampling day, the total remaining content of three Control and Turbulence flasks (from 100 to 230 ml depending on the day) were filtered through GF/F fiber filter (Whatman) and kept frozen at -80ºC until analysis. Thus, after day 7, the number of flask replicates of each treatment was reduced from 6 to 3. Filters were extracted with 100% methanol and palytoxin and ovatoxins were determined by UHPLC-HRMS using a Hypersil Gold C18 column (100 x 2.1 mm, 1.9 µm, Thermofisher Scientific) and a mobile phase gradient elution of acetonitrile : water (0.1% formic acid) for the chromatographic separation and coupled to a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermofisher Scientific) with electrospray as ionization source in positive ion mode.

In summary, two treatments were done, six 250-ml plastic culture flasks containing *O.* cf. *ovata* were maintained under still conditions, used as Control and six 250-ml culture flasks were permanently shaken after day 1 (Turbulence). Parameters measured were cell number, cell size and shape, toxin content and growth rate. The effect of turbulence on these parameters is discussed.

Results and Discussion

Ostreopsis cf. *ovata* showed the typical sigmoid growth curve and grew similarly under Control (still) and Turbulence (shaken) conditions (Fig. 1). However, the final cell numbers reached in the Controls (5300 cells·ml^{-1}) almost doubled the final yield reached by the Turbulence experiments (3000 cells·ml^{-1}). Furthermore, whereas *O.* cf. *ovata* growth rate was similar in both treatments (0.32 d^{-1} and 0.39 d^{-1} in Control and Turbulence, respectively), the exponential phase lasted for 11 days in the still flasks and only 5 days in the shaken ones. This result suggests some kind of disturbance on their reproduction or life history processes as has been observed previously in dinoflagellates (e.g. Berdalet *et al.* (2011) and references there in). In the natural environment, indeed, notably high *Ostreopsis* cell concentrations have been recorded during long lasting calm sea conditions (e.g. Giussani 2016, Accoroni & Totti 2016 and references there in).

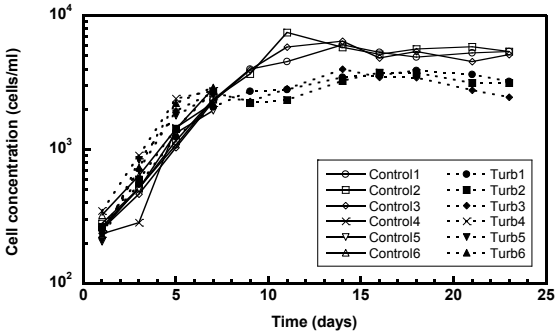

Fig. 1. Growth curves of *O.* cf. *ovata* in the Control (still) and Turbulence (shaken) treatments. Note that, for each treatment, there were 6 replicates until day 7, and 3 replicates until the end of the experiment. Each data point

corresponds to the average of two replicate cell counts per flask.

The mean cell size measured in the Turbulence treatment (see Table 1) was statisticaly significantly larger than cells measured in the Control ($p<0.01$) considering the four measured parameters.

Table 1. Cell sizes of Control and Turbulence treatments considering all the cells together (without taking account morphotypes).

	Control				Turbulence			
	n	Mean	Min-Max	SD	n	Mean	Min-Max	SD
DVt	97	32,7	22,1-51,0	5,3	101	34,0	20,2-49,9	5,0
DVc	101	26,4	14,7-45,1	5,5	103	28,2	17,6-41,7	4,4
Wt	97	24,4	16,0-43,0	6,1	101	24,9	13,6-41,8	4,5
Wc	101	20,4	11,8-39,1	6,0	103	20,9	10,7-37,5	4,3

Such increase in cell size under turbulence conditions has been described in other experiments and is related to interference of turbulence with cell division. However, as has already been reported by several authors, cultures of *Ostreopsis* show a large variability in shape and size (e.g. Accoroni *et al.* 2014). Bravo *et al.* (2012) classified cultured cells into three size-categories (25–35 µm, 35–50 µm and >50 µm in DV diameter). In this experiment, five morphotypes were identified at the beginning of the stationary phase based on their shape (drop-shaped to round-shaped cells), content (clear or dark cytoplasm), life history stage (vegetative or pellicle cysts), and cell size range (from 20 to 51 µm). The five morphotypes were designated as DropClear (DC), Dark (D), Round (R), Without theca (WT) and others (O). The three dominant morphotypes are illustrated Fig 2.

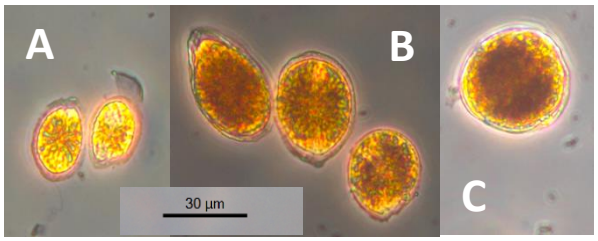

Fig. 2. Microphotographs of the three dominant morphotypes at the beginning of the stationary phase (day 11). A) Drop-shaped clear cells (”DC”), B) Dark cells (”D”) and C) Round cells (”R”).

DC comprised small cells (Table 2) that clearly dominated both treatments (61% in Control and 63% in Turbulence), followed by large dark cells (D) (16% in Control vs. 22% in Turbulence conditions); and finally, by rounded cells (R) (13% vs. 11%). The two last categories (WT and O) represented less than 10% of cell counts.

Table 2. Mean cell sizes (DVt, Wt) of the three dominant morphotypes in Control and Turbulence treatments. Standard deviation is indicated.

		Control	Turbulence
DropClear (DC)	**DVt**	31,0 ± 2,8	33,6 ± 3,7
	Wt	21,6 ± 3,2	23,0 ± 3,3
	n	62	65
Dark (D)	**DVt**	41,5 ± 5,7	38,2 ± 5,0
	Wt	34,4 ± 5,9	30,2 ± 4,0
	n	16	23
Round (R)	**DVt**	30,9 ± 4,3	27,3 ± 3,5
	Wt	26,8 ± 3,9	23,8 ± 2,6
	n	13	11

In this study we observed that whereas the most abundant DC cells were larger in the Turbulence treatments than in the Control ones (for the 4 parameters measured), D and R cells were larger in the Control than in the Turbulence flasks (Fig. 3). More studies are required to understand the specific role that each *Ostreopsis* morphotype plays in the life history of this organism.

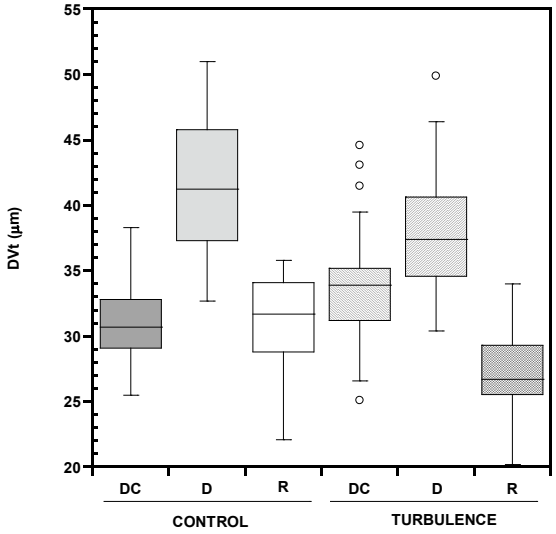

Fig. 3. Median (horitzontal line), 25 and 75 quartiles (box), minimum and maximum (whiskers) and outlier (point) of the dorsoventral diameter including the theca (DVt) measured for

the three dominant morphotypes (see Fig. 2) in Control and Turbulence treatments.

Regarding toxins, OVTXa was the dominant one with small amounts of palytoxin analogues such as OVTXb-g and putative palytoxin (not shown). *O.* cf. *ovata* toxin production was four times higher in the stationary phase than in the exponential one (Fig. 4); shaken cells had 30% lower toxin content (23 pg OVTXa·cell^{-1}) than the still cultures (32 pg OVTXa·cell^{-1}). In addition, intracellular toxin concentration was also lower in the Turbulence than in the Control flasks. Such trends have also been observed in *Alexandrium minutum* and *A. catenella* (Bolli *et al*. 2007) exposed to laboratory generated turbulence. These results reinforce the possible link of toxin production with reproduction processes.

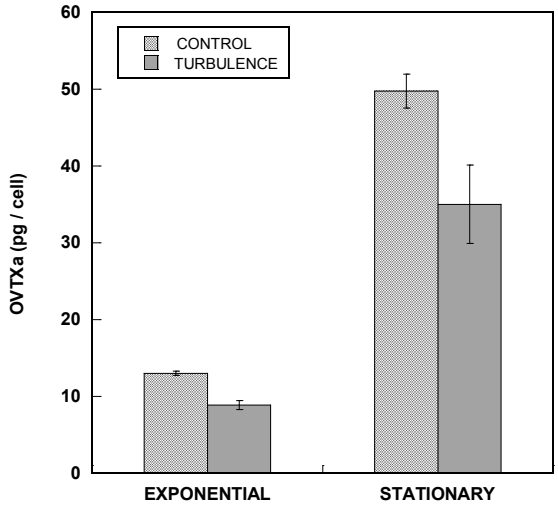

Fig. 4. Toxin content as OVTXa in Control and Turbulence conditions during the exponential and the stationary phase.

Respiratory outbreaks caused by suspected toxic aerosols seem to occur under low wind episodes (below 4 m·s^{-1}, Vila *et al*. 2016). In this study, the unshaken control treatments resulted in higher cell densities than the corresponding turbulence treatment. Control stationary phase cells also had higher toxin content per cell. Though it is not possible to extrapolate the results of this laboratory study to the natural events with certainty, the data do indicate that calm conditions may have allowed higher densities of more toxic stationary phase cells to accumulate. Release of these toxins from this higher biomass population, either by excretion or by lysis of senescent cells, accompanied by onshore wind direction, may account for observed intermitant respiratory illness. More work is needed, but results of this study do provides a new piece to the puzzle with respect to understand the *Ostreopsis* bloom and its negative effects on human health.

Acknowledgements

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Influence of environmental factors on the bloom dynamics of the benthic dinoflagellate *Ostreopsis* cf. *ovata* in the Mediterranean Sea

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Abstract

During the last few decades, recurrent and intense blooms of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* have been frequently reported during summer along several sections of the Mediterranean coast. In these areas, blooms have been associated with both noxious effects on human health and mortality of marine organisms, due to the production of palytoxin-like compounds. *Ostreopsis* grows on several types of benthic substrata (macrophytes, rocks, invertebrates, sands) forming a brownish, mucilaginous mat that can be easily resuspended in the water column. Blooms typically develop in sheltered, shallow coastal areas characterized by rocky bottom habitats. The role of environmental factors on the *Ostreopsis* bloom dynamics has been studied in the northern Adriatic Sea since 2006. Each year, maximum abundances are typically recorded in late summer-autumn with 10^6 cells g^{-1} fresh weight macroalgal thalli. *Ostreopsis* abundances show a significant decrease with depth, most likely related to light intensity. Substrate type and availability are also thought to influence *Ostreopsis* blooms; living substrata often host lower abundances of epibionts than other substrates, suggesting colonization is possibly limited by allelopathic interactions. The synergic effects of hydrodynamics, temperature nutrient availability, particularly both inorganic and organic phosphorus, are main factors triggering the bloom.

Keywords: palytoxin; harmful algae; Mediterranean Sea; hydrodynamics, temperature; nutrients

Introduction

Ostreopsis Schmidt (Schmidt, 1901) is a genus of benthic marine dinoflagellates widespread from tropical to temperate waters, and includes species that are able to produce toxins mostly belonging to the palytoxin group (Parsons *et al*., 2012). In the last few decades, large-scale changes in the summer microphytobenthos assemblages of Mediterranean coastal areas have occurred due to substantial increases in the number and intensity of blooms of *Ostreopsis* species (Accoroni & Totti, 2016). To date, three *Ostreopsis* species have been reported from the Mediterranean Sea: *O.* cf. *ovata*, *O.* cf. *siamensis* and *O. fattorussoi*, (Penna *et al*., 2012; Accoroni *et al*., 2016). *O.* cf. *ovata* produces a large array of palytoxin analogues, i.e. isobaric palytoxin (isobPLTX) and ovatoxins (OVTXs) namely OVTX–a to –h (Brissard *et al*., 2015; García-Altares *et al*., 2015). This toxic dinoflagellate has been often associated with mass mortalities of various marine organisms and a suite of human illnesses (e.g. fever, cough, dyspnoea, sore throat, rhinorrhoea, skin irritation, etc.) attributed to inhalation or cutaneous contact with cells or toxic aerosols (Migliaccio *et al*., 2016; Vila *et al*., 2016).

Despite the number of studies concerning the influence of environmental parameters on *Ostreopsis* blooms, the complexity of bloom development is far from being understood. In this study, we analyzed data from blooms in the Conero Riviera, northern Adriatic Sea, from 2006 to 2015, in order to compare the environmental factors associated with bloom onset, maintenance and decline. These observations show how environmental factors, including hydrodynamics, temperature and nutrient availability, and their possible synergistic effects, may affect bloom dynamics and account for the inter-annual variability of *O.* cf. *ovata* blooms in this region.

Materials and Methods

Sampling was carried out from spring to fall of 2006-2015 at the station of Passetto in the Conero

Riviera (Ancona, NW Adriatic Sea). This station is a shallow and sheltered site due to the presence of a natural reef and is characterized by a rocky benthic surface, and it is moderately affected by human impact during the summer season, mainly due to small holiday holdings that have been excavated into the cliff face, that often discharge wastewater directly into the sea water. Surface temperature (CTD) and wave height (Douglas scale) were recorded. Water samples for nutrient analysis were collected (in triplicate) at each site, filtered through GF/F Whatman and stored in dark polyethylene bottles (4 mL) at -22°C. Surface seawater was collected to analyze the abundance of dinoflagellates in the water column. Samples were preserved by adding 0.8% neutralized formaldehyde.

Several seaweed species and pebbles were sampled for the analysis of benthic dinoflagellates (in triplicate). All the benthic substrata were collected in order to avoid the loss of epiphytic cells and treated to obtain by using the protocol developed under the ENPI-CBCMED project M3-HABs (http://m3-habs.net).

Ostreopsis cells were identified and counted by inverted microscopy, according to the Utermöhl's method. The final data were expressed as cells g^{-1} fresh weight /dry weight (macroalgae), cells cm^{-2} (macroalgae, rocks) and cells L^{-1} (planktonic cells).

The analysis of dissolved inorganic nitrogen (DIN), inorganic phosphorus, i.e., Filtrable Reactive Phosphorus (FRP) and silicates was carried out following Strickland & Parsons (1972). Measures of total N and P were made on filtered (FTN and FTP) and unfiltered (TN and TP) samples following persulphate digestion (Langner & Hendrix, 1982). Dissolved organic N and P fractions (FON and FOP) were operationally determined as the difference between filterable total fractions and the inorganic fractions.

The analogue substrates *para*-nitrophenyl phosphate (*p*NPP) and bis-*para*-nitrophenyl phosphate (bis-*p*NPP) were used as substrates for the assays of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities, respectively. The procedure used largely followed that of Turner *et al.* (2001).

Results and Discussion

O. cf. *ovata* blooms occurring along the Conero Riviera seem to be among the most intense in the entire Mediterranean basin, with maximum abundances reaching 10^4 cells cm^{-2} (10^6 cells g^{-1} fw, 10^7 cells g^{-1} dw) in late summer (Fig. 1C).

Several studies considered hydrodynamic conditions as the main factor affecting *Ostreopsis* bloom trends (Mabrouk *et al*., 2012; Totti *et al*., 2010). Observations in the northern Adriatic Sea showed that there are significantly higher abundances in sheltered compared to exposed sites. It was shown that sites exposed to storm events can experience dramatic decreases in *Ostreopsis* cell abundances, with bloom density re-establishment only after an extended period of calm sea conditions.

Many authors suggested that *Ostreopsis* spp. need relatively high temperatures to proliferate, proposing that the global warming might have influenced *Ostreopsis* expansion in temperate areas such as the Mediterranean Sea (Hallegraeff, 2010; Granéli *et al*., 2011). But a more careful analysis of temperature data shows that the effect is not global (Accoroni & Totti, 2016). *Ostreopsis* blooms are generally summer events in temperate areas, but across several Mediterranean areas peaks in cell abundances have occurred from spring to autumn, with significant inter-annual variability (Mangialajo *et al*., 2011). Laboratory studies have shown that *O*. cf. *ovata* strains from different Italian coasts (in the Tyrrhenian and Adriatic Seas) displayed different growth temperature optima that coincided with the *in situ* temperature values typical of the blooming period of each strain (Guerrini *et al*., 2010; Pezzolesi *et al*., 2012).

It has been repeatedly shown that the highest abundances of *Ostreopsis* do not occur with the highest water temperature in all areas. The data indicate that a temperature threshold seems to be important to trigger a bloom; for example, the Conero Riviera bloom consistently peaked in late summer (between 18.8 and 24 °C), but the bloom onset was always observed at the maximum temperatures (25 to 28.6 °C). It was hypothesised that *Ostreopsis* may need to reach a high temperature threshold to initiate cyst germination that generally occurs at around 25 °C (Accoroni *et al*., 2014).

However, in the northern Adriatic Sea the bloom onset is often observed about 30 days after the reaching of the 25 °C-temperature threshold, suggesting that other environmental factors, besides temperature, may affect the development of *O*. cf. *ovata* blooms. In fact, our studies showed that *O*. cf. *ovata* blooms appear to be triggered by

a combination of optimal temperature and nutrient type and availability: the temperature threshold plays a key role on the germination of *O*. cf. *ovata* cysts and optimal nutrient conditions are necessary to allow cell proliferation.

Several studies have provided increasing evidence of a link between the nutrient enrichment of coastal waters (anthropogenic eutrophication) and harmful algal events (e.g. Glibert *et al.*, 2010). Although no clear relationship was found between nutrient concentrations and *O.* cf. *ovata* abundances in the Conero Riviera (a strongly P-limited area), it was observed that in the bloom onset period, inorganic N:P ratios were significantly lower than in the rest of the study period. In contrast, bloom development is observed when inorganic P decreases, suggesting that other adaptive mechanisms (e.g. ability to utilize organic P), would enable the maintenance of blooms (Accoroni *et al*., 2015a).

Ostreopsis displays high rates of both PMEase and PDEase in Conero Riviera (Fig. 1D), indicating a potential to rapidly use a wide range of organic P sources. Phosphatase activities were located both extracellularly (cell surface and within the EPS) and intracellularly (ventral cytoplasm). Rates of activity rapidly increased at the onset of bloom proliferation. This increase in activity coincided with a nutrient profile characterized by low FRP and high relative FOP concentrations. Our results highlighted that in Pi-poor environments, having hydrolytic enzymes within and closely associated with cells, would allow *Ostreopsis* to benefit from the prevailing nutrient conditions and to quickly take advantage of any high concentration nutrient pulses that likely occur.

Contradictory results are reported in the literature regarding salinity effects on the *Ostreopsis* bloom development. Salinity measured in the north-western Mediterranean Sea during the *Ostreopsis* spp. blooms showed values around 37–38 (Vila *et al*., 2001; Mangialajo *et al*., 2008). In the Conero Riviera, during the bloom periods of *Ostreopsis* (2006 to 2015), the salinity range was much wider (31.3 to 39.3). However no significant correlation was determined between cell abundances and salinity values (Accoroni *et al*., 2015a)

The role of water depth on *Ostreopsis* abundances along the Conero Riviera was investigated in 2007 (Totti *et al*., 2010) in target sites where samples were collected at various depths between 0.5 and 10 m. *O.* cf. *ovata* abundances decreased significantly with depth, suggesting a potential growth limiting effect of light intensity. This may explain why *Ostreopsis* blooms mainly develop in shallow waters.

Ostreopsis has often been indicated to be preferentially epiphytic on macroalgae (Vila *et al*., 2001), although it has been recorded on a variety of other substrata, including marine angiosperms, rocks, coral rubble, soft sediments and invertebrates (Accoroni & Totti, 2016). That *Ostreopsis* can be epiphytic, epilithic, or epizoic, indicates that it is not an obligate epiphyte. We showed that *O.* cf. *ovata* had significantly higher abundances on pebbles rather than macroalgae (e.g. Totti *et al*., 2010). That living substrata host lower concentrations of epibionts than any other substrates, it was hypothesized that allelopathic processes may be involved.

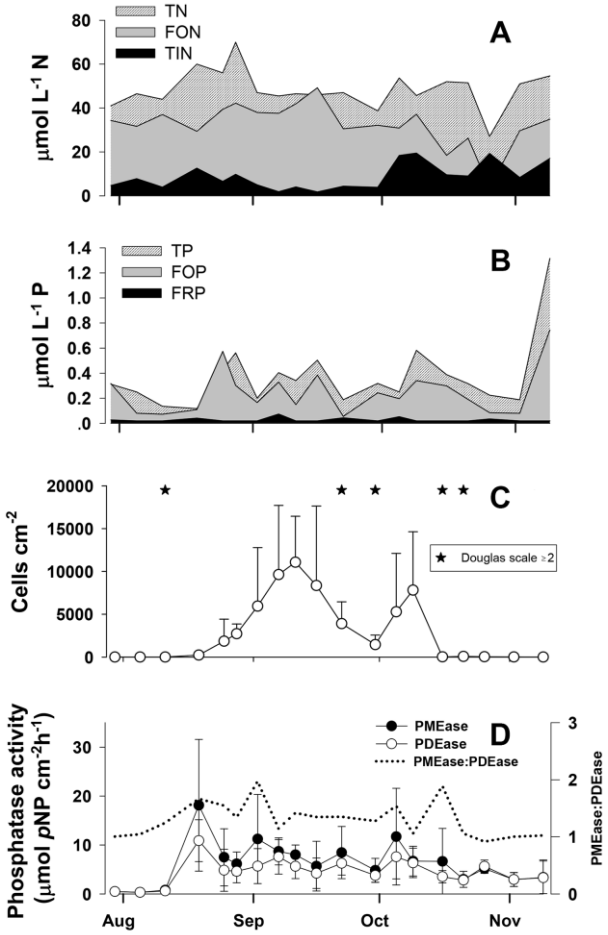

Fig. 1. Changes in nutrient concentrations (µmol L^{-1}) (A and B), *Ostreopsis* cf. *ovata* abundance (cells cm^{-2}) (C) and phosphatase activity (µmol pNP cm^{-2} h^{-1}) (D) in Passetto station, Conero Riviera (N Adriatic Sea) from 30 July to 9 November 2015. (A) Nitrogen fractions: Total Nitrogen (TN), Filtered Organic Nitrogen (FON) and Total Inorganic Nitrogen (TIN). (B)

Phosphorus fractions: Total Phosphorus (TP), Filtered Organic Phosphorus (FOP) and Filtrable Reactive Phosphorus (FRP). (C) *O.* cf. *ovata* abundances on macroalgae. * denotes dates when sea conditions could potential disturb the microepiphytic com-munity (Douglas scale ≥ 2). (D) Values of phosphomonoesterase (PMEase), phosphordiesterase (PDEase) activity and the ratios of PMEase:PDEase.

A study was also conducted to assess any possible allelopathic interactions between *O.* cf. *ovata* and macroalgae. It was shown that all the investigated seaweeds (*Dictyota dichotoma*, *Rhodymenia pseudopalmata* and *Ulva rigida*) had negative effects toward the benthic dinoflagellate, with the highest inhibitory effect observed from *D. dichotoma* and the lowest in *R. pseudopalmata* (Accoroni *et al*., 2015b).

In conclusion, the research to date has elucidated many of the environmental aspects involved in *Ostreopsis* bloom formation. However, we are still some way from fully understanding the causal factors driving *Ostreopsis* through its full bloom cycle. In particular, while factors causing bloom onset are relatively well understood, those driving bloom development and decline are not. Understanding bloom development and decline will require more in depth studies examining both biotic and abiotic factors. The only biotic studies done to date examined the effects of bacteria (Vanucci *et al*., 2012) and diatoms (Pichierri *et al*., 2016) on bloom dynamics. The potentially important role of viruses, bacteria and parasites in cyst formation and bloom termination (e.g. Garcés *et al.*, 2013; Mizumoto *et al*., 2008) have yet to be investigated and could prove to be fundamental in *Ostreopsis* bloom dynamics.

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Distribution of cyanobacteria blooms in the Baltic Sea

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Abstract

Blooms of diazotrophic cyanobacteria in the Baltic Sea are common mainly in summer months. The dominant genera are *Aphanizomenon*, *Dolichospermum* (syn. *Anabaena*) and *Nodularia* of which the latter produces the hepatotoxin nodularin. Surface accumulations of cyanobacteria are observed offshore, along coasts and in archipelagos. The blooms are of concern to society in several ways, e.g. they affect tourism, ecosystem services and provide input of atmospheric nitrogen that may increase primary production leading to hypoxia in deep water. In this study, cyanobacteria distribution was examined by water sampling and microscope analysis and by automated measurements of fluorescence of the pigment phycocyanin using a Ferrybox-system as instrument platform. The *in situ* data were combined with ocean colour data from satellite remote sensing (Aqua-MODIS) to describe bloom distribution and spatial and temporal variability. Satellite observations showed a large inter-annual variability in the distribution of near surface cyanobacteria accumulations compared to other data. The *in situ* observations showed a different pattern in cyanobacterial bloom distribution with regular blooms most years. Dissimilar cloud cover in different years may bias the satellite-based results.

Keywords: Cyanobacteria, nitrogen fixation, Baltic Sea, *Nodularia*, Ferrybox, remote sensing

Introduction

The Baltic Sea is a brackish water area in northern Europe and may be considered as a large sill fjord. The area is strongly influenced by riverine input. In the northernmost part, the Bothnian Bay, the surface salinity is approximately 3 and in the middle part, the Bothnian Sea, it is around 5. Further south, in the Baltic Proper, the surface salinity varies between approximately 6 and 10.
Water exchange with the North Sea – Skagerrak - Kattegat is through the Sound (Öresund) and the Belt Sea between Sweden and Denmark. Replenishment of the deep water occurs intermittently, often with several years in between. The Baltic Sea is considered to be eutrophic. Algal blooms are recurrent phenomena and a large part of the sea floor has low oxygen or anoxic conditions. Cyanobacteria blooms were observed already in the mid 19th century. During the second part of the 20th century, the frequency of cyanobacteria blooms may have increased. Surface accumulations consisting mainly of the diazotrophic (nitrogen fixing) genera *Aphanizomenon*, *Dolichospermum* (syn. *Anabaena*) and *Nodularia,* are a nuisance to tourism and also a health problem. *Nodularia spumigena* Mertens ex Bornet & Flahault produces the hepatoxin nodularin. Mortalities of dogs are regularly reported and small children should avoid surface scums. Picoplanktonic *Synechococcus*-like cyanobacteria are numerically dominant in summer in the Baltic Sea and are sometimes dominant in biomass. Dinoflagellates, diatoms and large cyanobacteria most often dominate the biomass. During summer nitrate and ammonium is often depleted in near surface water and the diazotrophic species have a competitive advantage compared to other phytoplankton. Nitrogen fixation adds nitrogen to the system and may increase eutrophication.
The aim of this article is to address the distribution of cyanobacteria blooms using a multi method approach. Satellite remote sensing, i.e. ocean colour measurements, is used to give a large horizontal coverage of surface accumulations detected, and an automated observation system on a merchant vessel, i.e. a Ferrybox system, provides data on the fluorescence of chlorophyll and phycocyanin, as proxies for total phytoplankton biomass and the biomass of certain cyanobacteria. Water sampling from research vessels and subsequent microscope

analysis of phytoplankton gives detailed taxonomic information and biomass based on cell volume measurements. By combining the different data sources a broad view of the cyanobacteria bloom distribution can be constructed.

Material and Methods

Water sampling and microscopy
Water samples were collected monthly between 1999-2015 as part of the Swedish National Marine Monitoring programme by SMHI and the University of Umeå. A tube was used to collect water from 0-10 m depth. Samples were preserved using Lugol's iodine and the whole phytoplankton community except for autotrophic picoplankton was analysed using the Utermöhl method. Cells were measured and cell volumes were estimated according to Olenina et al. (2006). Data was downloaded from the Swedish National Oceanographic data Centre at SMHI and processed using the Plankton Toolbox software (Karslon et al. 2014). Data were plotted using the R software and the ggplot2 library for box and whisker plots, details are available at http://www.r-project.org and http://ggplot2.org/.

Ferrybox – phycocyanin flurescence
The merchant vessel TransPaper was used as an instrument platform for a Ferrybox system described by Karlson et al. (2016). The fluorescence of phycocyanin was used as a proxy of cyanobacteria biomass (Seppälä et al. 2007). The ship covers a large part of the Baltic Sea twice a week (Fig. 1).

Satellite remote sensing
Ocean colour data was used to detect surface accumulations of cyanobacteria during cloud free condtions in the months June, July and August during the period 2002-2015. Data from the MODIS sensor on satellites Aqua and Terra were downloaded from NASA and processed by SMHI according to Öberg and Karlson (2014) using a method originally described by Kahru (2007). Areas shallower than 10 m were excluded in the analysis due to problems with artefacts related to the sea floor. In the Gulf of Finland and the Gulf of Riga, the data from areas shallower than 30 m were excluded due to high sediment content.

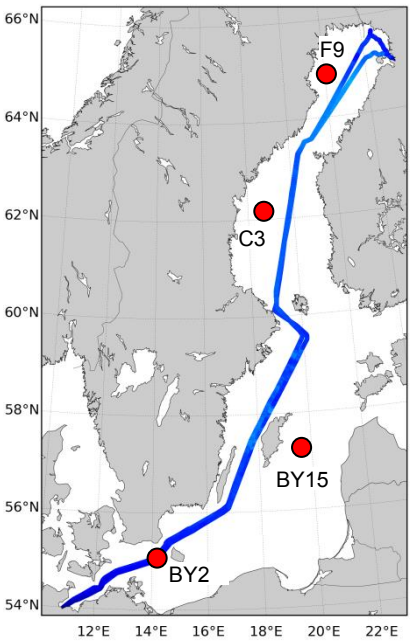

Fig. 1. Red symbols represent sampling locations for phytoplankton data presented in Figs. 4 and 5. The blue line indicate the route of ship TransPaper in 2015. Data from latitude 54-64° N are presented in Fig. 3.

Results and Discussion

The satellite remote sensing data are summarized in Fig. 2. The number of days with observed surface accumulations in summers (Jun. - Aug.) for years 2002-2015 is illustrated. The highest number of surface accumulations were observed in the Baltic proper, only a few observations were made in the Bothnian Sea and none in the Bothnian Bay. Blooms in the south-western Baltic Proper were in general few except for year 2006. An example of Ferrybox data on phycocyanin fluorescence is presented in Fig. 3. When comparing results between phycocyanin fluorescence and satellite observations of surface accumulations of cyanobacteria from year to year (data not shown) there was no consistent pattern found. This may be due to that the water intake of the Ferrybox system is at 3 m depth while satellites mainly detect near surface accumulations of cyanobacteria. During windy conditions cyanobacteria are mixed down in the water column and do not form surface scums. During cloudy conditions detection of ocean colour from satellites is not possible. Thus satellite observations may underestimate the frequency of observed cyanobacteria blooms.

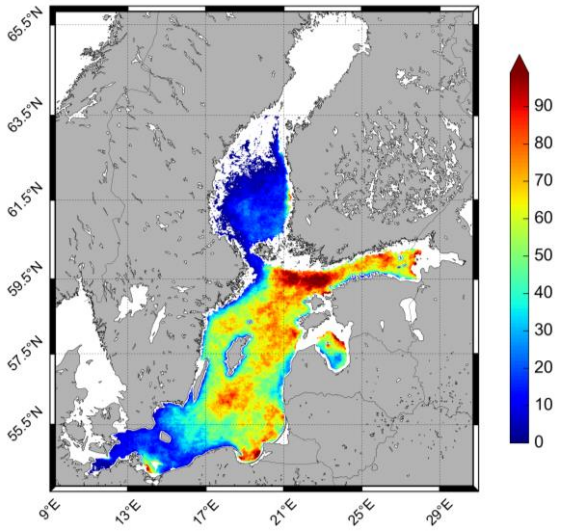

Fig. 2. The number of days of detected surface accumulations of cyanobacteria in June-August using satellite remote sensing in years 2002-2015, NASA Aqua-MODIS processed by SMHI.

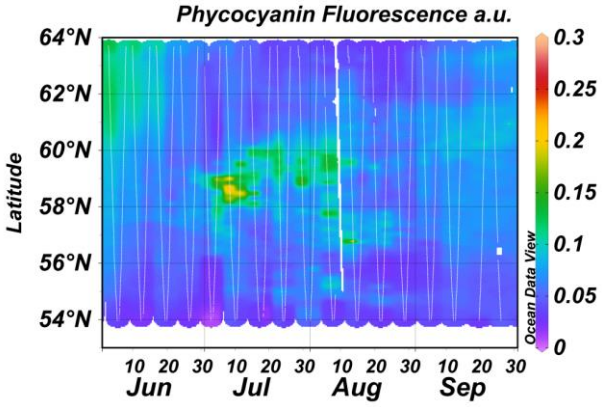

Fig. 3. Phycocyanin fluorescence detected using sensor on Ferrybox-system. See Fig 1. for route of ship.

Data on the distribution of selected cyanobacteria from water sampling and subsequent microscope analysis are summarised in Fig. 4 and 5. The genus *Nodularia* was mainly observed in June-August in the Baltic Proper and only occassionally in the Bothnian Sea. It was almost absent in the Bothnian Bay. *Aphanizomen* sp. was common both in the Baltic Proper and in the Bothnian Sea and absent in the Bothnian Bay. Salinity is likely to be a structuring factor for the distibution of cyanobacteria in the Baltic Sea. Experiments using cultures of *Nodularia spumigena* and *Aphanizomenon* sp. support this (Rakko and Seppälä 2014).

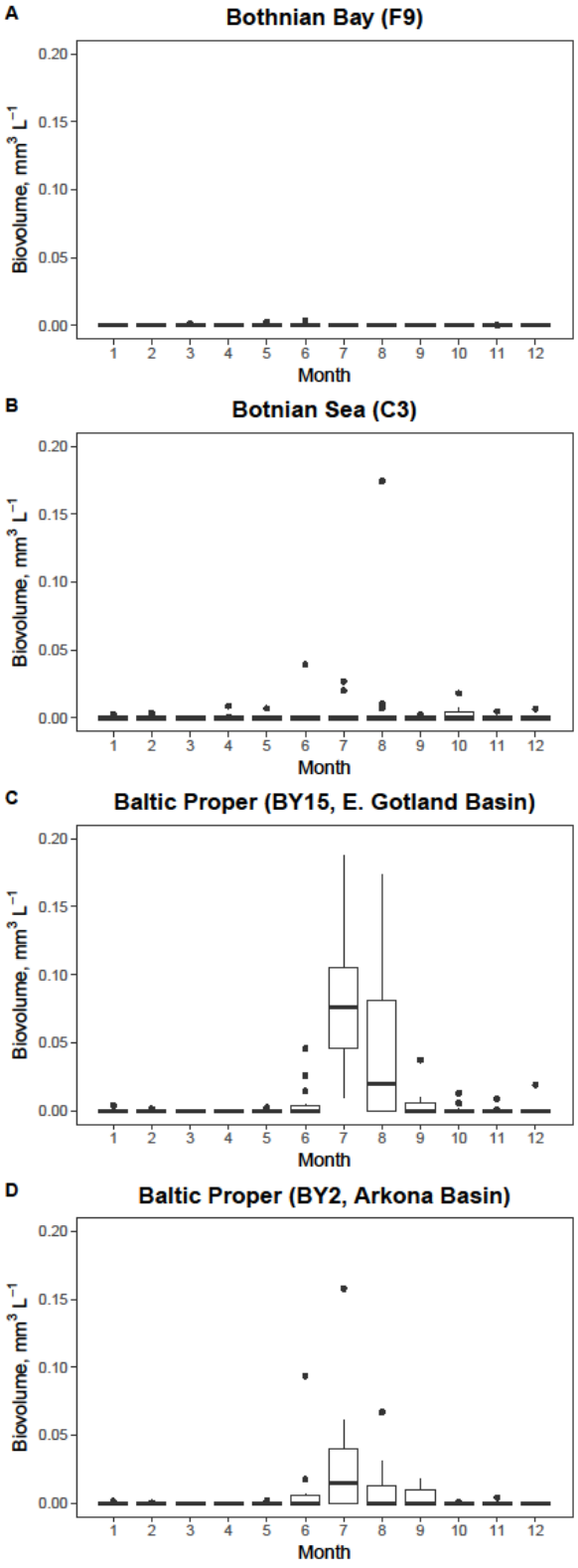

Fig. 4. Monthly distribution of *Nodularia* at four stations in the Baltic Sea. Data is based on a time series 1999-2015 with monthly sampling. Mid of box is median, lower hinge represents 25% quantile and higher 75% quantile. See Material & Methods for details.

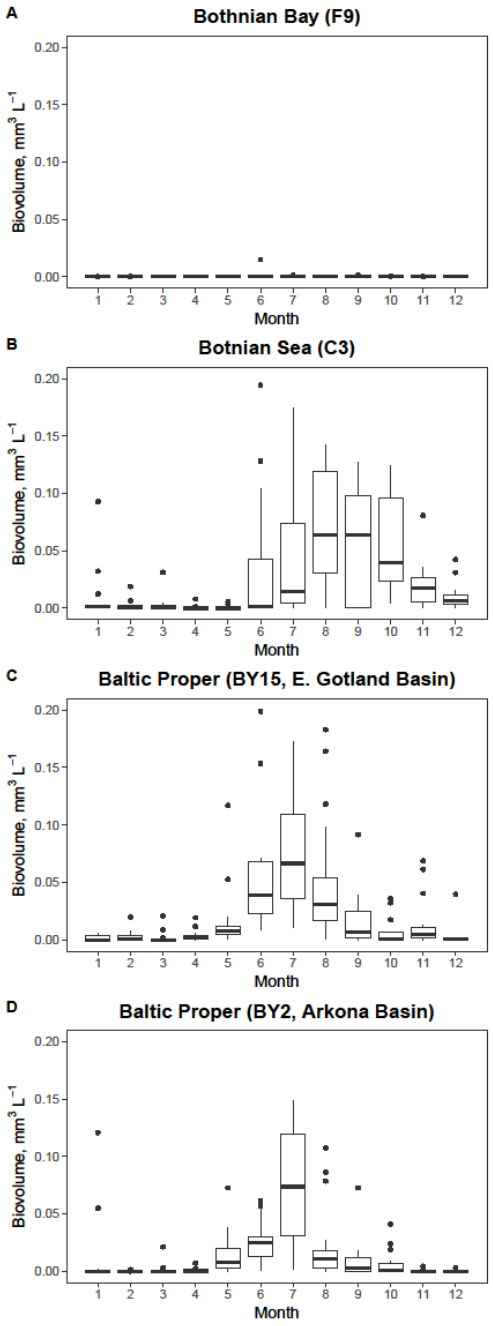

Fig. 5. Monthly distribution of *Aphanizomenon* spp. at four stations in the Baltic Sea. Data is based on a time series 1999-2015 with monthly sampling. Mid of box is median, lower hinge represents 25% quantile and higher 75% aquantile. See Material & Methods for details.

Acknowledgements

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Occurrence of nodularin in a cyanobacterial bloom in a shrimp farm in South Brazil

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Abstract

Since 2010, blooms of the brackish cyanobacteria *Nodularia spumigena* were recurrent in the shrimp growth tanks of the Marine Aquaculture Station during summer in Southern Brazil. Cyanobacterial growth led to a decrease of 37% in the white shrimp *Penaeus vannamei* Boone 1931, productivity. In the summer of 2014, a *Nodularia* bloom was collected from the tanks; filaments were separated by flotation and washed thoroughly twice in F/2 culture medium. Healthy filaments were lyophilized and the powder used for nodularin quantification by HPLC-DAD and immunoassays. Nodularin containing lyophilized powder was also tested for toxicity against the brine shrimp *Artemia salina* post-larvae and the lyophilized *Nodularia* powder contained 1.88 mg of the toxin nodularin g^{-1} d.w. Its toxicity was confirmed in bioassays with *Artemia salina* and *Penaeus vannamei* giving a LC50 of 1.22 and 2.50 μgL^{-1} of nodularin, respectively. This paper describes the occurrence and the toxicity of nodularin in South Atlantic coastal waters with consequences for shrimp farming.

Keywords: cyanobacteria, *Penaeus vannamei*, *Nodularia spumigena*, nodularin, shrimp farm, toxicity tests.

Introduction

Early registration of scum, or colored waters, consistent with cyanobacterial blooms refer back to at least 1853. In a perceptive and prescient paper in Nature, the Adelaide assayer and chemist George Francis reported on stock deaths at Milang on the shores of Lake Alexandrina in South Australia. Francis attributed the deaths to the ingestion and toxicity of scums of the cyanobacterium *Nodularia spumigena* (Francis, 1878).

Later, dog and cattle poisoning have also resulted from nodularin ingestion in the Baltic Sea (Kruger *et al*. 2009) and more specifically in the Gulf of Finland. Considerable losses in the North Atlantic flounder *Platichtlys flexus* populations were documented following a *Nodularia* bloom collapse (Drevs *et al*. 2007).

Nodularia cells produce the pentapeptide nodularin whose toxicity and lethal concentration (i.p.) in mammals are similar to microcystins (Carmichael, 1989). The main target organ of nodularin and potentially MC-LR is the liver (Svircev *et al.* 2010), which expresses high levels of many uptake transporters, including OATPS, organic anions transporting polypeptides (Roth *et al.* 2012). One of the important molecular toxicological mechanisms of these toxins is the inhibition of serine/threonine specific protein phosphatases PP1 and PP2A (MacKintosh *et al.* 1990). This in turn leads to hyperphosphorylation of proteins, ultimately resulting in deterioration of cellular integrity.

A marine shrimp farm located on the south coast of Brazil (Cassino Beach, RS, Brazil) uses the "bioflocs" system of intensive cultivation, in order to achieve a high production of shrimp associated with high air flow with this microbial aggregate (Wasielesky *et al.* 2006). The Microbial aggregation was stimulated by the extra addition of organic carbon sources; however, at outdoor shrimp tank systems this carbon input stimulates photoautotrophic organisms. Moreover, shrimp excrement and excess food portions, under sunlight exposure (open systems), led to a microbial growth (Hargreaves 2013).

Due to its geographical location the Aquaculture Marine Station (EMA) from the Federal University of Rio Grande (32º 12' S, 52º 10' W) runs the intensive shrimp (*Penaeus vannamei)* growth mainly at the end of spring and before autumn. In this period high light intensities and drought turns environmental conditions ideal for the shrimp growth. On the other hand, these were also ideal conditions for cyanobacterial (*Nodularia spumigena*) (Fig.1) growth leading to a decrease on shrimp growth and survival in some outdoors tanks (Fig.2)

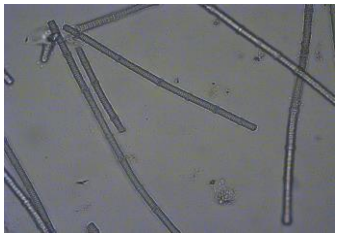

Fig 1. Cyanobacterium *Nodularia spumigena*

The presence of *Nodularia* filaments in the outdoor tanks was first identified and reported by Costa *et al.* 2013 there are indications that productivity may be affected In up to 37% (Table 1). However the precise estimation and identification of the toxin content and toxicity to shrimp is necessary and this is the objective of the present work. The present work also described and summarized methodological procedures which can be followed to monitor the occurrence of this toxic pentapeptide in aquaculture farms.

Fig 2. Open shrimp tanks next to the shoreline at the Cassino beach where EMA is located. Intense blue-green color of cyanobacterial blooms in the water.

Table 1. Productivity of the white shrimp *Penaeus vannamei* in the tanks with and without *Nodularia* (NOD) blooms.

Productivity (kg ha^{-1})		
Years	**Tanks**	**Tanks with NOD**
2010	13600	7100
2011	5630	2200

Material and Methods

Cyanobacterial sampling

The bloom analysed in this work occurred in the tank number 3 during the south hemisphere summer from 2013 to 2014. Samples were collected using buckets and taken to the laboratory as a total volume of 10 liters. Floating filaments of *Nodularia* were separated from the water, algae and other organisms by the use of a slow centrifugation LKB centrifuge at 2.000 rpm and separating a 2 liters sample each time. Floating healthy filaments were predominant and were pipeted into a 50 µm phytoplankton net and fully washed 3 times with the F/2 culture media prepared to marine cyanobacteria salinity. The resulting scum had a high dominance of *Nodularia* cells. The scum was frozen and totally transferred to a lyophilizer (Micromodulyo-Edwards®).

Sample treatment of nodularin analysis

A 500 mg of *Nodularia* lyophilized powder was weighed in a precision balance (Marte, Brazil). A volume of 7 mL of Milli-Q water plus 8 mL of methanol were added to it and let to extract during 24h on an orbital shaker (Aros 16O, Thermolyne-USA®) at room temperature. The whole extract was transferred to a rotaevaporator and left to concentrate during 1 hour. The resulting sample was homogenized using an ultrasonic device (Hielsher, Germany®) three times during 30 second, with 30 seconds intervals. This sample final volume was collected into a glass cylinder and set to 6 mL, with the initial mixture of Milli-Q water: Methanol (7:8). This extract was used for NOD concentration analysis by HPLC-UV and immunoassays for microcystins and nodularins.

The preparation of the solutions for HPLC-DAD was performed using only the reagents with HPLC grade and ultrapure Milli-Q water in

preparing solutions. NOD standards and their reference material certificates were obtained from SIGMA-Aldrich® and from Abraxis® (USA).

The two mobile phases used were 0.05M trifluoroacetic acid (Merck®) in acetonitrile (Merck®) and 0.05M acid trifluoroacetic in ultrapure Milli-Q water.

The chromatograph HPLC-DAD (Shimadzu, Japan) consists of a controller CBM- 20A, a detector SPD-M20 (200-400 nm), a deuterium lamp (D_2), two binary pumps LC-20AD and CTO-20A column oven at 40°C controlled through Labsolution 5.41.240 software. The analytical column used for analysis was a Luna C18 (2), (250 x 4.6mm, 5µ) (Phenomenex®).

The detection limit, resulting from SIGMA-Aldrich® standard analyzed in HPLC-DAD for nodularin was 0.025 µg L^{-1} .

Elisa analysis for NOD was also done using the specific immunoassay test for nodularins in the range from: 0.25 to 1 gL^{-1} (Beacon® (ME, USA)) following instructions of the supplier.

Toxicity Tests

NOD containing lyophilized powder was used in the toxicity assays with larvae of the brine shrimp *Artemia salina* (Fig 4.) and the 35 days old post-larvae of the white shrimp *Penaeus vannamei*. Both organisms were supplied by the Marine Aquaculture Station (EMA-FURG). Both tests were run without water replacement but with a strict control of the nodularin contents in all triplicates during 24h and 92h length experiments.

Thus, the NOD containing lyophilized powder was used in tests at seven concentrations ranging from 0 to 5.0 mg. $m.L^{-1}$ powder in a 500 mL glass bottle with aeration for white shrimp *Penaneus vannamei*. The NOD containing lyophilized powder was also used in seven concentration tests ranging from 0 to 5.0 mg. mL^{-1} for the brine shrimp *Artemia salina* in a 1 mL well of a 96 well plate kept inside a 28ºC incubation chamber.

The data obtained with the death of the organisms in the acute tests were analyzed through the program Trimed Sperman Karber 1.5. This treatment correlates the observed death or effect data in toxicological tests, and gives Lethal Concentration estimates for 50% of organisms (LC_{50}), in addition to their maximum and minimum confidence intervals.

Posteriorly, an analysis of variance, such as normality and homogeneity, were verified. To meet the assumptions, the results of the NOD were transformed into log (x). A analysis of variance was used to identify significant differences between the control (concentrations 0) and toxic (different concentrations) at a significance level of 5 %.

Results and Discussion

Nodularin identification.

Lyophilized powder from blooms in the tanks was used for identification of the presence of nodularin in the samples. Analysis by High Performance Liquid Chromatography attached to a UV-DAD detector after methanol extraction, and concentration, reveals the presence of a single peak. A single peak was detected at a retention time from 6.1 to 6.3 minutes which corresponds to an identical peak of the Nodularin standards supplied by Sigma-Aldrich® or Alexis Biochemicals®.

Elisa analysis for nodularin (NOD): the *Nodularia* bloom was also analyzed for the presence of nodularin using the specific test for nodularins in the range from: 0.25 to 1µg L^{-1} supplied by Beacon® (ME, USA). A value of 0.4 mg per liter of the dried lyophilized powder corresponds exactly to the highest value of the kit calibration interval. Thus, giving a positive response to the presence of nodularin in the sample and a possible nodularin concentration of 2.5 µg L^{-1} NOD mg dry lyophilized bloom powder.

Toxicity Tests.

The toxicity of the *Nodularia* lyophilized powder was confirmed in acute toxicity tests with the brine shrimp *Artemia salina* larvae. In three tests performed using a minimum of a hundred organisms the lethal concentrations which kill 50% of the organisms varied from 0.65 to 2.61 mg of the lyophilized *Nodularia* powder per mL of seawater ($p<0,05$). Equally the same powder was tested against 35 days old post-larvae of the white shrimp *Penaeus vannamei* giving a CL_{50} of 1.33 $mg.mL^{-1}$ ($p<0,05$).

Wherefor, taking into account that the nodularin concentration detected by HPLC in the powder was 1.885 mg g^{-1} d.w from the lyophilized material. The nodularin amounts that killed 50% of *Artemia* and *Penaeus* larvae were 1.22 and 2.50 µg L^{-1}, respectively.

The present work reports the development of *Nodularia* bloom caused by high light intensities the excess of fertilizers in the tanks of a shrimp farm. The bloom was strongly toxic to the white shrimp growth in the tanks as well as in bench tests using 35 day old post-larvae of the same shrimp. The reference marine toxicity test using *Artemia salina*, also confirmed the *Nodularia* bloom toxicity. Using certified standards supplied from two laboratories, a single peak of the pentapeptide Nodularin (NOD) was identified in the sample.

NOD is toxic pentapetide with a similar spectrum of action as the hepatoxin microcystin to mammals (Carmichael, 1989). The potencies of nodularins (Rinehart *et al.* 1988) and microcystins-LR (Carmichael, 1989) are the same, having LD_{50} of 60 µg/kg (ip, mice) and a CL50-18h of 4.79 $\mu g.mL^{-1}$ in *Artemia salina*. Equally an CL50-24h for *Microcystis aeruginosa* lyophilized powder to the local pink-shrimp *Farfantepenaeus paulensis* was 0.91 mg mL^{-1} (Yunes, 2009). Reports on their inhibitory activity against protein phosphatases 1 and 2A render these compounds as important toxins to be aware of. However, as a typical brackish water growing organism, the cyanobacteria *Nodularia* impact shallow areas of coastal regions in the Gulf of Finland (Kankaanpää et al, 2001) and South Australian marine farms (Stewart *et al*. 2012).

NOD has caused the death of several marine organisms, including fish of the genus *Gaterosteus aculeatus* (Three-spined stickleback) and its concentration in the animal tissues reached 170 µg NOD kg d.w. (Stewart *et al*. 2012). Also, it was suggested as the cause of the death of sea mullets reported for Australia. The NOD animal tissue concentration was 43.6 mg/kg (Stewart *et al*. 2012). Therefore, a proper surveillance in the tissue of shrimp farm animals reported in this paper must be considered.

Although several aquatic environments can maintain cyanobacteria, the aquaculture environment can also provide favorable conditions for the development of these organisms, among them Nodularia. This can reach high biomass and form blooms.

Blooms, in turn, can reach toxic levels to NOD, with harmful consequences for cultivable species, for animals that may drink water from these environments, plant irrigation and recreation. Aquaculture farmers are at risk of severe intoxication of fish and crustaceans as a result of the lack of an adequate surveillance method, now available here. This communication describes the occurrence of toxic NOD in Brazilian waters with consequences to the shrimp farming in South American Atlantic Coast.

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Monitoring of cyanobacterial populations and the detection of cyanotoxin- genes in Billings Reservoir (Diadema/São Paulo, Brazil)

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Abstract

The cyanobacterial biomass dominance over phytoplankton in Billings Reservoir is a problem that arouses attention of researchers and authorities responsible for water treatment as these organisms are capable of producing blooms and cyanotoxins. The aim of the study was to monitor and identifify the populations of cyanobacteria present in Billings Reservoir and detect possible genes responsible for cyanobacterial toxin production (microcystin and anatoxin-a). The samples used were collected monthly between June 2014 and July 2015, at a fixed point in Billings Reservoir (Diadema/SP, Brazil). The sample for detection of cyanotoxin genes was collected in November/2015. The total biovolume fluctuated between 0.76 mm3 L-1 (Dec.14) and 9.2 mm3 L-1 (Sept.14). Cyanobacteria were dominant (relative biovolume > 50%) over other phytoplankton groups during 57% of time. The most frequent species were *Aphanocapsa annulata*, *Cylindrospermopsis raciborskii*, *Merismopedia punctata*, *Microcystis aeruginosa*, *Microcystis panniformis* and *Planktothrix agardhii*. The molecular analysis demonstrates that the genes for microcystin and anatoxin production were present, suggesting that the cyanobacteria in this studied point were potential microcystin and anatoxin producers.

Keywords: Cyanobacteria, microcystin, anatoxin;

Introduction

The Billings Reservoir (São Paulo, Brazil) built in 1927, is a eutrophic reservoir with a wide-ranging use, providing water supply to the public, energy generation, flood control and recreational activities (Carvalho, 2003). The eutrophic condition and the presence of cyanobacteria in the reservoir have been highlighted (Carvalho et al., 2007; Moschini-Carlos et al., 2009). Cyanobacteria blooms can be very harmful to the aquatic environment since they can alter properties of the ecosystem, such as light penetration and oxygen concentration. It is known that some cyanobacteria species are potential toxin producers, including some that are also found in Billings Reservoir (Moschini-Carlos et al., 2009).

Usually, cyanotoxins are detected in water using chemical methods, such as HPLC and ELISA (Carmichael et al., 2001), which are very expensive and requires high concentration of cells or toxins. Recently, cyanotoxin detection in water bodies has been evolving. Using molecular biology, researchers are now trying to detect within the genetic pool of cyanobacteria fragments of the genes responsible for the production of toxins (Rantala et al., 2004; Barón-sola et al., 2012). Cyanobacteria are capable of producing multiple types of cyanotoxins, such as microcystins and anatoxin a, which were chosen as targets in the present study.

This new approach is important because using morphological identification cannot differentiate between harmful (capable of producing toxins) and non-toxic blooms, while this can be addressed using total DNA extractions from water samples to investigate the presence of cyanotoxin producing genes. Exposure (chronic or acute) to cyanotoxins can be very hazardous to both humans and animals. In 2001, 76 patients died at a dialysis center in Caruaru (Brazil) caused by

poisoning with microcystins and cylindrospermopsin (Carmichael et al., 2001).

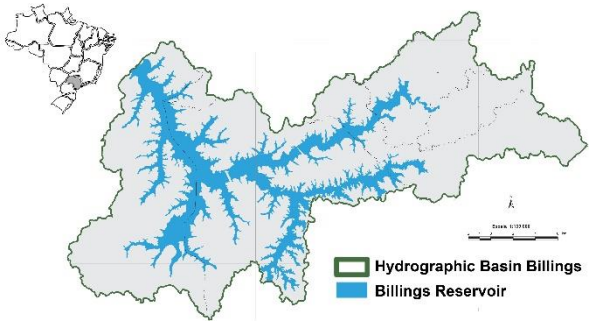

Fig. 1: Map showing Billings Reservoir in São Paulo State, Brazil (Adapted from Cobrape, 2010).

Material and Methods

Sampling

Sampling for phytoplankton analysis was collected during one year (June 2014 to July 2015), using a plankton net (identification) and a Van Dorn sampler (quantification). Samples used for molecular biology assays were collected on November 2015: a surface sample (S1) and two column samples (C1 and C2).

Identification

The samples used for phytoplankton identification were preserved with 4% formalin. Taxonomical analysis was based on traditional literature (Komárek & Anagnostidis, 1998; 2005; Komárek 2013).

Quantitative analysis

Samples used for quantification were preserved with 1% Lugol's solution. Countings were conducted with sedimentation chambers (Utermöhl, 1958; Sant'Anna et al., 2006), using an inverted microscope (Axio Vert.A1, Carl Zeiss), 400x magnification. Determination of dominant and abundant species was done following criteria established by Lobo and Leighton (1986).

DNA Extractions

The samples C1 and C2 were divided in two: extraction with floating cyanobacteria and one after centrifugation (4.000 rpm), generating the samples C1D and C2D. Only one extraction was done using S1 sample, as it was collected from the surface. The DNA extractions were carried out using Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), following the protocol established by the manufacturer.

PCR assays

Cyanotoxin -genes were detected using two sets of primers: mcyE-F2 and mcyE-R4 (Rantala et al., 2004) for microcystin and anaC 5247F and anaC 6107R (Rantala-Ylinen et al., 2011) for anatoxin a.

The PCR mix (25 µL) contained 2.5 µL 10x Buffer (ABM), 0.5 µL $MgSO_4$ 25 mM (ABM), 0.5 µL dNTP mix 10 mM (Invitrogen), 0.5 of each of the primers 10 pmol, 0.25 µL Taq polymerase (ABM), 19.25 µL of sterile water (Invitrogen) and 1 µL of DNA.

As a positive control for genetic material from cyanobacteria, the reactions were carried out using PCβF and PCαR primers (Neilan et al., 1995), which are responsible for the amplification of intergenic space in the phycocyanin gene of cyanobacteria.

All DNA were visualized on 1,5% agarose gels, stained with ethidium bromide or Brilliant Green (Neotaq). All reactions were carried out on a Veriti 96-well thermal cycler (Applied Biosystems).

Results and Discussion

Forty-three cyanobacterial species were identified, among them *Aphanocapsa annulata, Cylindrospermopsis raciborskii, Dolichospermum planctonicum, Merismopedia punctata, Microcystis aeruginosa, M. panniformis, M. protocystis, Planktothrix agardhii, P. isothrix, Raphidiopsis brookii* and *Sphaerocavum brasiliense* were the most frequent species. All of these species have commonly been found in Billings Reservoir by different researchers (Carvalho et al., 2007; Sant'Anna et al., 2007; Gemelgo et al., 2009; Moschini-Carlos et al., 2009). Some of the species described also were previously associated as cyanotoxin producers from other compartments of the reservoir (Carvalho et al., 2007; Moschini-Carlos et al., 2009).

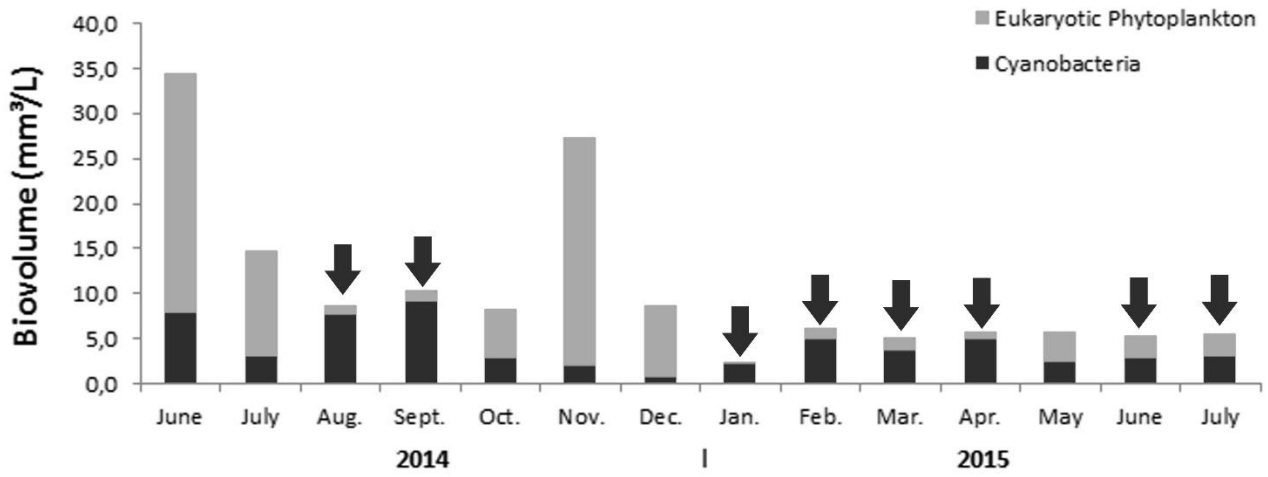

Fig. 2: Biovolume values for the months June 14 to July15 for eukaryotic phytoplankton and cyanobacteria. The arrows represent months when cyanobacteria biovolume was higher than 50%.

The total cyanobacteria biovolume fluctuated between 0.76 mm /L (Dec 14) and 9.2 mm /L (Sept 14) (Fig 2). Although cyanobacteria were dominant (relative biovolume > 50%) over the other phytoplankton groups in 57% of the period, the biovolume values did not exceed levels of 5.0 mm /L. This value was the limit established by CONAMA Brazilian Resolution n° 357/05 for Class II freshwater. *Microcystis aeruginosa* is recurrently a dominant species in Billings Reservoir (Gemelgo et al. 2009; Cortez, 2013). Though this species was not dominant in the study period, it was present in 100% of time. It is important to emphasize their genetic background and monitoring their presence, as they are potential toxin producers.

The PCR run as positive control for cyanobacteria genetic material was realized using PCβF and PCαR primers (Neilan *et* al., 1995) showed that cyanobacteria genetic material was present in all samples (Fig 3.)

The detection of anatoxin-a -genes carried out using anaC 5247F and anaC 6107R primers indicated the presence of the genes responsible for the synthesis of the toxin in all samples analyzed (Fig 4).

Few works discuss the presence of potentially anatoxin-a producers in Brazilian reservoirs. There is only one account about the presence of this cyanotoxin in a reservoir in São Paulo state (same as Billings Reservoir), which discuss the detection of anatoxin-a in Taiaçupeba Reservoir, probably concerning the presence of *Dolichospermum sp*. (Yunes et al., 2003). In Billings Reservoir, previous research found no presence of this toxin (Moschini-Carlos et al., 2009). In the present work, during the phytoplankton monitoring, we found potential anatoxin-a producing genera, such as *Aphanizomenon* and *Phormidium* (Ballot et al., 2010; Wood et al., 2011) and this can be related to the presence of the anatoxin-a producing-gene in the genetic pool of the cyanobacteria in Billings Reservoir.

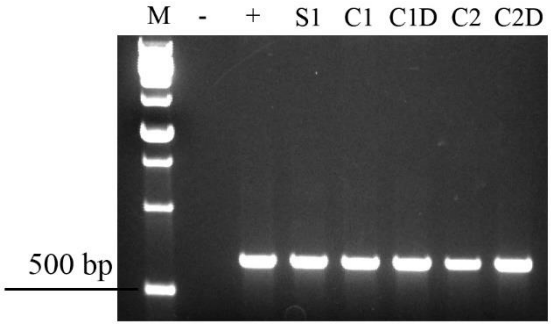

Fig. 3: PCR used as positive control to Cyanobacteria genetic material. Amplification using the PCβF and PCαR primers.

Regarding microcystin -genes, the research with mcyE-F2 and mcyE-R4 primers also indicated the potential capability of the production of microcystins (Fig 4). The detection and quantification of microcystins in Brazilian

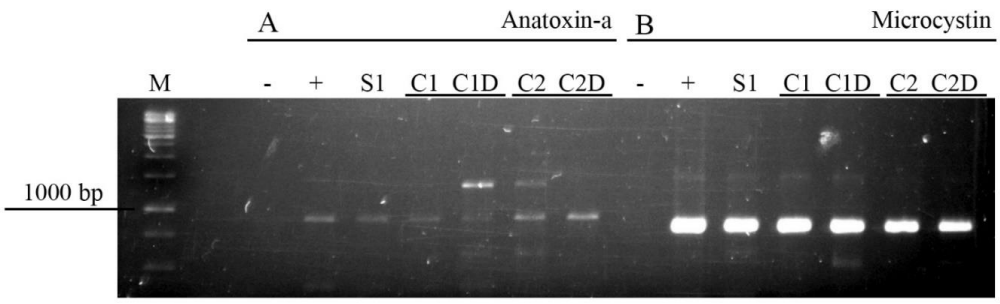

Fig. 4: (A) Amplification of the anatoxin-a gene, using anaC 5247F/6107R primers, 861 bp. (B) Amplification of the microcystin gene, using mcyE-F2/R4, 812 bp. M – 1kb molecular weight ladder.

reservoirs has been elucidated, with various works revealing microcystins presence in various samples, including Billings Reservoir (Carvalho et al., 2007; Dos Anjos et al., 2006; Moschini-Carlos et al., 2009). The existence of the genes responsible for the production of this toxin in the genetic pool of Billings' cyanobacteria, can be related to the dominance and presence of well known cyanotoxin-producing genera, such as *Microcystis* (Chorus and Bartram, 1999) and *Planktothrix* (Christiansen et al., 2003).

In conclusion, data acquired showed the existence of potential cyanotoxin-producing cyanobacteria in Billings Reservoir capable of producing microcystin (confirmed by other works) and new evidence of potential anatoxin-a producers in the reservoir.

Acknowledgements

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Chemical and analytical sciences in a whirlwind of global change

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Abstract

Oceans and coastal ecosystems provide a number of services to humans, including the provision of seafood and drinking water, waterways for the transportation of goods, recreational spaces and increasingly renewable energy. Harmful Algal Blooms (HABs) potentially impact negatively on any of these services. Detecting toxins has always been a challenge due to the difficult predictability of algal blooms and the need for rapid reaction due to consumption of seafood as a fresh commodity. Global change (including climate change, redistribution of organisms through ballast water etc.) further accentuates this challenge, leading to an increased need for the detection of a wider range of toxins than ever. Recent discovery of tetrodotoxin in echinoderms, gastropods and bivalve molluscs exemplifies the re-discovery of known compounds in unusual ecosystem compartments. Consequently, the use of non-targeted analysis as a tool to screen for not just dozens but hundreds of compounds in a single analysis gains importance. Recent developments in passive sampling and the combination of non-targeted analysis and bioanalytical tools for the discovery of yet undescribed toxins are discussed. Finally, remaining challenges in method validation, risk evaluation and regulatory monitoring are reviewed.

Keywords: Global change, non-targeted analysis, metabolomics, food safety, food security, algal toxins.

Introduction - Drivers of change

Since the industrial revolution in the mid-1800s and advances made in medicine in the 20th century, the climate on earth has undergone a rapid change and the human population has grown faster compared to any period before. Climate change includes many parameters impacting oceanic ecosystems and the atmosphere: seawater temperature, acidity, changeable weather, storms, flooding, ice coverage and currents to name but a few (IPCC, 2014). Increase in population and life expectancy have resulted in numerous societal challenges, spanning from the simple increased need for food and drinking water to more complex issues such as the diversification in feeding behaviour and diversity, the denser population of coastal zones and their usage and increased maritime transportation. Almost 30% of marine fish stocks were overexploited in 2011 (FAO, 2014).

Factors directly impacting on the initiation and the frequency of Harmful Algal Blooms (HABs) have been reviewed recently (Lassus et al., 2016) and can be summarised as follows: i) temperature increase of seawater may lead to both range extension and increased frequency of HABs; ii) eutrophication may lead to increased severity of HABs, and also through change in nutrient ratios to change in HAB-species; iii) transportation of harmful organisms by ships and through live relaying of shellfish also may contribute to the establishment of alien species and thus cause novel problems; iv) overfishing also heavily impacts on marine food chains and has been suggested as a factor increasing HABs; v) increased use of coastal zones is another complex phenomenon which may contribute to localised nutrient changes through dredging or aquaculture, or to contamination from mining or fossil fuel production. Unfortunately, all these factors interact with each other and lead to difficult-to-model scenarios. Finally, assessment of the evolution of HABs is also confounded by increased awareness and monitoring. The following review examines to what extent these drivers of change have led to improved understanding of the chemistry of algal toxins and the methods used for their detection.

Disruptive technologies – response to change

The most powerful driver for the introduction of novel methods had been the major HAB events causing paralytic, neurotoxic, diarrhetic and

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azaspiracid seafood poisoning: These shellfish poisoning incidents have led to the introduction or re-validation of bioassays (APHA, 1970; Hess et al., 2009; Schantz et al., 1957; Sommer and Meyer, 1937; Yasumoto et al., 1978). In the case of domoic acid, the amnesic shellfish poison, a HPLC-method was developed rapidly as the existing bioassays did not have sufficient detection capability (Quilliam, 1989). These methods were rapidly implemented and allowed for efficient protection of public health. However, some of them, such as the lipophilic mouse bioassay (Yasumoto et al., 1978) also led to a large number of false positive assays and impeded productivity in the shellfish sector. Hence, this methodology was refined (Yanagi et al., 1989; Yasumoto et al., 1984), and later replaced in Europe by instrumental methods using liquid chromatography coupled to tandem mass spectrometry, i.e. LC-MS/MS (Anonymous, 2011). In France, the introduction of LC-MS/MS methodology for lipophilic toxins has resulted in an average reduction from 27 to 5% of false positive results (2003 - 2008 versus 2010 - 2015, respectively), and also reduced up to four-fold the closure periods in certain production areas, e.g. Arcachon Bay (unpublished data).

However, global change, as abovementioned potentially leads to the introduction of new species in different areas of the World Ocean, extended range and abundance of certain species and unusually large blooms (Lassus et al., 2016). Interesting examples for range extension can be found for STX-producers on the US East- and West-coasts where datasets had been acquired over sufficiently long periods (1944 – 1983) to allow for trend analysis (US National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution, 2011) or for both *Noctiluca scintillans* and Ciguatoxin-producing species along the Australian East coast (Hallegraeff, 2014). The recent discovery of known toxins in different phyla or ecosystem compartments may also indicate that such changes are already well underway. For instance, detection of tetrodotoxin (TTX) in gastropods (Jen et al., 2014; Rodríguez et al., 2008), sea slugs (McNabb et al., 2010) and bivalves (Turner et al., 2015; Vlamis et al., 2015) poses problems to recently interlaboratory validated methods that are specific for paralytic shellfish poisons (PSP) and impede replacement of PSP mouse bioassay (MBA) in many areas. Similarly, the discovery of analogues of palytoxin, originally known from *Palythoa toxica* (Moore and Scheuer, 1971), in micro- and macro-algae exemplifies the complexity of toxins in the marine food web. Also the confirmation of the constitutive production of domoic acid in macro-algae (Jiang et al., 2014), in addition to its production by *Pseudo-nitzschia* diatom spp. (Quilliam and Wright, 1989) and other micro-algae (Lassus et al., 2016), outlines the spread of toxins across different phyla and thus the need for more comprehensive analysis of natural bioactive compounds in seafood.

Furthermore, the need for better understanding of the role of toxins in the biology of the producing organisms and their ecological function suggests that analysis of a wide range of compounds may be necessary. In analogy of biomolecular methods of analysis for the detection of organisms, e.g. through metagenomics (S. Nagai, this conference) or proteomics (Wang et al., 2014a; Wang et al., 2014b), such a wider scope of methods for the detection of metabolites can be achieved through non-targeted analysis, e.g. metabolomics.

Non-targeted analysis & passive sampling

Low resolution mass spectrometry (LRMS) only allows for quantitation of targeted analytes, even if recent instrument developments allow for the rapid acquisition of data. Therefore, several recent studies have explored the capability of high resolution mass spectrometry (HRMS) to detect a large number of compounds from a single analysis, including marine, and specifically algal toxins (Blay et al., 2011; Domènech et al., 2014; Gerssen et al., 2011). These studies have all used Orbitrap technology which allows for the use of very high resolution (> 100,000) but has limitations in both scan speed and mass range. Therefore, we have recently introduced HRMS using quadrupole-Time-of-Flight (Q-ToF) technology for the analysis of marine toxins (Zendong et al., 2015). In that study the sensitivity, resolving capability and detection limits for lipophilic toxins were evaluated for both Orbitrap and Q-ToF technology. While sensitivity and detection limits of the Q-ToF for algal toxins in shellfish matrix were essentially similar to LRMS and Orbitrap technology, the resolving power of the Q-ToF and Orbitrap led to similar mass accuracies observed (<1 to < 3ppm), (Zendong et al., 2015).

Passive sampling (SPATT devices) was first introduced by MacKenzie et al. (2004) and further studied for its detection capability by several groups (Fan et al., 2014; Fux et al., 2010; Kudela,

2011; Lane et al., 2010; Lefebvre et al., 2008; Li et al., 2011; MacKenzie, 2010; Rundberget et al., 2011; Stobo et al., 2008; Zhao et al., 2013). Zendong et al. (2014) were the first to use passive sampling in combination with HRMS as a detection technique. The main advantages of using SPATT devices as compared to bivalve molluscs are that i) profiles are simpler, show less matrix influences and reflect more closely algal footprints (as algal metabolites on SPATTs do not undergo shellfish metabolism) and that ii) they can be deployed in any location (Zendong et al., 2016a).

Examples of environmental metabolomics

A deployment of SPATTs in Ingril, France?, showed that the algal metabolic footprint allows tracing of a number of species, e.g. *Dinophysis* spp., *Prorocentrum lima*, *Alexandrium ostenfeldii* and *Vulcanodinium rugosum*, i.e. both pelagic and benthic micro-algae (Zendong et al., 2014). A more recent survey of French coastal waters allowed us to establish the capability of non-targeted screening to detect spatio-temporal changes in coastal waters (Zendong et al., 2016a). This study showed that both geographical and temporal trends can be easily detected using the combination of passive samplers.

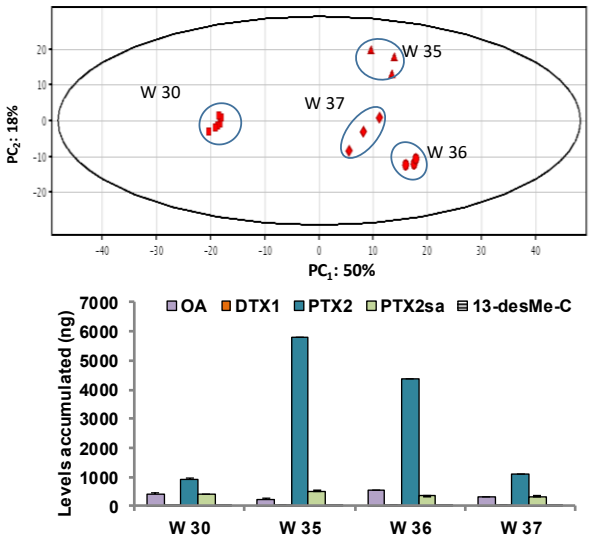

Fig. 1. Principal component analysis of non-targeted analysis of SPATT devices (top) and lipophilic toxins at Scoré, Atlantic coast of France (bottom).

Principal component analysis (PCA) is typically used to reduce data complexity and analyse the trends in the non-targeted data. Thus, temporal trend analysis of the French data set showed that weekly differences in non-targeted data were related to an appearance of *Dinophysis* and its toxins (Fig.1). Similarly, Zendong et al. (2016b) showed that this trend analysis was repeatable in Nigerian waters: rain and dry season showed significantly different metabolite profiles, temporal differences at every station were discernible but smaller than overall regional differences and geographic differences between stations were also observed for every single week. Also, the toxin profiles indicated the occurrence of *Dinophysis* spp., which was indeed confirmed through the microscopic observation of *D. caudata* in the water column (Fig. 2). Interestingly, the non-targeted data set in Nigeria also pinpointed the presence of cyanobacterial metabolites, which is coherent with the overall low salinity of the area (2-20) during the study period, due to the high freshwater input from the Niger delta.

Comparative metabolomes and bioassays

At a previous meeting, we showed that comparison of algal metabolomes can reveal toxins and serve

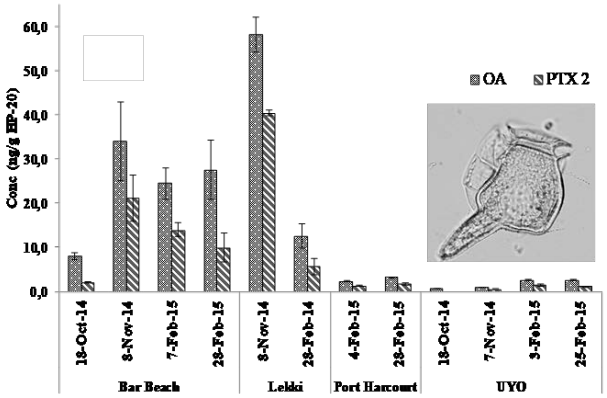

Fig. 2. Geographical differences in background levels of *Dinophysis* toxins in Nigerian waters. Bar Beach and Lekki are areas of higher salinity (18-20) and contain more OA. Inset: *D. caudata* identified at Bar Beach, a potential toxin producer.

as a taxonomic tool for classification of micro-algae (Hess et al., 2012). Combination of HRMS with miniaturised bioassay allows for dereplication of metabolites and thus to focus on novel analytes (Geiger et al., 2013a; Geiger et al., 2013b). Ongoing work in our laboratory also exemplifies the usefulness of this combination for

detection of bioactive analogues in other micro-algal species, e.g. *Gambierdiscus excentricus*, where a simple two-step bioassay (N2a)-guided fractionation allowed for 25-fold simplification of the crude extract (Pisapia et al., 2016).

Remaining challenges

Even though HRMS has enabled a number of advances in our understanding of the fate of toxins in marine ecosystems, a number of challenges remain to be solved, both in terms of technology and in terms of risk evaluation and management.

One of the greatest challenges is the integration of scientific knowledge generated in different disciplines. Studies on genomes or transcriptomes start to verify whether the proteins predicted are actually biosynthesized (Allan Place, this conference). However, very few links are made between transcriptomics, proteomics and metabolomics. The software to benefit from a multi-omic approach is already available (e.g. Agilent), however, there has been no studies on toxin-producing dinoflagellates.

Also classical, targeted methods still require significant improvement, mostly in terms of sensitivity, e.g. for saxitoxins (STXs) and ciguatoxins (CTXs). Even though UHPLC-LRMS/MS has been adapted for the detection of STXs (Boundy et al., 2015), the method requires inter-laboratory validation before possibly being applied in regulatory monitoring. An additional major advantage of that method is that it deals adequately with detection of the emerging TTXs. Progress required in the area of CTXs goes well beyond the issue of sensitivity, since many of the algal precursors of the metabolites in fish have not even yet been identified. An international strategy is being developed in this area as an integrated effort between IOC, IAEA, FAO and WHO (IOC/IPHAB, 2015).

Many hurdles also remain in the area of risk evaluation and management. At least two toxin groups have been regulated in the EU (YTXs, yessotoxins; and PTXs, pectenotoxins), both indirectly and explicitly, without any evidence for actual human poisoning related to these toxin groups over the last two decades. Even though recent data from chemical analysis show that the risk for PTXs being involved in any poisoning is negligible, risk assessors have not been able to come up with recommendations for risk management to deregulate these toxin groups.

There are also still a number of poorly understood risks, including the occurrence of cyclic imines (Hess et al., 2013) and ß-methyl-amino alanine (BMAA) in shellfish. BMAA is suspected to be an environmental contributor to amyotrophic lateral sclerosis (ALS), a neuro-degenerative disease which is fatal, on average within 3 years of first diagnosis (Murch et al., 2004). Since it had been postulated to be ubiquitously occurring in cyanobacteria (Cox et al., 2005), recent efforts have also confirmed its occurrence in shellfish (Masseret et al., 2013). This finding has triggered further research, allowing for the refinement of methods and confirming the levels of BMAA in shellfish and other compartments of the Thau Lagoon ecosystem, France (Reveillon et al., 2014; Réveillon et al., 2015). Interestingly, this small non-proteinaceous amino acid is not only found in that lagoon but also in shellfish from all other coastal areas of mainland France, and other areas in Europe (Réveillon et al., 2016b). This finding can be better understood following the results from a most recent study showing that BMAA is produced by several very abundant species of diatoms, thus making BMAA one of the basic metabolites of the marine food web (Réveillon et al., 2016a). An international effort should be made to review these findings independently to allow the productive sectors to continue to thrive and consumers to enjoy seafood.

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Biotransformation and chemical degradation of paralytic shellfish toxins in mussels

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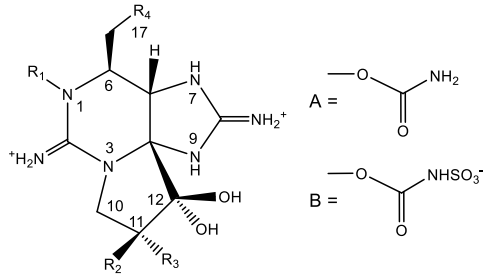
Abstract

Five new paralytic shellfish toxin analogues, M1 to M5, were reported previously in mussels that had been exposed to an intense bloom of *Alexandrium tamarense*. Structures of M1 to M4 have been published. The structure of M5 is revealed in this paper. In addition, it is shown that several additional related analogues were detected by LC-MS/MS analyses. Some of these were produced after feeding mussels with cultured toxic algae, while others were produced through chemical degradations, which can occur in stored mussel tissues. Tentative structures for the additional analogues are proposed. The formation of these analogues appears to begin with conversion of the 11-hydroxysulfate group in the C2 toxin to a hydroxyl function to form M1, which can then be further converted to other analogues through a combination of enzymatic transformations and/or chemical degradations. A corresponding set of N-hydroxy analogues was also revealed.

Keywords: paralytic shellfish poisoning, toxins, metabolism, structure determination

Introduction

Over 50 paralytic shellfish toxins (PSTs) have been reported in the literature (Wiese *et al.*, 2010). Structures of the principal PSTs typically observed in shellfish are presented in Fig. 1.

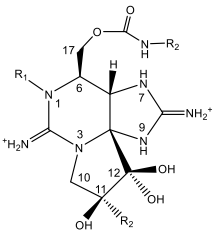


R_1	R_2	R_3	R_4 = A	R_4 = B	R_4 = OH
H	H	H	STX	GTX5	dcSTX
OH	H	H	NEO	GTX6	dcNEO
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 1. Structures of the 18 common PSTs.

Only a few of these toxins are produced by marine dinoflagellates (e.g., *Alexandrium*, *Pyrodinium* and *Gymnodinium* genera) but, when accumulated in shellfish, they can undergo transformations to various analogues through enzymatic and chemical mechanisms (Bricelj & Shumway, 1998). These include: (a) the epimerization of 11-hydroxysulfate functions (e.g., C2⇌C1); (b) reductions (e.g., NEO → STX; GTX3 → STX); (c) decarbamoylation (e.g., C2 → dcGTX3); and (d) *N*-desulfation (e.g., GTX5 → STX). Five unusual PST analogues, M1 to M5, were reported in mussels that had been exposed to an intense bloom of *A. tamarense* (Dell'Aversano *et al.*, 2008). These metabolites have been observed in several other bivalve species including scallops, clams and cockles (Vale, 2010, Li *et al.*, 2012).

M1 to M4 have been identified (Fig. 2) but M5 has remained uncharacterized due to insufficient quantity being available for structure elucidation.



R_1	R_2		
H	SO_3^-	M1β	11β-hydroxy-*N*21-sulfocarbamoyl-saxitoxin
H	H	M2β	11β-hydroxy-saxitoxin
OH	SO_3^-	M3	11,11-dihydroxy-*N*21-sulfocarbamoyl-saxitoxin
OH	H	M4	11,11-dihydroxy-saxitoxin

Fig. 2. Structures of metabolites M1 to M4.

M1 to M5 were presumed to be metabolites as they were absent in algae and only present in shellfish. They appear to have been produced from C2, as that toxin was predominant in the algae but was at a much lower relative levels in mussel tissues. The mechanism of formation appears to begin with an *O*-desulfation, in which the 11-hydroxysulfate is converted to a hydroxyl to form M1. Insertion of an additional hydroxyl at C11 leads to M3. *N*-desulfation of M1 and M3 lead to M2 and M4, respectively. It is possible that M1 to M5 may all have been produced partly or entirely through chemical degradation.
The objectives of the present study were to determine the structure of M5, to search for related transformation products, and to confirm metabolism by feeding toxic algae to mussels.

Material and Methods

Calibration solution reference materials for PSTs were obtained from the National Research Council Canada (Halifax, NS). LC-MS/MS analyses were conducted on an Agilent 1200 LC coupled via electrospray ionization source to a Sciex 4000 QTRAP instrument. The column used was a TOSOH TSKgel Amide-80 hydrophilic interaction liquid chromatography (HILIC) column as reported previously (Dell'Aversano *et al.*, 2008). Metabolites were purified on a Bio-gel P-2 open column (Laycock *et al.*, 1994) followed by preparative HILIC. NMR spectra (^{1}H and ^{13}C) were measured on a Bruker DRX-500 spectrometer as reported previously (Dell'Aversano *et al.*, 2008).
Chemical conversion experiments of C1/2, M1 and M3 toxins were conducted at 37°C and pH 8.5 (aqueous solutions adjusted with NH_4OH).
A toxic *A. fundyense* (GTCA) culture was fed to mussels (*Mytilus edulis*) in laboratory feeding experiments. Mussels were acclimatized and fed with nontoxic algae for 7 days before beginning experiments. Samples were taken at several intervals after toxic culture feeding began and the feeding continued for 168 h.

Results and Discussion

Chemical conversion of C1/2 with base revealed that, in addition to the production of dcGTX2/3, M1, M3, M5 and two new compounds M6 and dcM6 are also formed as side-products. In addition, it was found that M1 could transform to M3 and M5 under the same conditions. However, M5 was not formed from M3. These observations suggested that the M1 to M5 conversion may be at least partly due to chemical transformation in stored tissue samples. The observation that M5 can be formed by chemical reaction allowed the production of a larger quantity for detailed MS and NMR studies.
M5 was initially thought to be an isomer of M1 with an $[M+H]^+$ ion at *m/z* 396. The product ion mass spectrum (*m/z* 396>316>) of M5 was very different than that of M1 (Fig. 3), indicating a substantial difference in structure such as an opened ring system.

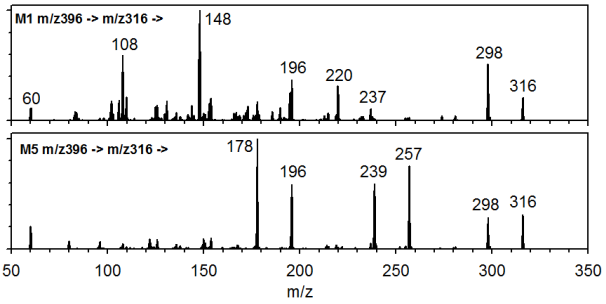


Fig. 3. Positive ion electrospray MS/MS product ion spectra for M1 (top) and M5 (bottom).

Further investigations revealed that a very weak ion at *m/z* 414 was the true $[M+H]^+$ ion, fragmentation of which gave the same spectrum as in Fig. 3 (bottom). This very strong loss of H_2O from the $[M+H]^+$ ion is quite unusual in electrospray MS on Sciex 4000 instruments. High resolution MS gave a free base molecular formula of $C_{10}H_{19}N_7O_9S_1$. NMR measurements (^{1}H, ^{13}C) indicated a novel structure in which one ring of M1 has been opened (Fig. 4). Further details of the structure elucidation will be detailed elsewhere.

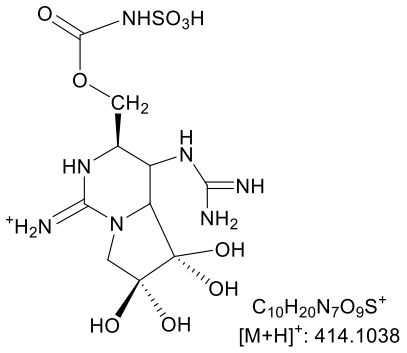


Fig. 4. Proposed structure of M5.

Feeding experiments revealed a drastic change in toxin profile after ingestion of algae by the mussels (Fig. 5). The kinetics of uptake and conversions will be presented in a separate paper. From the data in Fig. 5, it can be observed that epimerization of C2 to C1, GTX4 to GTX1, and GTX3 to GTX2 occurred. The sum of C1+C2 was reduced significantly, which supported C2 being the precursor of the metabolites. M1 and M3 were the principal compounds present in the mussels at 43% and 6.5% mole percent, respectively. M2 and M4 were observed. M5 was not present initially but did appear after sample storage (~5 d).

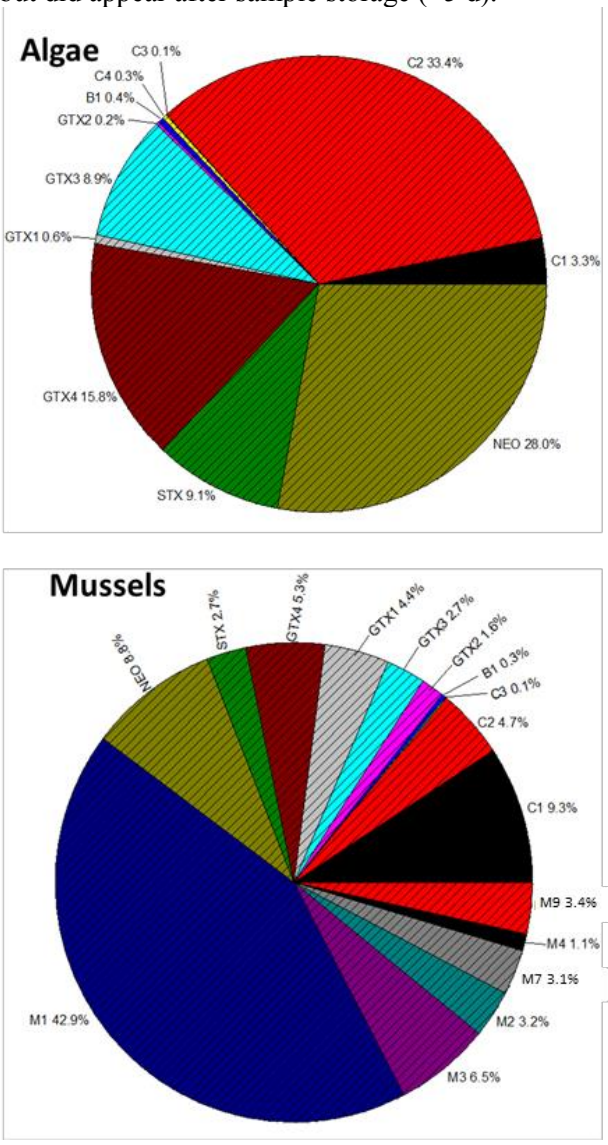


Fig. 5. Toxin profiles plotted as mole % in algal culture and in mussels 168 h after initiation of feeding.

Two additional compounds were detected in this experiment, coded as M7 and M9. LC-MS/MS data indicated that these compounds were N1-hydroxy analogues of M1 and M3, respectively. Preparative isolation of M9 and NMR analysis confirmed the structure as 11,11-dihydroxy-N21-sulfocarbamoyl-neosaxitoxin. M7 and M9 were probably formed from C4, which was present in the alga.

Additional chemical conversion experiments and analyses of older, stored mussel tissues revealed a number of other analogues (see Table 1) such as M6 and dcM6, which would result from *N*-desulfation and decarbamoylation of M5, respectively. Decarbamoyl analogues of M2 and M4, coded as dcM2 and dcM4, have also been detected. The N1-hydroxy analogues of all these compounds have been detected and given codes as M7-M12, dcM8, dcM10 and dcM12. The highlighted section of Table 1 shows the compounds that have not been reported previously.

A proposed mechanism of formation for analogues M1-M6, dcM4 and dcM6 is presented in Fig. 6. The same approach would apply to the N-hydroxy series. The production of M1 and M3 is probably due to a combination of enzymatic and chemical transformations, while the production of M5 is primarily due to chemical conversion.

Conclusions

Many new metabolites related to M1-M4 have been discovered and structures were postulated based on LC-MS/MS analyses and a combination of chemical and *in vivo* conversions. Full structure elucidation of both M5 and M9 has been achieved using NMR. This work has established that the metabolism of PSTs is very complex. It is valuable to know the identities of these compounds as they may contribute to shellfish toxicity. It is also important for a full understanding of the uptake, metabolism and depuration of PSTs after ingestion of toxic algae by shellfish.

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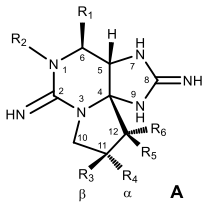
Table 1. Structures of common and new PSTs. The α analogue structures in parentheses have not been observed directly as they do not resolve from the β isomers but both are expected to be present. The highlighted section indicates new compounds not previously reported.



					R1:	N-sulfocarbamoyl	Carbamate	Decarbamoyl
Ring	R2	R3	R4	R5	R6			
A	H	H	H	OH	OH	GTX5	STX	dcSTX
A	H	H	OSO3H	OH	OH	C1	GTX2	dcGTX2
A	H	OSO3H	H	OH	OH	C2	GTX3	dcGTX3
A	OH	H	H	OH	OH	GTX6	NEO	dcNEO
A	OH	H	OSO3H	OH	OH	C3	GTX1	dcGTX1
A	OH	OSO3H	H	OH	OH	C4	GTX4	dcGTX4
A	H	H	OH	OH	OH	(M1α)	(M2α)	(dcM2α)
A	H	OH	H	OH	OH	M1β	M2β	dcM2β
A	H	OH	OH	OH	OH	M3	M4	dcM4
A	OH	H	OH	OH	OH	(M7α)	(M8α)	(dcM8α)
A	OH	OH	H	OH	OH	M7β	M8β	dcM8β
A	OH	OH	OH	OH	OH	M9	M10	dcM10
B	H	OH	OH	OH	OH	M5	M6	dcM6
B	OH	OH	OH	OH	OH	M11	M12	dcM12

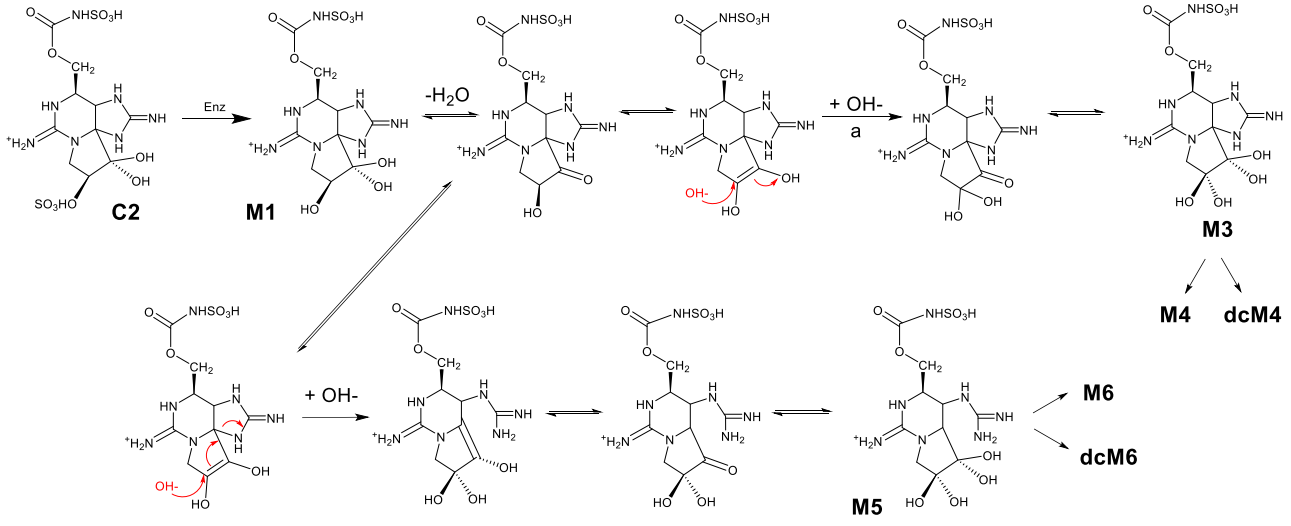


Figure 6. Proposed mechanisms of formation of the various metabolites.

Benzoyl analogs of the dinoflagellate *Gymnodinium catenatum* from the Gulf of California and the Pacific coast of Mexico as characterized by LC-MS/MS and NMR

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Abstract

Gymnodinium catenatum is a chain-forming marine dinoflagellate, notorious for formation of harmful algal blooms (HABs) and production of paralytic shellfish toxins (PSTs), including a wide array of neurotoxic analogs of saxitoxin (STX). Enhanced research efforts linked to improved detection and structural elucidation techniques have led to discovery of a new group of STX derivatives, named GC toxins or benzoyl analogs. We fractionated extracts of *G. catenatum* cultures by column chromatography and analyzed semi-purified extracts by hydrophilic interaction liquid ion chromatography coupled with tandem mass spectrometry (HILIC-MS/MS) and nuclear-magnetic resonance (NMR). We confirmed the presence of 15 of the 18 theoretical benzoyl toxins in isolates from the Pacific coast of Mexico. This is the first record of such high relative and quantitative richness of the benzoyl toxins among cultured isolates. Benzoyl toxins are not routinely monitored in shellfish and this might be a risk in seafood safety programs that rely exclusively on chemical analytical methods.

Keywords: *Gymnodinium catenatum*, benzoyl toxins, HILIC-tandem mass spectrometry, emerging toxins

Introduction

The naked chain-forming dinoflagellate *Gymnodinium catenatum* Graham is a neurotoxin-producing marine planktonic microorganism, capable of forming harmful algal blooms (HABs). Blooms of this dinoflagellate have caused significant poisonings of marine fauna and human consumers of bivalve mollusks that accumulated the toxins from the plankton they fed upon (Morey-Gaines, 1982). The species is distributed in both hemispheres, but occurs primarily in temperate and sub-tropical coastal waters; in Mexico, for example, it frequently produces HABs along the Pacific coast (reviewed by Band-Schmidt et al., 2010).

Among global populations, *G. catenatum* has been reported to produce a diverse array of saxitoxin (STX) analogs, defined by their chemical structures as carbamoyl, decarbamoyl and sulfocarbamoyl analogs. A total of 18 relatively hydrophilic STX analogs were previously found among various *G. catenatum* cultured strains and natural populations. Cell toxin content and compositional profiles vary widely among different strains, apparently depending on physical oceanographic conditions, growth stages and nutritional factors (Hallegraeff et al., 2012). Nevertheless, the toxin profile within individual strains is genetically determined and tends to remain more or less consistent over time and under various culture conditions (Bustillos-Guzmán et al., 2012). No known isolate produces the complete toxin spectrum, although all isolates typically produce more than one, and usually several, analogs (Cembella, 1998).

Enhanced search efforts and improved detection and structural elucidation techniques, has led to the recent discovery of a new group of benzoyl analogs produced solely by this dinoflagellate species. The benzoyl GC toxins are considered to

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be relatively "hydrophobic" analogs (Negri et al., 2003). These 18 hypothetical GC analogs contain a benzoyl moiety on the lateral chain instead of the carbamoyl group present in STX (Fig. 1). Benzoyl analogs have been found in varying proportions in most *G. catenatum* strains analyzed from locations around the world, (Negri et al., 2007; Vale, 2010; Hallegraeff et al., 2012), including from the Gulf of California and the Pacific Coast of Mexico (Bustillos-Guzmán et al., 2012; Durán-Riveroll et al., 2013).

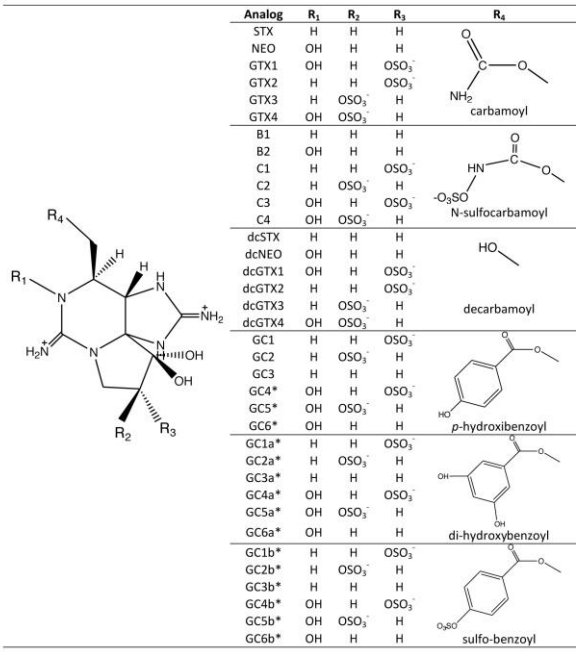

Fig. 1. Chemical structures of saxitoxin and analogs produced by *Gymnodinium catenatum.* Structures marked with (*) have not been fully characterized in previous studies but their probable structures were inferred based upon mass spectrometry by Vale (2008).

The objective of this study was to identify and report on the richness of benzoyl analogs from *G. catenatum* isolates from the Gulf of California and the Pacific coast of Mexico.

Materials and Methods

Isolates from the Gulf of California and the Pacific coast of Mexico were collected from natural samples and cultured in GSe medium (Blackburn et al., 2001) in seawater, supplemented with earthworm soil extract. Culture medium was prepared with chemically sterilized seawater (17 mg L^{-1} $Ca(ClO)_2$, 90%). Nutrients were autoclaved at 121°C for 15 min, and vitamins were filter-sterilized and added afterwards. Cultures were maintained at 24 ± 1 °C on a 12:12 h light:dark regime under 150 µmol m^{-2} s^{-1} illumination from cool-white fluorescent lights in a temperature-controlled growth room. Cell biomass of 300 L culture was harvested with 20 µm mesh and pooled. The average cell concentration was 1.5 x 10^6 cells L^{-1}, 4.5 x 10^8 total cells. Cell suspension was centrifuged at room temperature for 15 min and 1500 x g.

Toxin extraction was performed by ultrasonicating with 28 mL of 0.17 M acetic acid, and subjecting the biomass to two freeze-thaw cycles. The extract was centrifuged for 15 min at 1500 x g and the supernatant was lyophilized.

The toxin extract was suspended in deionized distilled water and filtered (Whatman GFF 24 mm). A chromatographic glass column (70 cm height x 4.5 cm i.d.) packed with 100 g C-18 silica gel (Bulk C-18 Flash, FCO140100-0, Agela Technologies, USA) was used for the first fractionation. Fraction 1 (F1) was obtained with 100% deionized distilled water; F2 with 20% v/v MeOH; and F3 with 100% MeOH. The aqueous residues of the fractions were lyophilized and MeOH was removed by rotary evaporation at 40 °C.

Mass spectral experiments to detect GC analogs were performed on a triple quadrupole mass spectrometer (API 4000 QTrap, AB Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray interface, coupled to a liquid chromatograph (1100, Agilent, Waldbronn, Germany). The liquid chromatograph included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B) and temperature-controlled column oven (G1316A), with a 5 µm 150 x 4.6 mm ZIC-HILIC column stationary phase held at 35 °C. Mass spectrometric analyses for GC analogs were carried out by addition of GC-toxin transitions to the method of Diener et al. (2007).

Fractions 2 and 3 (20 and 100% MeOH, respectively) were refractionated by preparative chromatography on a Knauer chromatograph, equipped with a 10 mL pump, exchange valve, degasser and mixing chamber. The same ZIC-HILIC column was loaded with consecutive 50 µL injections, and operated at ambient temperature (~20 °C), with otherwise identical parameters as in the previous analyses. The flow rate was 0.7 mL min^{-1} for a total run time of 45 min.

Fractions were collected and lyophilized prior to further analysis by HILIC-MS/MS to determine

toxin composition for continued stepwise preparative fractionation.

^{1}H- NMR experiments were performed at 25°C in a Varian 500 spectrometer with 50 transients for each experiment, according to standard Varian ^{1}H sequence pulses. Exchanged MeOH intense signal was suppressed by presaturation at 4.54 ppm.

Results and Discussion

In Fractions 2 and 3 (F2 and F3), we identified 15 out of the 18 benzoyl analogs proposed by Vale (2008). Fraction 1 (F1), obtained with 100% water, was not analyzed because it presumably contained only the called "hydrophilic" analogs (carbamoyl, decarbamoyl, and sulfocarbamoyl analogs), and we were looking specifically for the benzoyl derivatives. Even though GC toxins have been considered and reported as "hydrophobic analogs" in almost all literature, we demonstrated that this terminology is inaccurate. The chromatographic behavior of benzoyl analogs revealed that they are not strictly hydrophobic, with certain "hydrophilic" analogs, such as C toxins showing retention times characteristic of slightly less hydrophilic behavior than most benzoyl analogs (except for GC3).Given the hydrophilic characteristics of all STX analogs, we propose to group them based upon structural consideration of their respective side chains, i.e., as carbamoyl, decarbamoyl, sulfocarbamoyl, or benzoyl analogs. The latter group can be further sub-divided into hydroxybenzoyl, di-hydroxybenzoyl and sulfo-benzoyl analogs.

Unequivocal identification of all GC analogs was not possible by mass spectrometry alone because some analogs share the same mass-to-charge (*m/z*) ratio. For example, analogs GC4 - GC5 and GC1a - GC2a, all show *m/z* of 489 because they all have the same substituent but at different positions in the molecule.

Fraction 2 (F2, 20% MeOH) yielded 12 GC analogs, namely GC1/2 (*m/z* 473), GC4/5, GC1a/2a (*m/z* 489), GC4a/5a (*m/z* 505), GC1b/2b (*m/z* 553), and GC4b/5b (*m/z* 569) (Fig. 2).

Several GC toxin analogs were present in the four sub-fractions; these were therefore pooled and re-fractionated in an attempt to improve purity. Two of these pooled sub-fractions (Ps-f 3 and 4), showing *m/z* 489, were subjected to ^{1}H-NMR spectroscopy analysis to determine if their structures correspond to the analogs GC4/5 or GC1a/2a. ^{1}H-NMR results of Ps-f 4 allowed confirmation of the di-hydroxylated benzoyl derivatives (Durán-Riveroll et al., 2017). Results from this fraction showed a probable AMX pattern for three protons, consistent with the presence of a 3,4-dihydroxylated benzoyl ring. These findings could be interpreted to correct the 2,4-dihydroxylated structure previously proposed (Vale, 2008) for the GCa benzoyl analog series, but more research is needed. These structures could also correspond to new benzoyl analogs, produced by these Mexican strains and different from those produced by *G. catenatum* isolates from Portugal, from where the structures were first proposed.

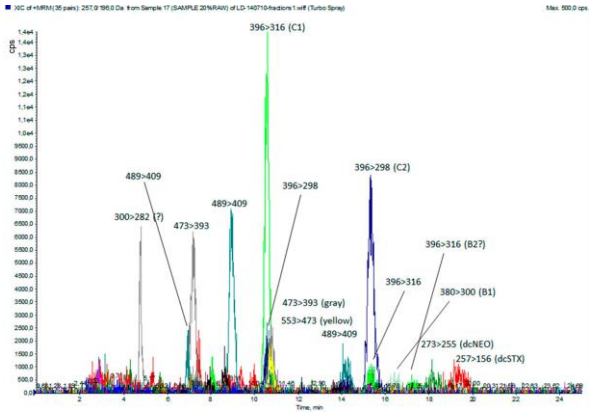

Fig. 2. HILIC-MS/MS chromatogram of F2 (20% MeOH) with selected reaction monitoring (SRM) in positive ion mode. Not all the *m/z* values reported in the Results are indicated in this chromatogram because some analogs were only detectable after further purification.

Fraction 3 (F3, 100% MeOH) yielded 3 benzoyl analogs: GC3 (*m/z* 377), GC6 and GC3a (*m/z* 393) (Fig. 3). None of these sub-fractions were subjected to ^{1}H-NMR.

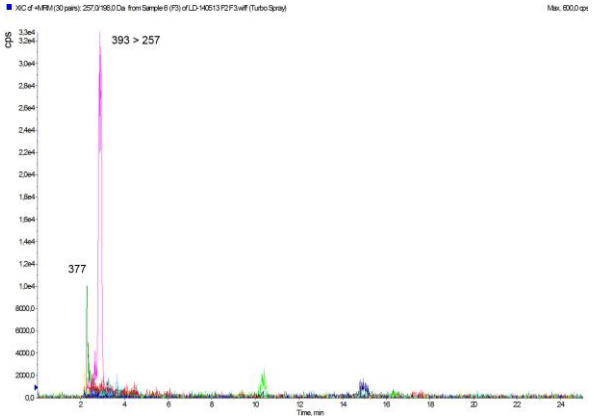

Fig. 3. HILIC-MS/MS chromatogram of F3 (100% MeOH) with selected reaction monitoring (SRM) in positive ion mode. The detected *m/z* corresponding to toxins are indicated.

Altogether, the confirmed benzoyl analogs from *G. catenatum* isolates from the Gulf of California and the Pacific coast of Mexico contribute 15 benzoyl derivatives to the known array of naturally produced toxins by this species in Mexican waters. This is the first record of such high relative richness of the benzoyl toxins among populations of *G. catenatum*.

Application of HILIC-MS/MS provides the opportunity to explore toxin variation among populations of *G. catenatum* that include a GC-toxin component. Previous research on these analogs has been hampered mainly by the lack of analytical standards (Vale, 2008; 2010).

Our current chromatographic approach coupled with tandem mass spectrometry and proton NMR spectral analysis of the GC-toxins contributes to the scarce structural information about these benzoyl analogs from *G. catenatum*. To our knowledge, this work represents the first confirmation and at least partial structural elucidation of 15 of 18 putative GC analogs from this marine dinoflagellate.

Mammalian toxicity of the GC analogs remains unknown, but some studies *in vitro* (Llewellyn, 2004) and *in silico* (Durán-Riveroll et al., 2016) have suggested potent neurotoxicity. This highlights the necessity for further research on purification and structural elucidation of these emerging toxins to determine mammalian toxicity, general bioactivity, chemical properties and ecological risks.

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Physico-chemical and functional characterization of Portimine purified from *Vulcanodinium rugosum* strain IFR-VRU-01

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Abstract

Vulcanodinium rugosum, found in the Mediterranean lagoon of Ingril (France) and in the coastal zones of New Zealand, Australia Japan and China, may now be spreading through maritime commerce. While a French *V. rugosum* isolate produces pinnatoxin-G and South Pacific *V. rugosum* strains produce pinnatoxin-E, F, G and H, the novel *V. rugosum* isolate isolated from a ship ballast tank in the USA mainly produces portimine, a toxin that is common to several *V. rugosum* isolates. Portimine is a polycyclic ether toxin containing a five-carbon imine ring (m/z 402.22804). Although less toxic to mice by intraperitoneal injection than other cyclic imine toxins, portimine exhibits potent cytotoxicity against different cell lines. We have found for the first time that *V. rugosum* (French isolate) also produces portimine. Bio-guided purification of portimine was performed on dinoflagellate SPE fractions using a non-radioactive receptor-binding assay and preparative HPLC-UV. We have purified milligrams of portimine and characterized it by HRMS and 2D-NMR. Quantification of portimine was performed by NMR using chloroform and benzene as internal standards. Two-electrode voltage clamp recordings on *Xenopus laevis* oocytes showed that portimine acts as antagonist of human neuronal α7 nicotinic acetylcholine receptors.

Keywords: Portimine, cyclic imine toxins, pinnatoxin-G, nicotinic acetylcholine receptors, *Vulcanodinium rugosum*

Introduction

Two decades after the discovery of pinnatoxin-A (PnTX-A) from extracts of the shellfish *Pinna muricata* in Japan, (Uemura et al., 1995) a peridinioid dinoflagellate was identified as the producer of PnTX-E and -F in Northland, New Zealand (Rhodes et al., 2010). Nézan and Chomérat (2011), purified and characterized a dinoflagellate named *Vulcanodinium rugosum* IFR-VRU-01 isolated from Ingril lagoon, France. Then, *V. rugosum* was linked to PnTX outbreaks in New Zealand, Japan, Australia (Rhodes et al., 2011) and France (Hess et al., 2013). Since then, *V. rugosum* was reported in China (Zeng et al., 2012), Mexico (Hernandez-Becerril et al., 2013), Spain (Satta et al., 2013), United States (Garrett et al., 2014) and Qatar (Al Muftah et al., 2016).

V. rugosum IFR-VRU-01 (Fig. 1) is known to produce PnTX-G (Hess et al., 2013), while South Pacific *V. rugosum* strains produce PnTX-E, -F, -G, -H, and portimine (Rhodes et al., 2011; Selwood et al., 2014; Selwood et al., 2013). A strain of *V. rugosum* isolated from a ship ballast tank in USA produces portimine (Garrett et al., 2014). A common feature of cyclic imine toxins (CiTXs) (gymnodimines, spirolides, pinnatoxins, pteriatoxins, prorocentrolides, spiroprorocentrimine and portimine) is the presence of a five-, six- or seven-carbon imine ring, responsible for their potent antagonism towards nicotinic acetylcholine receptors (nAChR) (Araoz et al., 2011; Bourne et al., 2015; Stivala et al., 2015). CiTXs are neurotoxins of global distribution produced by *Karenia selliformis* (gymnodimine), *Alexandrium ostenfeldii* (spirolide) and *V. rugosum* (pinnatoxin, portimine). Shellfish accumulate CiTXs in their digestive glands and edible tissues constituting a primary vector for their transfer to humans. CiTXs are fast acting neurotoxins provoking mice death by respiratory arrest through blockade of nAChR-mediated neurotransmission after intraperitoneal. or oral administration.

nAChR are cation-selective transmembrane pentameric proteins activated by acetylcholine. Muscle nAChRs mediate fast neuro-transmission at the neuromuscular junction, leading to skeletal muscle contraction necessary for escape or

respiration. Therefore, they are primary targets for a wide array of toxins including CiTXs. At the CNS, nAChRs modulate neurotransmitter release participating in fundamental aspects of synaptic plasticity. Neuronal α7 and α4β2 nAChRs are validated drug targets for several disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia, drug addiction, (Albuquerque et al., 2009; Changeux, 2010; Dani et al., 2007).

Herein, we monitored the fractionation of a *V. rugosum* (IFR-VRU-01) extract with a microplate receptor-binding assay coupled to MS analysis and found that this strain produces PnTx-G as well as portimine. Portimine was purified and characterized by HR-MS and 2D-NMR. We show that portimine is a weak antagonist of nAChRs.

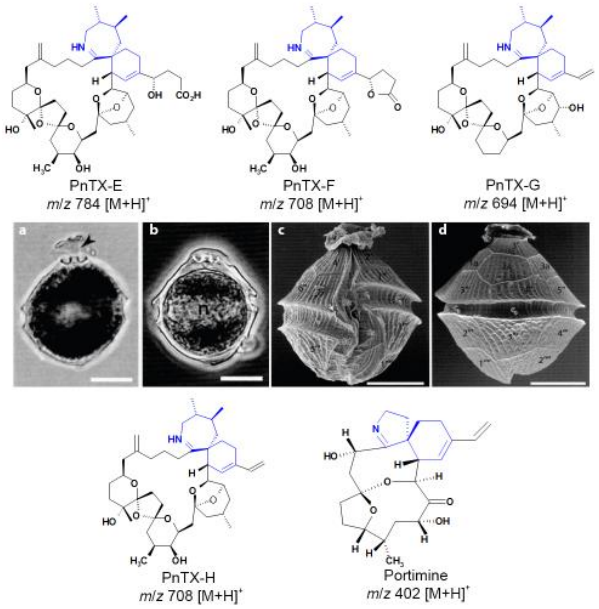

Figure 1. *V. rugosum* produces PnTX-E, -F, -G, -H and portimine. Central panel: Light micropgraphs of **a**. *V. rugosum* showing an apical mucous matrix extrusion, and **b**. a cell showing the nucleus (n). **c** and **d**: scanning electron micrograph of ventral and dorsal view of *V. rugosum*. (Adapted with permission from Nézan, E. & Chomérat, N. (2011).

Material and Methods

Culture conditions and Toxin extraction

V. rugosum IFR-VRU-01 was cultured in batches of 200 ml in L1 medium (Mediterranean sea water filtered with a 0.2 µM filter unit) at a photon flux density at 200 µmol m^{-2} s^{-1} and 16 h light/ 8 h dark. Cells from stationary phase cultures were harvested by centrifugation (3500g, 20 min, 4°C), lyophilized and stored at -20°C. One g of lyophilized cells was disrupted in 100 ml ethanol by sonication. The homogenates were centrifuged (5000*g*, 15 min, 4°C) and the supernatants were passed through 0.2 µm filters. The extracts were stored at -20 °C until use.

Bio-guided portimine purification

Aliquots of 100 ml of 10-times diluted extracts were loaded by gravity onto 5g-C18 SPE columns previously conditioned with 60 ml MeOH and 100 ml H_2O. The SPE columns were successively washed with 60 ml of H_2O, 10%, 25% and 100% MeOH. After drying, the fractions were dissolved in 5 ml MeOH. Microplate-receptor binding assay for detection of CiTX was done as described (Aráoz et al., 2012). Analytical HPLC was done using an X Bridge BEH 300 C18 column (5 µm 4.6 × 250 mm). Preparative HPLC was done using an X Bridge Prep C18 column (5 µm OBD 19 × 150 mm) with UV monitoring at λ 230 nm and a flow rate of 10 ml min^{-1}. The retention time of portimine in the preparative column was 20 min when separated with an isocratic gradient of 20% acetonitrile, 0.1 % TFA.

Mass spectrometry

Positive SPE fractions by microplate receptor-binding assay were analyzed by microLC/ESI-MS using a Thermo Accucore C18 column (2,6 µm 300 Å, 2.1 x 50 mm) at a flow rate of 200 µl min^{-1} and a gradient of 5-95% **B** in 20 min (**A**: 0,1% TFA/ H_2O; **B**: 0,09% TFA/ACN) controled by an Agilent 1100 Series LC system (Santa Clara, CA) coupled on-line to an Esquire HCT ion trap mass spectrometer equipped with an Electrospray Ionization (ESI) source (Bruker Daltonik, GmbH, Germany). Purified portimine was structurally characterized by infusing the sample at a flow rate of 3 µl min-1 in ESI-MS^n positive mode using the same MS ion trap system. ESI was done in positive mode with a nitrogen nebulizing gas set at 9 psi, a drying gas at 5 µL min-1 and a drying temperature at 290°C. Ionization and mass analyses conditions (capillary high voltage, skimmer and capillary exit voltages and ion-transfer parameters) were tuned for optimal detection of compounds in the 50-500 m/z range. In MSn mode, precursor ion was isolated with a 2 *m/z* width, fragmentation process was optimized using Bruker SmartFrag technology. Full scan MS and MS^n spectra were acquired with Esquire Control software processed with Data Analysis software (Bruker Daltonik, GmbH, Germany).

NMR

NMR experiments were performed at 20°C on a Bruker DRX700 spectrometer equipped with a triple resonance cryoprobe. NMR data were processed with Topspin 1.3 software (Bruker Biospin, Germany) and analysed with CcpNmr

Analysis software34. ^{1}H and ^{13}C resonance of portimine were previously assigned by Selwood et al. (2013). ^{1}H NMR quantification of portimine was performed as described by Dr. Armen Zakarian (Araoz et al., 2011). Briefly, 7.0 µl of $CHCl_3$ and 7.0 µl of C_6H_6 were added to 7.0 ml of CD_3OD. Portimine was dissolved in 0.5 ml of the previous solution in a 2 ml glass flask. The whole was added to a NMR tube for quantification.

Two-electrode voltage-clamp

Oocytes, were harvested by repetitive laparotomy from *Xenopus laevis* females anesthetized with 0.1% tricaine as described (Araoz et al., 2011). Oocytes were injected with mRNA coding for α7 nAChR sub-unit. Automatic two-microelectrode voltage clamp recordings (HiClamp, MCS GmbH., Reutlingen, Germany) were performed after 1-3 days injection. An oocyte is withdrawn from a 96-wells oocyte-microplate and placed in a silver wire basket, which is also the reference bath electrode. Two microelectrodes filled with 3 M KCl (50 MΩ) automatically impaled the oocyte and imposed a holding potential of -60 mV. The oocyte is then transferred into a 96-wells test-microplate, filled with 200 µl ACh or portimine.

Results and Discussion

Bio-guided fractionation of a extract of *V. rugosum* IFR-VRU-01 followed by MS analysis showed a main analyte at *m/z* 402.22804. Further analysis of this purified compound by HR-MS and 2D-NMR led us to conclude that portimine was the major toxin produced by IFR-VRU-01 strain. PnTX-G was also present, but as a minor component. Portimine is common to several *V. rugosum* isolates described to date and the strain IFR-VRU-01 seems not to be the exception. Indeed, dereplication strategy based on HR-MS in full scan mode was used to unveil the chemical identity of bioactive molecules produced by the axenic *V. rugosum* strain IFR-VRU-01I (Geiger et al., 2013). In that exercise, the major compound within the crude extract and fraction F2 was a molecule with a *m/z* of 402.2281 $[M+H]^+$. Dereplication results suggested three isobaric compounds: nakijiquinone, N-carboxy-methyl Smenospongine and stachybotrin A (Geiger et al., 2013). As portimine had just been described by the time of that study (Selwood et al., 2013), it was not present in in-house or public databases.

To determine the accurate mass and fragmentation pattern of portimine, the toxin was subject to HR ESI MSn (MS 2-4) in the positive mode. We used the Thermo Scientific Mass Frontier spectral interpretation software for interpreting the resulting fragment ions.

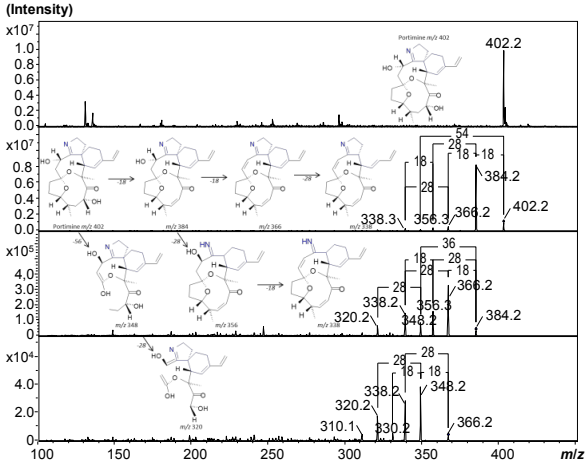

Figure 2. Hierarchical fragmentation pathway of portimine by MSn.

The first stage of ionization gave portimine molecular ion at *m/z* 402.22804. The fragmention of the molecular ion gave a daughter ion at *m/z* > 384.2 ($[MH - H_2O]^+$). Given the selective resonance of the precursor ion in the ion trap, the fragment ions do not fragment and were used as precursor ions for the next stage of ionization/ fragmentation. The fragmentation of *m/z* 384.2, gave a series of ions at m/z 366.2 $[(MH - H_2O) - H_2O]^+$, 356.3 $[(MH - H_2O) - C_2H_4]^+$ and 338.2 $[(MH - H_2O) - C_2H_4 - H_2O]^+$. Fragmentation of the ion *m/z* 366.2 gave three ions at m/z 348.2, 338.2, and 320.2 (Fig.2). The MS^n approach gave us an insight on the hierarchical fragmentation of portimine, which is important for structure authentication and is consistent with CID MS/MS spectrum of portimine by Selwood et al (2013).

NMR signal assignments for ^{1}H and ^{13}C were in concordance to Selwood et al. (2013). The slight differences in the chemical shifts of ^{1}H and ^{13}C were probably related to the solvent used for NMR: $CDCl_3$ by Selwood et al (2013), and CD_3OD in the present study. Eighteen of twenty three ^{13}C resonances of portimine are depicted in Fig. 3 including four of five olefinic carbons, a hemiacetal carbon (111 ppm), four oxygenated carbons (84, 79, 70, and 68 ppm) including a methylene carbon adjacent to the imine group (50.5 ppm). Alltogether, our HRMS and NMR data are consistent with the physicochemical features of portimine by Selwood et al. (2013). We quantified portimine by NMR using $CHCl_3$ and C_6H_6 as internal standards. The purification yield was 10 mg portimine 100 ml^{-1} ethanolic extract of *V. rugosum* IFR-VRU-01I.

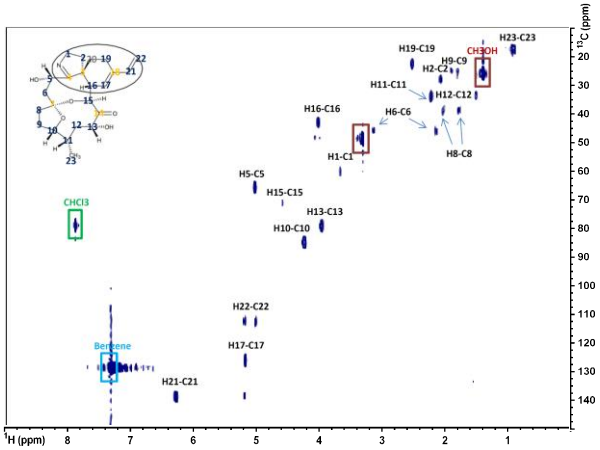

Figure 3. 2D 1H-13C HSQC NMR spectrum of portimine acquired at 700 MHz and 20°C.

We evaluated the biological activity of portimine on *Xenopus* laevis oocytes expressing the human neuronal α7 nAChR using automated two-electrodes voltage-clamp electrophysiology (Fig. 4). Maximal peak amplitude of the acetylcholine-induced current was reached less than 100 ms after immersion of the impaled oocyte in the ACh wells containing 100 µM ACh, after which α7 nAChRs became rapidly desensitized. Micro-stirrers ensured active exposition of the nAChRs to the test compound. Portimime by itself did not elicited nicotinic current responses showing that it is not an agonist of this type of receptors. But, in the presence of the 100 µM ACh, portimine reduced the peak amplitude in a dose-dependent manner. Complete blockade of ACh-induced current occured at 100 µM portimine (Fig. 4).

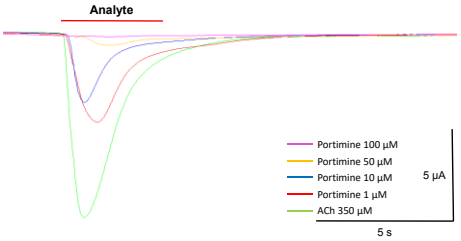

Fig. 4. Dose-response antagonistic activity of portimine towards α7 nAChR.

The microplate-receptor binding assay coupled to MS characterization led us to the finding that portimine (m/z 402.22804) was the major toxin produced by *V. rugosum* IFR-VRU-01 strain. The unknown compound from *V. rugosum* extracts and fraction F2 (m/z 402.2281) from the previous study (Geiger et al., 2013) associated with *in-vitro* cytotoxicity and genotoxicity in Neuro2 ad KB cells was thus probably portimine. This finding highlights the importance of using bioassays coupled to MS for the discovery of new bioactive compounds (Aráoz et al., 2012). Although less toxic to mice than other CiTXs, portimine antagonizes the neuronal α7 nAChR.

Acknowledgements

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Five years of application of the Receptor Binding Assay (RBA) on seafood products and threatened turtles during outbreaks HABs in El Salvador

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Abstract

In El Salvador, toxin producing harmful algal blooms have occurred annually over the last five years. With the establishment of response capabilities for species identification and phycotoxin analysis, public managers were able to make informed and timely decisions. Despite two extremely toxic episodes, no human fatalities were recorded during this period. Since 2011, the Laboratory of Marine Toxins, has implemented the use of the PSP receptor binding assay, an AOAC official method of analysis (OMA 2011.27), to determine concentrations of PSTs in bivalve molluscs. High concentrations of saxitoxins were measured across multiple shellfish species, in some cases exceeding 35 x the regulatory level of 80µg/100g. Saxitoxin activity was as high as 2,800 µg STX equivalents/100 g tissue in oysters (*Crassostrea iridiscens*) and 771 µg STX eq./100 g mussel (*Modiolus capax*) tissue. The PSP receptor binding assay has great versatility in its application, and was also used in El Salvador to measure STX in crabs, fish and phytoplankton during HABs. This method was successfully also applied to analysis of PSTs in samples from sea turtles during a 2013 mass mortality event, revealing STX-like concentrations as high as 730 µg STX equivalents/100 g intestines in *Chelonia mydas*.

Keywords: biotoxins, saxitoxin, red tide, receptor binding assay, bivalve, HAB, seafood safety

Introduction

El Salvador, located on the Pacific coast of Central America, has experienced 10 harmful algal bloom (HAB) events in the last 10 years. HABs in El Salvador are responsible for both human illness and deaths (Barraza 2009), and mortalities of sea turtles (Amaya *et al*. 2014). The phytoplankton species responsible for these events primarily has been *Pyrodinium bahamense* var. *compressum* (Espinoza *et al.* 2014).

Since 2011, the Marine Toxins Laboratory of the University of El Salvador (LABTOX-UES) has successfully implemented testing using the saxitoxin receptor binding assay (RBA), with the financial and technical support of the IAEA and NOAA, to provide a timely response to toxic HAB events. Data provided crucial information for making policy decisions based on the quantification of saxitoxins found in molluscs, which is vital to impose bans on their collection, sale and consumption to protect human health.

The saxitoxin RBA, the AOAC official method of analysis (2011.27), is a functional biological method used to determine the total saxitoxin-like activity in a sample. It is analogous to the mouse bioassay, listed as type IV method by the Codex Alimentarius Commission, and an accepted method by the United States Interstate Shellfish Sanitation Commission. At the same time, this method helps state institutions to control, inspect and/or regulate the toxicity of marine products destined to safeguard the health of the population.

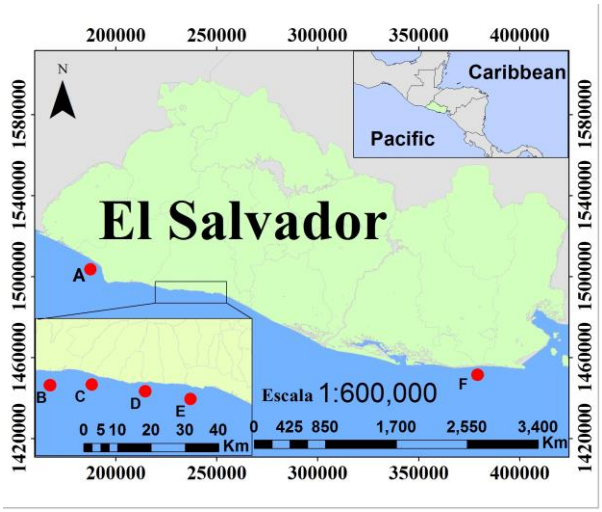

Fig. 1. The coastal area in El Salvador showing sites where saxitoxin contaminated samples were collected including oysters, crabs, mussels, sea snails, and turtles. The points indicate sampling sites on the beaches: **A**) Metalio, **B**) Mizata, **C**) Taquillo, **D**) El Zonte, **E**) El Pimental and **F**) El Cuco.

Material and Methods

Samples were collected in coastal areas affected by *Pyrodinium* HABs and were collected in collaboration with the National Red Tide Commission (CONAMAR), which is comprised of governmental institutions that focus on fisheries (CENDEPESCA-MAG), environment (MARN), and public health (MINSAL) work, and the non-governmental organization FUNZEL, which is committed to the conservation and protection of endangered species. Sampling criteria were based on collecting species of a size and weight that have a commercial value. Samples were collected from directly from impacted sites and in seafood markets. Sample types included oysters, mussels, clams, snails, and crabs. Additionally, different tissue types from pufferfish and sea turtles were analyzed.

The extraction of shellfish toxins was performed in LABTOX-UES using the acid extraction in the standard mouse bioassay for saxitoxins, modified only by scale: 5mL of 0.1M HCl was added to 5g of tissue homogenate (IAEA TECDOC 1729 2013). The saxitoxin RBA assay was performed as described with minimal variations for sample matrix (Van Dolah *et al.* 2012)

The saxitoxin receptor binding assay (RBA) is based on the interaction between saxitoxins and voltage-gated sodium channels (receptors). Tritiated saxitoxin competes with unlabeled saxitoxins in the extracts for a finite number of available receptor sites in a brain membrane homogenate. Unbound saxitoxins are removed by filtration and the remaining tritiated saxitoxin is measured with a scintillation counter. The amount of radioactivity present is inversely related to the amount of saxitoxin-like activity in the sample.

The essential equipment and materials needed to conduct this assay were: a microplate scintillation counter, variable volume single (1-1000 µL) and eight channel (5-200 µL) micropipettors, 96-well microtiter filter plates and a Multiscreen vacuum manifold. The reagents used were: [3H] STX standard solution (American Radiolabeled chemicals), STX di-HCl standard solution, 0.003M HCl, a MOPS/choline chloride buffer, rat brain (2011-2013) or porcine brain (2014-2015) membrane homogenates, and Optiphase liquid scintillation cocktail. Data analysis was conducted using Graphpad Prism software using non-linear regression with a variable slope, which is based on the Hill equation for competitive binding assays.

Results and Discussion

The range of concentrations of PSP toxins measured in molluscs using the saxitoxin RBA was 25-3000 µg STX equivalents / 100 g tissue. (Fig.2) The saxitoxin RBA was also able to provide a rapid response to mortality of marine turtles poisoned by saxitoxins during September 2013. A permanent monitoring program for marine phytoplankton has been established, along the country's 320km coastline; to date more than 300 species of microalgae have been identified along with their respective cellular concentrations, allowing to establish the seasonality and greater probability of occurrence of these phenomena. In 2011, this method was implemented to measure analytical concentration of paralyzing toxins, known as Receptor Ligand Assay (AOAC/OMA 2011.27). With this method, more than 200 analyzes have been performed on: shells, oysters, crabs, mussels, snails, sea turtles, and other species of marine origin. Analytical concentrations of paralyzing toxins up to 2800 µg eq STX/100 g were found in oyster tissue (Crassostrea iridiscens) exceeding 35 times the regulatory level (80 µg eq STX/100 g). Additionally the method was adapted to analyze

saxitoxins in dead sea turtles, demonstrating that turtle mortality was due to the consumption of toxic microalgae in its food chain occurred in 2013. To date we are in the process of accreditation of the method under the international ISO standard 17025: 2005.

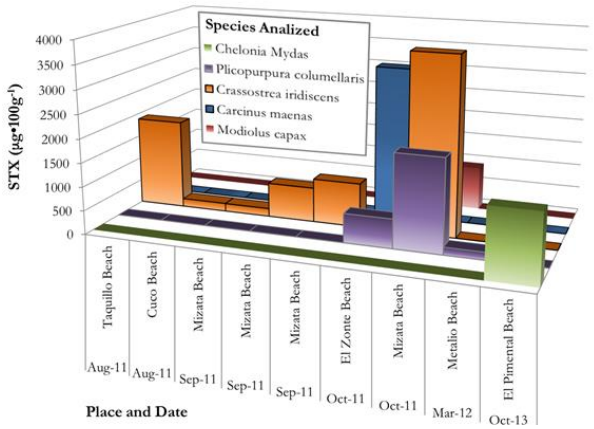

Fig. 2 Saxitoxin concentrations measured in molluscs and sea turtles using a saxitoxin RBA

The saxitoxin RBA has been successfully implemented in El Salvador at LABTOX-UES for measuring PSP toxins in shellfish and other important species, and has been providing information to governmental organizations for the last 5 years, this information strengthens the production and commercialization of molluscs to improve the economics of small producers engaged in the aquaculture industry.
In 2016 we participated in a laboratory inter-comparison proficiency test for PSP toxins in shellfish using the saxitoxin RBA. (WEPAL-QUASIMEME). The goal of LABTOX-UES is to pursue accreditation to ISO 17025/2005 standards, so fisheries products can be qualified and offered for sale in international markets. Harmful algal blooms have caused deaths, millions of losses to the local economy and serious environmental damage in El Salvador and Central America. Fortunately after creating the Marine Toxins Laboratory in 2007, El Salvador does not report deaths by Red Tides despite having suffered at least 5 toxic episodes and 6 ichthyotoxic episodes, occurring in the last decade (2007-2016).

Acknowledgements

This work was supported by the International Atomic Energy Agency (IAEA) Technical Cooperation regional project RLA7020 (with participants from 10 countries in the Central America and Caribbean Region), the IAEA National Technical Cooperation project ELS7007, and NOAA provided technical and scientific support to the program "Monitoring of the Marine Environment in El Salvador". This method has contributed to mitigate HAB impacts on human health and is being considered as a method for regulation of toxicity of marine products.
The International Atomic Energy Agency is grateful to the Government of the Principality of Monaco for the support provided to its Environment Laboratories.

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Paralytic Shellfish Poisoning and Pet Dogs in Southern Chile

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Abstract

An atypical *Alexandrium catenella* bloom in April-May 2016 along the Pacific coast (43°-39°) was associated with Paralytic Shellfish Toxins (PST), stranding of bivalve mollusks (*Mesodesma donacium*), bird and marine mammals' mortalities and significant social and economic impacts. At Maicolpué beach (40°34'52.3"S; 73°44'13.6"W), Los Lagos region, four pet dogs became intoxicated after licking kelp fronds (*Macrocystis pyrifera*; *Durvillaea antarctica*), suggesting PSP intoxication. No saliva, tissues nor stomach contents of dogs were collected but stranded kelps were examined. The fronds and stipes were colonised by the barnacle *Lepas australis*. Mouse bioassay and HPLC analysis on barnacles and wash water of kelp+barnacles, were conducted. The barnacles had between 107-178 µg STX eq. 100 g^{-1}, the wash water had 0.5 µg eq STX 100 g^{-1}. In *L. australis*, 95% of the PST were GTX2-3 and 5% STX; in the wash water, 53% were GTX2-3 and 48% STX. No *A. catenella* vegetative cells nor cysts in the wash water or adhered to barnacles or fronds surface were observed, but PSP might also be explained by exudates from shellfish tissues. Our results suggest that the licking of toxic stranded kelp+barnacles and the ingestion of toxic barnacles might explain the dogs' intoxication and the death of two of them.

Keywords: PSP, Pet dogs, barnacles, kelp seaweed, intoxication, *Alexandrium catenella*

Introduction

Along the open coast of the Pacific Ocean (43°-39°), to the North of the Chilean fjords area, an unexpected *Alexandrium catenella* bloom during April-May 2016 was associated with Paralytic Shellfish Toxins (PST). It took about forty years from South (55° S) to North since 1972 (Guzmán *et al*. 1975), for *A. catenella* and PST, to be detected as noxious events along the entire Chilean fjord system (55°- 41° S) (Lembeye *et al*., 1997; Molinet *et al*. 2003; Guzmán *et al*., 2016).
This unusual bloom along the open coast, caused PST contaminated shellfish, stranded bivalve mollusks, and mortality of birds and marine mammals, together with a significant social and economic impact.

In the past five years, walking along the beach with pet dogs has been a common occurrence in Chile, but until now there was no evidence of intoxications linked to macroalgae and invertebrate consumption on the beach. Since PST affects higher vertebrates such as birds and mammals, it is hypothesized that kelp fronds (*Macrocystis pyrifera*, *Durvillaea antarctica*) colonised by barnacles (*Lepas australis*) which were licked and eaten by affected pet dogs were contaminated with Paralytic Shellfish Toxins from a *A.catenella* bloom, causing the intoxication and deaths of two out of four pet dogs.

Material and Methods

Dog intoxication occurred on the evening of 15th April, 2016 at Maicolpué beach. The case was comprised of two pet dogs and two dogs of unknown ownership. No samples of poisoned dogs were collected, but PSP intoxication was suggested by the dogs' symptoms and a certificate provided by a veterinarian.
On the 16th April stranded kelp samples were collected at Maicolpué beach and Mansa Bay (Fig. 1) in the Northern area of Los Lagos region. The fronds and stipes of both kelps were colonised by a barnacle species (Fig. 2).
Toxin analyses by AOAC mouse bioassay (MB) (kelp wash water) were conducted at the Environmental Laboratory, Health Department from Los Lagos region based at Puerto Montt city, and LC-FLD analyses at the Red Tide Laboratory,

Instituto de Fomento Pesquero located at Punta Arenas city, were performed. Acidic extracts of kelp wash water from barnacles and kelp+barnacles, and meat extracts from barnacles were analyzed.

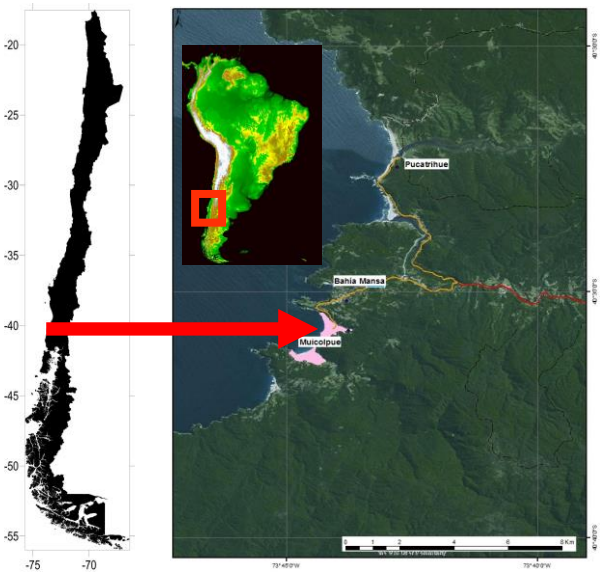

Fig. 1. Sampling area at Maicolpué beach near Mansa Bay, Northern sector of Los Lagos region (40°34'52.3"S; 73°44'13.6"W). Beach where the dogs were intoxicated.

Fig. 2. *Macrocytis pyrifera* fronds colonised by *Lepas australis*. Photographs taken by Hernán Pacheco.

Results and Discussion

The owner described, "in the last five years my dogs have licked and chewed macro-algae and eaten small bivalves from the beach, with no effects to my dogs", "After an hour ride, on our return, after five minutes one dog had vomiting, fainting, sudden cardiac arrest and death; other dog had vomiting and severe stomach pain, tremors and imbalance and taken to a veterinarian". The veterinarian description includes vomiting and acute abdominal pain with severe bowel spasms, and although food poisoning was suspected no further diagnostic was provided.

Of the other two dogs, which were not her property, one died with similar symptoms and the other presented acute abdominal pain. The dogs' intoxication was most unusual.

The stranded macroalgae were kelps, identified as *Macrocytis pyrifera* and *Durvillaea antartica*. The organism growing on top of kelp fronds and stipes were barnacles identified as *Lepas australis*.
The kelps were washed and no vegetative cells or cyst of *A. catenella* in the wash water were observed, but PST toxins were detected by HPLC and Mouse Bioassay.

According to HPLC and Mouse Bioassay barnacles had between 107-178 PSP 100 g^{-1} and between 163-181 µg eq. STX-2HCl 100 g^{-1}, respectively. Most of the toxins were gonyaulatoxins (95%; GTX 2-3) and a small amount of saxitoxin (5%; STX) (Table 1).

The wash water of kelp+barnacles presented 0.5 PST µg 100 g^{-1} (= 25 µg eq. STX-2HCL 100 g^{-1} by LC-FLD) or 118 µg STX eq. 100 g^{-1} (MB). The differences can be explained by extraction modes, being more intensive with Mouse Bioassay, since extraction considered 100 g of macroalgal tissue, maintained during 4 hours in fresh water and then extracted following the Mouse Bioassay protocol. In contrast HPLC analysis was conducted on water samples used to wash the external walls of kelp fronds and stipes, reflecting a lower toxin concentration. LC-FLD analyses showed 53% (GTX2-3) and 48% (STX) (Table 1).

The combined results suggest that both the licking of stranded kelp or kelp+barnacles and the ingestion of toxic barnacles can explain the dogs' intoxications and death of two of them. The mucilage exudated by macroalgae could serve as an attachment for motile stage and cysts of *A. catenella*, but negative results were obtained by microscopic analysis. But PST was detected by HPLC and Mouse Bioassay, and its presence might be explained by *A. catenella* cells adhering to barnacles and to the fronds' surface, and exudates from shellfish tissues.

Table 1. PST concentration (**A**) and Toxicity (**B**) in mouse units associated to an *Alexandrium catenella* bloom. Kelps: *Macrocystis pyrifera* (**Mp**); *Durvillaea antarctica* (**Da**); Barnacle: *Lepas australis* (**Lp**). Wash water (**Ww**). Percentages are indicated in parentheses.

A) PST-complex concentration (µg toxin 100 g^{-1})

Sample	Locality	STX	GTX2	GTX3	C1	C2	Total PSP
Ww (Mp+Lp)	Mansa Bay	0.2 (47)	0.1 (29)	0.1 (24)	-	-	0.5
Lp on **Mp**	Mansa Bay	21 (19)	5 (4)	82 (76)	-	-	107
Lp on **Da**	Maicolpué	23 (13)	11 (6)	143 (81)	-	-	178

B) PST-complex toxicity (µg eq. STX.2HCL 100 g^{-1})

Sample	Locality	STX	GTX2	GTX3	C1	C2	Total PSP
Ww (Mp+Lp)	Mansa Bay	17 (71)	3 (12)	4 (17)	-	-	25
Lp on **Mp**	Mansa Bay	55 (34)	3 (2)	104 (64)	-	-	163
Lp on **Da**	Maicolpué	44 (24)	6 (3)	131 (72)	-	-	181

The usual practice of walking with dogs for a relatively long time along the beach, without adverse effects for dogs and their usual behaviors of licking and eating remains of algae and invertebrates stranded on the beach, points that the intoxication linked to a bloom of *A.catenella* associated to PSP was an unusual. This event was the first *A. catenella* bloom along the Chilean Pacific coast beyond the fjord system, being the most intensive bloom of this species in the Northern area of the fjord and moved to the North by advective processes.

Acknowledgements

To Ms Carol Barrientos-Miranda from Maicolpué, owner of two dogs, for her interest and valuable reported information. The financial support to attend 17th ICHA at Florianopolis, Brazil, came from HAB Management and Monitoring Programme at Los Lagos, Aysén and Magellan regions, Tenth Stage 2016-17. MINECON- 2016, Chile.

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Phylogenetic Analysis of Acetyl CoA Carboxylases in Dinoflagellates

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Abstract

Dinoflagellates are a diverse group of organisms that produce potent bioactive secondary metabolites. The molecular mechanisms by which these compounds are produced are not yet understood. Acetyl CoA carboxylase (ACC), is a necessary protein in polyketide and fatty acid synthesis. In this study, we survey thirteen dinoflagellate transcriptomes in addition to chlorophyte green algae, haptophytes, diatoms, and alveolates to characterize ACC in these organisms. Our analysis reveals that dinoflagellates express only ACC with homomeric, eukaryotic domain architecture, including the eukaryote specific central domain. Streptavidin western blotting shows many biotinylated proteins as well as bands close to the calculated ACC size in three dinoflagellate species. Two major clades of dinoflagellate ACC were found, both within the previously defined plastidial type of homomeric ACC. One clade exclusively includes peridinin pigmented dinoflagellates. The other clade contains all thirteen dinoflagellate species including those without peridinin chloroplasts as well as non-photosynthetic dinoflagellates. Based on phylogeny and chloroplast transit peptides, these two clades likely represent two different ACCs where one is putatively plastid while the other is cytosolic.

Keywords: Dinoflagellates, Acetyl CoA Carboxylase. Phylogeny, Toxin Synthesis, Fatty Acid Synthesis

Introduction

Dinoflagellates are a genetically complex phylum including many toxin producing species that are major contributors to harmful algal blooms (HABs) worldwide (Gómez 2012; Place et al. 2012). The mechanisms by which these products are synthesized are still not known. A necessary component, and rate limiting step, of both fatty acid and polyketide synthesis is Acetyl CoA Carboxylase (ACC). ACC is responsible for the conversion of acetyl CoA to malonyl CoA which commits the substrate to go through the FAS or PKS cycles (Hopwood and Sherman 1990; Tong 2013). Recently a description of the ACC in the basal dinoflagellate *Amphidinium carterae* based on domain arrangement, expression profiles, and mass spectrometry proteomics has been made (Haq et al. 2017). As with other carboxylating enzymes, a biotin carrier is first charged followed by transfer of the carbon onto the acetyl CoA. In eukaryotes, a homomeric form exists as a large polypeptide consisting of sequentially arranged functional domains connected by a central domain unique to eukaryotic ACC (Hunkeler et al. 2016). The chloroplast and cytosolic versions can have different evolutionary histories in different algal groups. For example, in land plants the cytosolic and plastid ACC are relatively closely related, while in most stramenopiles and apicomplexans the cytosolic and plastid ACC are found in two distinct clades (Huerlimann et al. 2015). Characterization of ACCs in dinoflagellates will prove to be an important step in elucidating FAS/PKS pathways.

Material and Methods

The previously identified *Amphidinium carterae* ACCs contained the conserved domains, carboxy loading domain (cd 06850), biotin carrier (COG0439), the central domain (pfam 08326), and a carboxy-transferase domain (pfam 01039), and were compared against twelve of the available dinoflagellate transcriptomes (del Campo et al. 2014), including the non-photosynthetic *Crypthecodinium cohnii* and *Oxyrrhis marina*. In addition, two atypically pigmented groups were

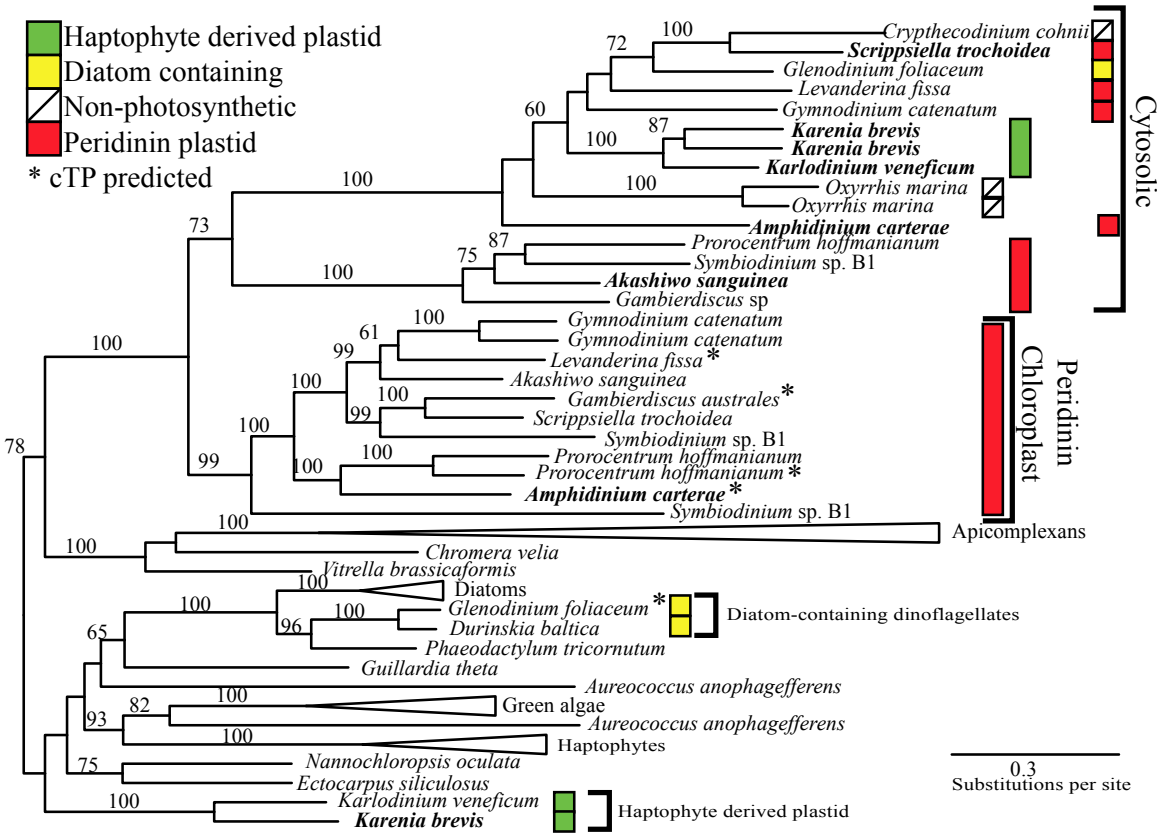

Fig 1: Maximum likelihood phylogeny of ACC from dinoflagellates, chromerids, apicomplexans, rapid bootstrap replicates above the branches when over 50%. Species marked with * indicate a chloroplast transit peptide (cTP) was found while species in **bold** indicate a spliced leader sequence was found. The bulk of the dinoflagellates contain peridinin-pigmented plastids, but two alternately pigmented groups were included with haptophtye and diatom derived symbionts. In addition, there are two non-photosynthetic species included in the tree.

sampled, the diatom-containing species *Glenodinium foliaceum* and *Durinskia baltica*, and the haptophyte-pigmented *Karenia brevis* and *Karlodinium veneficum* (Delwiche 1999). Initial analysis (data not shown) placed all the dinoflagellate ACC within the plastidial clade as defined by Huerlimann and Heimann in 2015; the plastidial sequences from additional outgroups included the apicomplexans, heterokonts, cryptophytes and green algae (Huerlimann et al. 2015). The ACC were aligned using Clustal Omega with the full-iteration mode and the alignment examined in Mesquite. The phylogenetic analysis was performed with RAxML using 1000 rapid bootstraps with the optimal JTT amino acid substitution matrix on an alignment of 2876 amino acids (TreeBase accession TB2:S21383). Amino acid sequences were analyzed using the ChloroP CBS predictive software to predict the presence of a chloroplast transit peptides. Sequences were analyzed and evaluated by cutoffs recommended by the CBS group (Emanuelsson et al. 2007).

The *A. carterae* (CCMP 1314) was grown in ESAW at a salinity of 32 (Berges et al. 2001), while *K. veneficum* (CCMP 2936) and *Akashiwo sanguinea* were grown in ESAW at a salinity of 15 in polystyrene culture flasks (Gunderson et al. 1999). The cultures were maintained under constant light at 50µm cm^{-2} s^{-1} with a 14:10 light:dark schedule. Cultures were grown to a high cell density and collected at mid-day time points. The cells from 50 ml of culture were centrifuged at 350 x g for 10 minutes at room temperature in a conical tube. The supernatant was poured off and the pellet was flash frozen in a bath of dry ice and ethanol and stored at -80°C. Cell pellets of *A. carterae*, *K. veneficum*, and *A. sanguinea* were re-

suspended in 1ml of 1X SDS sample buffer (50mM Tris-Cl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100mM dithiothreitol), boiled for 5 minutes at 95 °C, and centrifuged at 10,000 x g for 5 minutes. Samples were loaded at varying cell equivalents and separated on Novex NuPAGE 3-8% tris-acetate gels at 150 V (constant) for 1 hour. Proteins were transferred onto a PVDF membrane using the high molecular weight program on the Trans Blot Turbo Transfer System (Bio-Rad) for 14 minutes. The membrane was rinsed with deionized water and blocked with 1X iBind solution (Life Technologies) for 1 hour at room temperature. The membrane was incubated with a streptavidin-HRP conjugate (Invitrogen) using the iBind Western Device (Life Technologies) per manufacturers protocol at a dilution of 1:1000. The blot was incubated for 5 minutes in Clarity ECL Substrate (Bio-Rad) and imaged on the ChemiDoc Touch Imaging System by Bio-Rad.

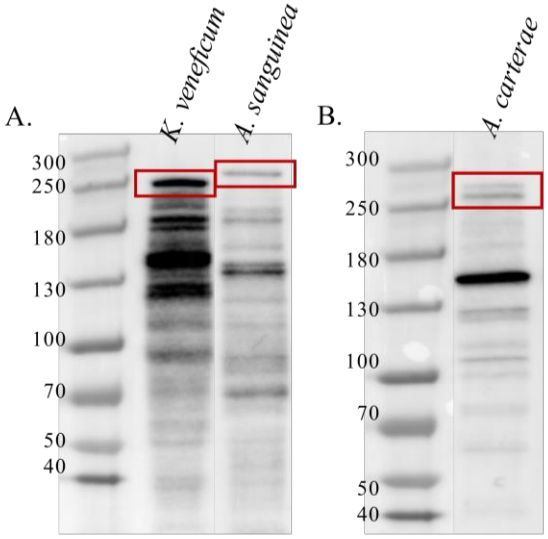

Fig 2. Natively biotinylated proteins were visualized with western blotting using streptavidin-HRP conjugate. **A.** *K. veneficum* and *A. sanguinea* were run at concentration of 304,000 and 14,400 cell equivalents respectively. **B.** *A. carterae* was run at a concentration of 193,200 cell equivalents. The putatively assigned ACC region is shown in a red box for all species.

Results and Discussion

The survey of thirteen dinoflagellate transcriptome datasets revealed between one and four full-length or near full-length putative ACC sequences per species. Searches against *Perkinsus marinus*, *Hematodinium* sp., and *Amoebophrya* sp. did not recover sequences with the correct domain architecture. In addition, sequences from the apicomplexan *Plasmodium* and *Cryptosporidium* were difficult to align and were on very long branches, so they were excluded from the analysis.

The ACC phylogeny contains a large number of poorly supported nodes, but specific clades such as the chlorophyte green algae (100 % bootstrap), haptophytes (100%), diatoms (100%) and alveolates (78%) were well supported (Fig. 1). The monophyletic alveolates, included the chromerids, apicomplexans and dinoflagellates and is generally consistent with overall organismal phylogeny. Within dinoflagellates, there were two major clades of ACC. One included all eight of the peridinin pigmented dinoflagellates (with duplications in three species) and none of the alternately pigmented or non-photosynthetic species. Of the 11 sequences in the peridinin-associated clade, 8 were full length sequences in the alignment, and 4 had a putative chloroplast transit peptide (cTP) with a cleavage site prior to the first conserved domain. Based on a restricted distribution to the peridinin-pigmented dinoflagellates and predicted cTP, this clade is proposed to contain the chloroplast-targeted ACC. Of the anomalously pigmented dinoflagellates only a single sequence from *G. foliaceum* had a cTP. Both the alternately pigmented diatom-containing species also had another ACC version imbedded with the diatoms, while the second ACC from the haptophyte pigmented *Karenia brevis* and *Karlodinium veneficum* did not group with the haptophytes in the phylogenetic analysis. A second clade contained sequences from all thirteen of the dinoflagellates surveyed. This second clade, included all the species surveyed whether non-photosynthetic or alternately pigmented, and is proposed as the cytosolic ACC. Of the 10 full length, only one, the *A. sanguinea* ACC had a score over cTP threshold but the cut site was inside the mature protein. Within the putative cytosolic clade a smaller, but well supported division is seen. Four relatively distantly related peridinin pigmented species, *Prorocentrum hoffmanianum*, *Symbiodinium* sp., *Akashiwo sanguinea*, and *Gambierdiscus australes* made up the smaller clade, while the remaining nine dinoflagellates, including all the alternately pigmented or non-photosynthetic species were in the larger clade. This division of the cytosolic clade into two parts is not congruent with current dinoflagellate phylogeny, nor in

general is there good bootstrap support within the two cytosolic clades. However, within the peridinin-pigmented chloroplast ACC most of the nodes are well supported (all but one have bootstrap support >90%), but the branching pattern is not consistent with current phylogenies of dinoflagellates using large numbers of concatenated proteins (Bachvaroff et al. 2014; Janouškovec et al. 2016).

Overall, the phylogeny suggests dinoflagellates duplicated a homomeric ACC present in the common ancestor of apicomplexans, chromerids and dinoflagellates, and retained one version for chloroplast-associated functions. This peridinin chloroplast version was then lost from the alternately pigmented and non-photosynthetic species, as part of chloroplast replacement. A second duplication with differential loss is also possible within the cytosolic ACC, with one distinct version found exclusively within four species.

Protein expression and biotinylation was confirmed using western blotting with a streptavidin-HRP conjugate to visualize all natively biotinylated proteins resulting in several prominent bands in three species surveyed. Focusing on high molecular weight bands closest to the calculated protein mass from transcriptome data we putatively assign bands shown in red boxes as ACC (Fig 2). The molecular weight of these proteins for *K. veneficum* was 251 kD*, A. sanguinea* at 276 kD*,* and two bands for *A. carterae* at 276 and 262 kD. These assignments were previously confirmed by mass spectrometry (MS) proteomics for *A. carterae* and are to be confirmed in future MS experiments for *K. veneficum* and *A. sanguinea* (Haq et al. 2017). This survey of dinoflagellate ACCs reveals that most species contain multiple ACC proteins that potentially localize to the plastid or cytosol.

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Detection of a gene encoding for saxitoxin biosynthesis (*sxtU*) in non-toxic *Alexandrium fraterculus*

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Abstract

Members of the genus *Alexandrium* are the most studied marine dinoflagellates due to their ability to produce potent paralytic shellfish toxins, among which saxitoxin (STX) is the best-known representative. STX-producing species of *Alexandrium* have shown to be lethal to humans, provoking food-poisoning events worldwide. However, some non-toxic species has been described for this genus, such as *A. fraterculus*. In this work, we evaluated the presence of *sxtU* gene (involved in STX biosynthesis in cyanobacteria) by real-time PCR of samples obtained from the Atlantic coast of Uruguay, two of them from an exceptionally large bloom of *A. fraterculus*. Although the bloom was non-toxic according to the mice bioassay, the *sxtU* gene was detected. These findings suggest that *A. fraterculus* harbors at least part of the cluster involved in STX biosynthesis, although gene expression and toxin production seem to be lost. Phylogenetic analyses based on the large subunit ribosomal gene of several toxic species of dinoflagellates showed that *A. fraterculus* belongs to the *A. tamarense* cluster, which implies that it would share a common ancestor with all toxic species.

Keywords: Alexandrium, qPCR, *sxtU,* Uruguay

Introduction

Alexandrium spp. are probably the most studied marine dinoflagellates due to their ability to produce potent toxins causing a syndrome known as Paralytic Shellfish Poisoning (PSP). PSP is provoked after the ingestion of shellfish containing high levels of neurotoxins, among which saxitoxin and analogs (STX) are the best-known compounds. STX are synthesized by prokaryotic (cyanobacteria) and eukaryotic (dinoflagellates) phytoplankters. Among the dinoflagellates, those belonging to *Alexandrium* genus are the most abundant and widespread saxitoxin-producers and are responsible for blooms worldwide (Hallegraeff, 1993; Taylor et al., 2003). The organisms that most frequently cause toxic blooms are those belonging to the *A. tamarense* species complex.

In Uruguay, the first PSP outbreak took place in 1980, while the identification of *A. tamarense* as the causative species was attained during a second outbreak in 1991 (Brazeiro et al., 1997). Nowadays, the species that produce most of the summer blooms is *A. fraterculus,* which has not been associated with PSP events and have not shown any evidence of STX production (MacKenzie et al., 2004; Martínez et al., 2016). These toxins are produced by a unique biosynthetic pathway, involving reactions that are rare in microbial metabolism (Mihali and Neilan, 2009). The genetic cluster involved in STX synthesis has been characterized in several species of cyanobacteria, such as *Cylindrospermopsis raciborskii*, *Raphidiopsis brookii* and *Aphanizomenon* sp., among others (Casero et al., 2014; Kellmann et al., 2008; Mihali and Neilan, 2009; Stucken et al., 2010). It has been found that the genetic mechanisms for saxitoxin synthesis in cyanobacteria and dinoflagellates are related but it is still not clear which were the evolutionary mechanisms implied (Mihali and Neilan, 2009; Moustafa et al., 2009). Although the genes involved in STX synthesis have not completely characterized in dinoflagellates, according to different authors the biosynthetic pathway would share some characteristics with cyanobacteria (Kellmann et al., 2008; Shimizu, 2003). Due to the large size of dinoflagellates genomes (ca. 60

times the size of the human haploid genome), genomic studies have been challenging (Lin, 2011) and most of the information regarding the genetic system to synthesize STX arises from transcriptomics or gene expression studies. In a transcriptome-based study of *A. fundyense* and *A. minutum*, Stüken et al. (2011) characterized *sxtA*, the starting gene of STX biosynthesis (Stüken et al., 2011). They showed that this gene is similar to cyanobacterial *sxtA*, but transcripts in dinoflagellates are monocistronic, suggesting that although encodes the same domains it differs in transcript structure. For example, two transcripts with different lengths were found in *A. fundyense,* a short one lacking the *sxtA*4 terminal aminotransferase domain and a longer transcript where all *sxtA*1-4 domains are present, similar to those found in cyanobacteria (Stüken et al., 2011). Moreover, these authors found homology between assembled transcripts libraries of *A. fundyense* and *A. minutum* and several *sxt* core genes from *Cylindrospermopsis raciborskii* T3. Other genes identified by transcriptomics as potentially involved in saxitoxin biosynthesis by *A. pacificum* and *A. tamarense* are *sxtD*, *sxtS*, *sxtU*, *sxtH/T*, *sxtI*, *sxtL*, *sxtN*, *sxtX*, *sxtF/M* and *sxtP* (Hackett et al., 2013). Therefore, there are several phylogenetically related genes in cyanobacteria and dinoflagellates that could be good candidates for the detection of the presence of these potentially dangerous organisms in marine water.

In this work we analyzed by real-time PCR the DNA extracted from bloom samples, either dominated by *A. tamarense* or *A. fraterculus*, to detect the presence of *sxtU*, one of the genes involved in STX biosynthesis encoding an alcohol dehydrogenase in *C. raciborskii* (Kellmann et al., 2008).

Material and Methods

Sampling and identification of *Alexandrium* spp. - Samples of *A. tamarense* were taken during a coastal cruise in October 2013 (sample E25, -34,7353º S, 53,4655º W) using a 25 µm net for qualitative samples and a Niskin bottle for quantitative samples. Samples from an *A. fraterculus* bloom were taken with a plastic bucket in March of 2015 during the HAB monitoring programme at surf zones of Punta del Diablo (PD) and Arachania (Ara) beaches, Uruguay (34.0423° S, 53.5473° W and 34.6161° S, 54.1516° W for PD and Ara, respectively). Qualitative samples were immediately fixed using 4% formaldehyde (FA). Quantitative samples were fixed with Lugol and settled in a counting chamber and inspected using an Olympus IM inverted microscope, at a final magnification of 100 x (Andersen and Throndsen, 2003).

DNA extraction and real-time PCR – FA-fixed samples (10 mL) were centrifuged at 5000 x g during 10 min (Thermo Scientific Sorvall RT1) and pellets were subjected to DNA extraction using the ZR Soil Microbe DNA MiniPrep (Zymo Research). After extraction, DNA quantity and quality were confirmed using a Nanodrop spectrophotometer (Thermo Scientific). Ten nanograms of DNA from each sample were applied to a Power SYBR Green PCR (Invitrogen) using primers sxtUF and sxtUR, which give a 79 bp amplicon (Martinez De La Escalera et al., 2014). Real-time PCR was run in a 96 FLX TouchTM thermal cycler (Bio-Rad) using the cloned *sxtU* gene as a positive control (Martinez De La Escalera et al., 2014). Ultrapure water was used as a negative control. Amplification efficiency was always ≥ 0.85. To identify *sxtU*-positive samples, results from real-time PCR were interpreted using a cut-off value of the threshold cycle (Ct) ≤ 36 (based on the Ct of the positive control). All PCR products were sequenced at Macrogen Inc. (Korea).

Phylogenetic analyses – Sequences of the large subunit ribosomal RNA gene of toxic and non-toxic dinoflagellates were retrieved from GenBank and aligned using the multiple sequence alignment program ClustalW. Phylogenetic trees were constructed by the maximum likelihood method (Felsenstein, 1981) and using 500 bootstrap repetition as a test of phylogeny (Felsenstein, 1985). All the analyses were performed in Mega (Tamura et al., 2011).

Results and Discussion

Taxonomic identification of the samples from Punta del Diablo and Arachania showed that PD and Ara samples were exclusively composed by *A. fraterculus* organisms (Fig. 1) (see Martínez et al., 2016 for details).

Although nucleic acids recovery from fixed samples was not very efficient (DNA concentration range was 5 – 10 ng μL^{-1}), when DNA obtained from E25, PD and Ara samples

was subjected to PCR using the *sxtU*-targeted primers, positive amplification was obtained from all samples. Ct values were 32, 34.1 and 34.5 for E25, PD and Ara, respectively, while for the positive control Ct was 19.5 and no amplification was observed for the negative control. The amplification of the targeted *sxtU* gene was confirmed by sequencing the PCR products (Table 1).

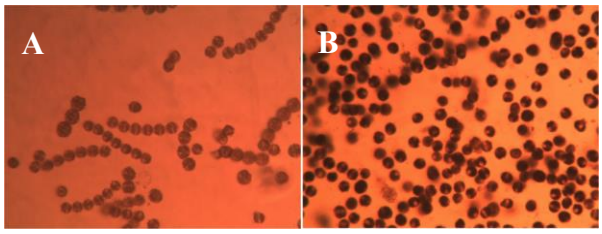

Fig. 1. Microscopy images of *Alexandrium fraterculus* samples obtained from PD (A) and Ara (B) blooms. 100x magnification.

Table 1. Results obtained from Blast using the amplicons sequences.

Sample/toxicity by mouse bioassay (Species)	GenBank homology (accession number)	% Identity (E-value)*
E25/toxic (*A. tamarense*)	. *L. wollei* PSP gene cluster (EU603711) . *C. raciborskii* T3 toxin biosynthesis gene cluster (DQ787200)	100 (2e-6) 100 (2e-6)
PD/non-toxic (*A. fraterculus*)	. *L. wollei* PSP gene cluster (EU603711) . *C. raciborskii* T3 toxin biosynthesis gene cluster (DQ787200)	100 (1e-12) 100 (1e-12)
Ara/non-toxic (*A. fraterculus*)	. *C. raciborskii* T3 toxin biosynthesis gene cluster (DQ787200)	93 (6e-8)

*The effective query length used by Blast for identity search were 52, 43 and 41 bp for E25, PD and Ara, respectively.

Our results showed that not only the samples containing *A. tamarense* organisms, but also those belonging to *A. fraterculus* blooms would harbour the *sxtU* targeted gene (Table 1). Our findings led us to hypothesize that the ability to synthesize STX could have been lost during speciation in *A. fraterculus*. It has been described that this species is closely related to other species of the *Alexandrium* genus (Anderson et al., 2012). Furthermore, in a previous study based on phylogenetic analyses of toxic and non-toxic strains of *A. tamarense* complex Lilly et al. (2007) suggested that the ability to produce toxins could either been acquired or lost on several occasions during the evolution of these organisms (Lilly et al., 2007). To test our hypothesis we performed phylogenetic analyses based on available ribosomal large subunit gene sequences from several dinoflagellates species known as STX producers. Our results showed that *A. fraterculus* would belong to a group composed by *A. tamarense* and *Pyrodinium bahamanense*, more specifically to the *A. tamarense* cluster (Fig. 2). Thus, non-toxic *A. fraterculus* would share a common ancestor with other toxic genera, contributing to the evidence for the evolutionary loss of the genetic system for STX production. Further research involving ecological studies and evolutionary genomics of *Alexandrium* species are required to reveal the ecological role of STX for these organisms and to infer how the toxin relates to speciation and niche adaptation.

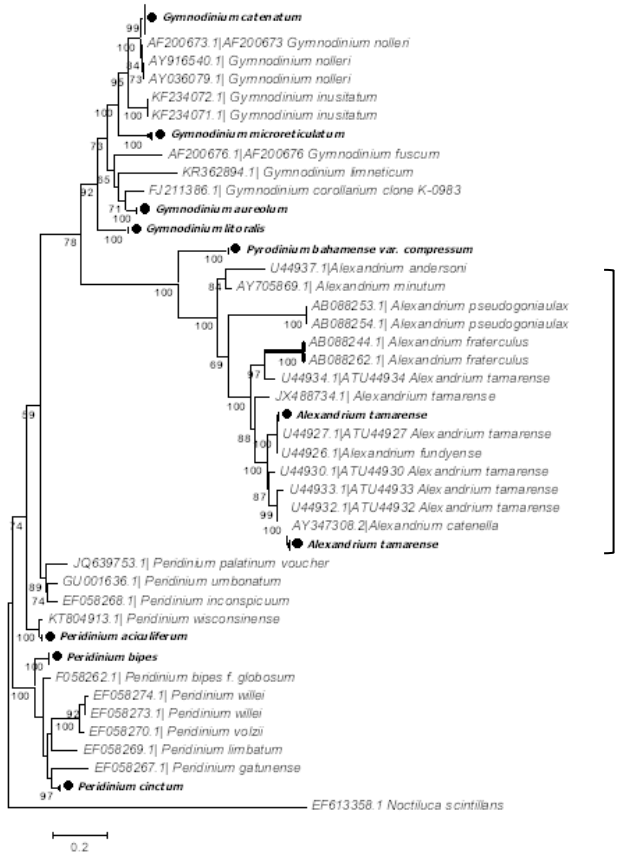

Fig. 2. Maximum Likelihood phylogenetic tree based on the large subunit ribosomal RNA gene from toxic dinoflagellates. The

bracket indicates the position of the *Alexandrium* cluster and thick lines shows *A. fraterculus* branch. In order to simplify the tree, several subtrees were compressed (black dots, bold letters). *Certium furcoides* was used as an outgroup. Bootstrap values are given at the nodes.

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Assessment of DNA extraction efficiency and quantification based on *Alexandrium* sp. cultures

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Abstract

Surveillance and management of Harmful Algal Blooms (HABs) relies on the identification and quantification of harmful and/or toxic species. Molecular techniques are currently supporting the results obtained by monitoring programs with optic microscopy, and have a great potential as fast, reliable and accurate analysis of phytoplankton communities. Molecular techniques are constantly evolving and improving, and the use of commercially available kits are facilitating their applicability; regardless of their analytical throughput and accuracy, all have common steps that can add bias to the final results.
Bias due to extraction efficiency and quantification accuracy of genetic material is usually overlooked, although both steps are basic for any molecular analysis and the development of new molecular-based methods. In the present work, the extraction efficiency of a commercial kit commonly used in microalgae analysis is assessed with samples from different *Alexandrium* sp. strains cultured in laboratory conditions. Different quantification methods of the extracted DNA are also compared.

Keywords: Alexandrium sp., molecular analysis, DNA extraction efficiency, Nanodrop™, Quantus™

Introduction

Harmful Algae are microalgae species able to bloom in aquatic environments, and in some instances produce toxic compounds, which are a risk for human health through consumption of contaminated water or aquatic organisms (i.e. seafood). Harmful Algal Blooms (HAB) cost hundreds of millions of $ yearly in losses for the marine aquaculture and fisheries sector worldwide (Kudela *et al.* 2015). The impact on human health is difficult to estimate, although it is accepted that, where monitoring programs are working, no serious intoxications are detected if controlled seafood or water is consumed (Fleming *et al.* 2007). It is expected that HAB negative impacts will increase in the climate change scenario (Edwards *et al.* 2006).

Research and monitoring of HABs tend to reduce the costs and time between the sampling and the results (Cornelisen 2013), in order to support decisions that affect human health, food / water safety and aquaculture and fisheries economy. The evolution of the density of toxic microalgae species in the environment is used as an early alert for HAB events. Utermöhl method (Utermöhl 1958), based on the identification at the optic microscope, is the most used by monitoring programs, although it is time-consuming and requires a high level of taxonomic expertise, among other drawbacks (Karlson *et al.* 2010).

Many molecular techniques have been tested for HAB monitoring, from Sanger sequencing and qPCR to DNA fingerprinting techniques, microarrays and High-Throughput Sequencing (HTS); nevertheless, none of these techniques is suitable for monitoring purposes because none fulfils the two required goals of identification and quantification of harmful algal species. Previous results with cyanobacteria populations have shown that HTS techniques allow the analysis of more samples than Utermöhl, they are very reliable at their final part (PCR amplification, DNA sequencing) and in the identification of microalgae species, but they are not reliable for the quantification of cyanobacteria cells present in the sample (Giménez Papiol *et al*. 2013).

The extraction of microalgal DNA is a bottleneck step, common to all the molecular techniques, which has a strong relationship with the success on microalgae quantification. A reliable, reproducible, fast, and low cost technique for this paramount step in molecular analysis is necessary for the transfer of these analysis to monitoring and management fields.

In the present work, a cosmopolitan marine microalgae genus, *Alexandrium* sp., which includes toxic and non-toxic species, has been used for the assessment of the extraction efficiency of a commonly used DNA extraction kit, and to compare the quantification obtained with two commonly used quantification methods. *Alexandrium* species are easy to keep in laboratory conditions, in autotrophic growth, and are the main producers of Paralytic Shellfish Poisoning toxins (PSP toxins), spirolides and other emerging toxic compounds which affect aquaculture industry and human health (Anderson *et al.* 2012).

Material and Methods

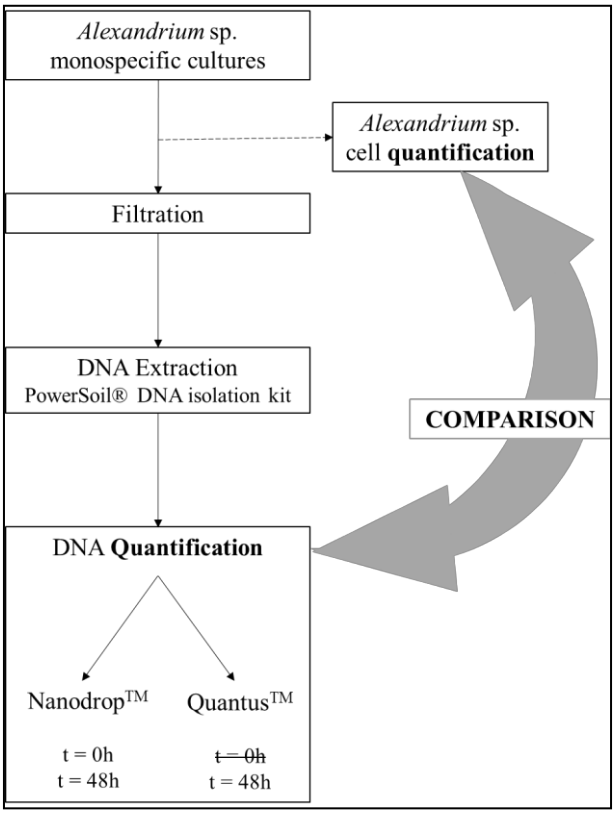

Figure 1. Steps followed in the assessment of DNA extraction efficiency and the comparison between quantification methods.

Figure 1 summarizes the steps followed in the present work

Alexandrium sp. cultures

Seven *Alexandrium* sp. strains were purchased at the Microalgae culture collection of the Instituto Español de Oceanografía (IEO-Vigo, Spain): *A. pacificum* (VGO566), *A. mediterraneum* (VGO654), *A. margalefi* (VGO661), *A. taylori* (VGO703), *A. minutum* (VGO722), *A. ostenfeldii* (VGO956) and *A. tamarense* (VGO1042). Strains were acclimated at laboratory conditions for 1 month before scaling up their cultures in 50 mL plastic pottles. Microalgae strains were cultured separatelly in Guillard's marine water enriched solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) diluted in artificial seawater (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), at room conditions (temperature and light conditions).

Alexandrium sp. cell densities

Five mL of culture was fixed with 50 µL iodine lugol. Triplicates of 1mL were counted on a Sedgewick Rafter chamber (PYSER-SGI Limited, Kent, UK) at the optic microscope (Olympus Corporation, Tokyo, Japan). Cell densities (cells mL^{-1}) were the average of these triplicates.

DNA extraction

Forty mL per culture were filtered on a GF/C glass microfiber filter (Whatman, GE Healthcare Life Sciences UK Limited, UK) and kept at -20ºC until DNA extraction.

Each filter was divided into two subsamples, extracted with PowerSoil® DNA isolation kits (MoBio Laboratories Inc., Carlsbad, USA) following the manufacturer's protocol, and the final extracted DNA from each subsample from the same *Alexandrium* sp. culture were mixed.

DNA quantification

Extracted DNA was quantified at time 0h with Nanodrop™ (Thermo Fisher Scientific Inc., Waltham, USA), kept at -20ºC for 48h, and quantified again with Nanodrop™ and Quantus™ (Promega Corporation, Madison, USA).

Table 1. Cells per sample and quantifications obtained at t = 0h (NanodropTM) and t = 48h (Nanodrop^{TM} and Quantus^{TM}) from each *Alexandrium* sp. culture.

Alexandrium species	Strain	Cells per sample	DNA (ng µL^{-1}) t = 0h Nanodrop^{TM}	DNA (ng µL^{-1}) t = 48h Nanodrop^{TM}	DNA (ng µL^{-1}) t = 48h Quantus^{TM}
A. pacificum	VGO566	19,271	12.3	4.8	2.89
A. mediterraneum	VGO654	238	4.8	2.3	0.61
A. margalefi	VGO661	2,967	5.6	2.3	1.42
A. taylori	VGO703	274	8.7	1.4	1.45
A. minutum	VGO722	2,312,317	17.5	7.6	4.24
A. ostenfeldii	VGO965	2,168	5.2	3.0	1.04

Results and Discussion

Utermöhl methods use sample volumes of 50 mL maximum. In this test, the sample volume used for DNA extraction, 40 mL, falls within the standard sample volume used for monitoring purposes.

Alexandrium sp., as all thecate dinoflagellates (Fensome *et al.* 1999), has a cellulose cover difficult to break by the standard procedures used in molecular analysis (Akcha *et al.* 2008). The amount of DNA extracted with the commercial kit and following standard laboratory protocols is close to the detection limits of Nanodrop^{TM} and Quantus^{TM}, even when obtained from highly dense microalgae cultures such as *A. minutum* culture (Table 1).

Alexandrium sp. cell densities in the samples ranged from low, far from the alert levels (i. e. *A. mediterraneum*, *A. taylori*), to close but below these levels (i. e. *A. pacificum*) and over the alert levels (i. e. *A. minutum*, 58-times more concentrated than the bloom alert level). The amount of cells per sample varied in orders of magnitude: aprox. 10^2 in *A. mediterraneum* and *A. taylori*, aprox. 10^3 in *A. margalefi*, *A. ostenfeldii* and *A. tamarense*, aprox. 10^4 in *A. pacificum* and aprox. 10^6 in *A. minutum*. But the amount of extracted DNA did not show this variability, there is no clear relationship between the amount of extracted DNA and the amount of cells or cell density in the sample.

Nanodrop^{TM} quantification was not consistent between data obtained at t = 0h and t = 48h (Table 1). All quantifications were lower at t= 48h compared to the ones obtained at t = 0h, but the reduction did not present a good linear regression ($R^2 < 0.9$, Figure 2).

Quantus^{TM} quantification was lower than the obtained with Nanodrop^{TM} (Table 1). Both methods present a better correlation (Figure 3) than the obtained using Nanodrop^{TM} at different quantification times (Figure 2), but still not a good linear regression ($R^2 < 0.9$).

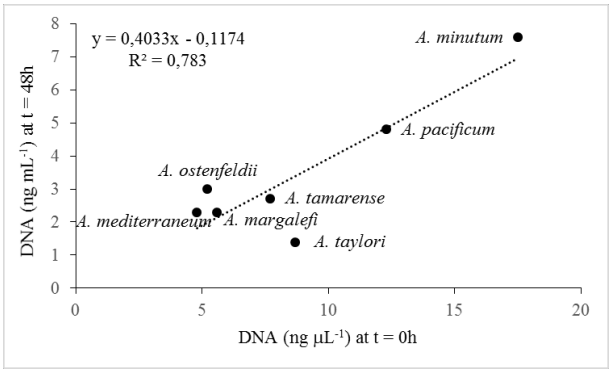

Figure 2. Linear regression between the quantifications obtained with Nanodrop^{TM} at t = 0h (x axe) and t = 48h (y axe).

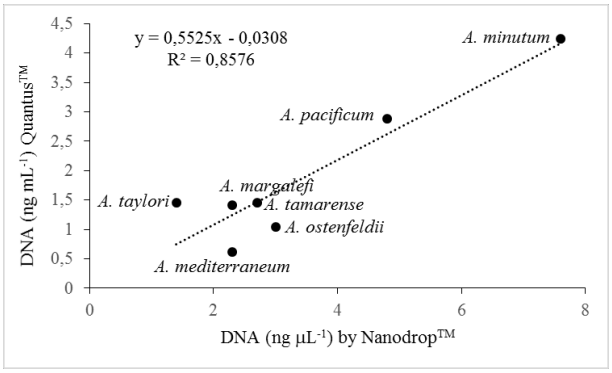

Figure 3. Linear regression between the quantifications at t = 48h obtained with Nanodrop^{TM} (x axe) and Quantus^{TM} (y axe).

Extraction of DNA in samples with higher amounts of *Alexandrium* cells than in natural samples is not efficient, and varies among species extracted. There are no significant differences between samples containing 10^2 to 10^3 cells. Quantification methods for DNA, even when quality control (advised by each manufacturer) points out an optimal performance, do not agree in the amount of DNA. These results should be taken into account when comparing results obtained with different quantification methods, even when they are validated, standardized and considered reliable.

Acknowledgements

Alexandrium sp. strains were purchased at the Microalgae culture collection of the Instituto Español de Oceanografía (IEO-Vigo, Spain); Thanks to Dr Santi Fraga for his advice and help. Molecular analysis by Dr Helena Torrell, from Centre for Omic Science (COS, Universitat Rovira i Virgili, Spain).
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Review of Progress in our Understanding of Fish-Killing Microalgae: Implications for Management and Mitigation

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Abstract

Fish-killing algal species are responsible for much greater global economic impacts than HAB species leading to seafood biotoxin contamination. Yet the precise mechanisms of how microalgae kill fish remain poorly understood. Progress has been hindered by the use of widely different bioassay systems and lack of analytical methods to quantify and characterize so-called "ichthyotoxins". All high biomass blooms even of nontoxic phytoplankton can cause significant stress for finfish contained in intensive aquaculture systems. Highly potent fish-killers include the taxonomically unrelated flagellate groups *Cochlodinium, Karenia, Chattonella, Pseudochattonella, Heterosigma, Prymnesium* which all readily lyse upon impact on the sensitive gill tissues of fish. A key mechanism for fish-gill damage being proposed is HAB cell lysis releasing free fatty acids (EPA, DHA, OPA) which in synergism with reactive oxygen species generate labile (min to hrs) lipid peroxidation products. Cell lysis is critical for *Karlodinium* and *Alexandrium* ichthyotoxicity, and high ROS producing strains (eg. *Chattonella*, Chilean *A .catenella*) cause greatest gill damage. With perhaps a single exception (Florida *Karenia brevis*), none of these ichthyotoxins are of human health significance, meaning that recently killed fish are still fit for human consumption. Finely ground bentonite clays at environmentally acceptable concentrations can effectively mop up ichthyotoxins and offer great potential as a HAB emergency response tool.

*Keywords:*Fish-killing algae; Lipid peroxidation; Reactive Oxygen Species; Emergency Harvest Strategy.

Introduction

Finfish held captive in intensive aquaculture systems are extremely sensitive to harmful algal blooms. Wild fish have been observed to change their migratory path to avoid HABs (Savage 1930) but captive fish do not have such choice. Fish-killing algal species are responsible for much greater global economic impacts on human society than HAB species leading to seafood biotoxin contamination. For many years the record fish kill HAB event was the USD 500M loss suffered by the yellowtail finfish aquaculture industry in 1973 in the Japanese Seto Inland Sea (Okaichi 1983). The 2016 "Godzilla Red Tide" in Chile is estimated to have caused USD 800M loss to salmon and the broader fishing industry. Other severely impacted economies include Korea (USD 95M in 1995; Fig.1 bottom), Australia (AUD 45M in 1996; Fig. 1), Hong Kong/China (USD 32M in 1998), British Columbia (USD 35 during 1980-1990), as well as New Zealand and Norway (Table 1).Ever increasing societal impacts force us to rethink our responses to such HAB events.

Table. 1. Summary of financial impacts of large fish-killing algal blooms in different parts of the world.

HAB species	Country	Financial Losses
Chattonella Heterosigma	Japan	USD500M, 1973 USD135M,1980/90
Cochlodinium polykrikoides	Korea, China Canada	USD95M, 1995 USD2M, 1999
Heterosigma Chaetoceros	British Columbia	USD35M, 1980-90
Heterosigma	New Zealand	NZD12M
Karenia digitata	Hong Kong	USD32M, 1998
Karenia mikimotoi	Norway	USD6M, 1988
Alexandrium catenella Pseudochattonella/ A. catenella	Chile	USD60M, 2002 USD800M, Jan-Mar 2016
Chattonella Karenia	South Australia Tasmania	AUD45M, 1996 AUD3M, 2003

Proença, L. A. O. and Hallegraeff , G.M. (eds). Marine and Fresh-Water Harmful Algae. Proceedings of the 17th International Conference on Harmful Algae. International Society for the Study of Harmful Algae 2017.

It is surprising that so little progress has been made in our understanding of how HABs kill fish. Progress has been hindered by the use of different bioassay systems and lack of analytical methods to quantify and characterize "ichthyotoxins". Assay systems have included *Artemia* or *Daphnia* assays, fish or mammalian erythrocytes, and a wide range of juvenile or adult fish (damselfish, sheepshead minnow, mountain minnow, zebrafish, salmon, sea bass), tested under different exposure times. The lability and short life span of ichthyotoxic fractions is problematic. Differential behaviour of ichthyotoxic compounds in plastic or glass containers and light and dark conditions can confound results (Dorantes-Aranda *et al.* 2014).

High biomass algal blooms

All high biomass algal blooms can cause mechanical stress to the sensitive gill tissues of fish. This results in gill irritation and excess mucus production. Mucus is produced by the fish themselves and to lesser extent also by HABs. Examples of high biomass algal blooms (>100,000 cells/L) and associated fish mortality are diatom blooms by the (non-toxic) *Pseudo-nitzschia delicatissima*-complex in Chile and Tasmania, Australia. High density diatom blooms of *Leptocylindrus, Rhizosolenia* and *Thalassiosira* have also been implicated in fish kills in Chile (Mardones & Clement 2016). Dinoflagellate blooms of *Tripos* (*Ceratium) fusus* (600,000 cells/L) have caused yellow-tail mortality in Japan. Similarly, dinoflagellate blooms of *Tripos furca* (10,000,000 cells/L) have caused tuna farm mortality in Mexico (Orrellana-Cepeda *et al.* 2004).

Better studied are diatom blooms by *Chaetoceros concavicornis* and *C. convolutus* in British Columbia and Chile that can kill salmon at 5,000 cells/L (Yang & Albright 1992). Closely related diatom blooms by *C. criophilum* and *C. danicus* (belonging to "subgenus *Phaeoceros*") cause annually recurrent problems for the salmon industry in Tasmania, Australia (Hallegraeff, 2016). The widely accepted explanation is that hairy diatom setae break off and penetrate the gill tissues of fish with the barbs preventing them from coming out (Bell 1961). This can lead to bleeding fish gills and secondary infections. Dense blooms by other *Chaetoceros* species belonging to the subgenus *Hyalochaeta* can also be problematic, albeit less severe.

High biomass blooms by the dinoflagellate *Noctiluca scintillans* can irritate fish (notably via high ammonia levels; Okaichi & Nishio 1976) but rarely cause mortality. In Tasmania in 2002 4m thick pink *Noctiluca* surface slicks accumulated within the confines of circular salmon net pens. This stopped fish from surfacing and feeding, a situation which was remedied by the installation of Ventura aeration systems to clear slicks from the surface of the pens. Demise of high biomass HABs can generate low oxygen conditions in sheltered waters which generate kills of both fish and marine invertebrates.

In sea water and microalgal cultures viscosity increases with algal biomass, but modified by species composition (Jenkinson & Sun, 2010; Jenkinson *et al.*, 2015) and physiological state (Seuront *et al*., 2006). Measurements of flow of fish-killing cultures of *K. mikimotoi* and *K. selliformis* (as *maguelonnense*) over the gills of freshly dead fish confirmed that flow was significantly reduced, but ROS-induced haemolytic activity was also present (Jenkinson & Arzul, 2002). In blooms of some *Karenia* spp., viscous, sticky slime may provoke gill malfunction and mortalities, perhaps interacting with toxins or fish-produced mucus, or both.

Highly potent fish-killing algae

Fig. 1. Mass mortality of bluefin tuna in South Australia, caused by *Chattonella marina* blooms. Higher potency of Australian blooms (kills at 66,000 cells/L) compared to Japan (500,000 cells/L) was attributed to higher sensitivities of tuna but also higher ichthyotoxicity by Australian high-light adapted algal strains (Dorantes-Aranda *et al.* 2013).

Of much greater concern for the aquaculture industry are the highly potent taxonomically unrelated flagellate groups *Cochlodinium,*

Karenia, Chattonella, Pseudochattonella, Heterosigma, Prymnesium. One feature that these algal groups have in common is that they all comprise fragile cells, which can lyse even upon impact on the gills of fish, and more so during the end of blooms, under the influence of algal parasites (E. Garces, pers. comm.) or conditions of osmotic stress in estuaries or during upwelling. For other fish-killing algae such as *Karlodinium* or the armoured *Alexandrium* the conditions that cause cells to exude ichthyotoxins and/or cause cell lysis are critical, and ichthyoxicity by these genera tends to be more variable.

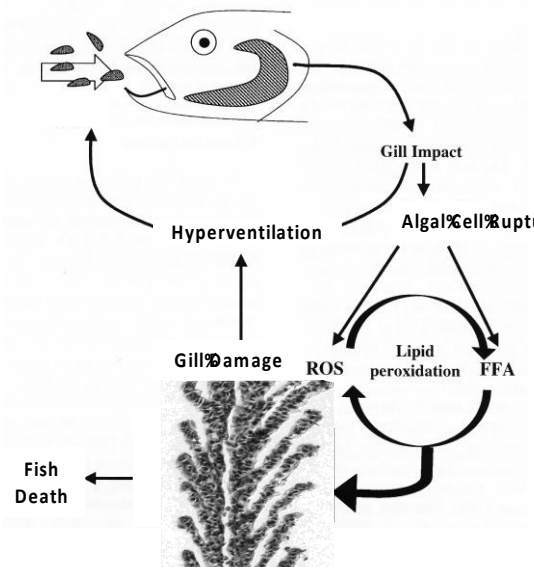

Fig. 2. Diagrammatic representation of algal bloom mediated fish kills, involving rupture of algal cells to release a cocktail of Reactive Oxygen Species (ROS), Free Fatty Acids (FFA) but which rarely involve true phycotoxins such as brevetoxins or karlotoxins.

The first point of attack by all above algal groups are the fish gills (Fig. 2), resulting a generalized necrotizing degeneration of the epithelium of the secondary lamellae with associated sloughing. This is often accompanied by swelling and pyknosis of the primary lamellar epithelium and congestion of branchial vessels (Roberts 1983). Fish gills can only respond in a single way to ichthyotoxic challenge: different algal groups therefore produce comparable gill pathology, and this is similar in different fish species such as rainbow trout, tuna, yellowtail fish, damselfish etc. (Roberts *et al.* 1983, Shimada *et al.* 1983, Marshall *et al.* 2003, Munday & Hallegraeff 1998, Deeds *et al.* 2006). Once the fish gills are compromised, gill ventilation is impaired, and a loss of Bohr effect impacts on the blood haemoglobin's oxygen binding affinity (Okaichi 1983). Furthermore, algal neurotoxins (if present) can penetrate the blood stream, and secondarily can cause fish behavioural changes.

What is the precise mechanism causing fish gill damage?

Several competing theories have been proposed as to the precise mechanism of how algae kill fish. These include: free fatty acids, reactive oxygen species, phycotoxins such as brevetoxins, karlotoxins, gymnocins, or varying combinations thereof. The novel application of a standardised and highly sensitive and reproducible rainbow trout RTgill-W1 cell line assay in our laboratory has allowed significant progress to be made (Dorantes-Aranda *et al.* 2011, 2015; Figs. 3& 4).The assay has been automated in a plate reader measuring cell viability dyes, and been successfully applied to screen natural seawater samples from fishkill events.

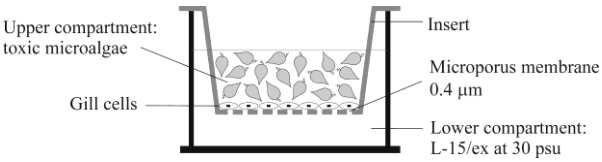

Fig. 3. Standardised ichthyotoxicity assay of microwells with permeable inserts upon which RTgill W1 cells areseeded, supported by growth medium in lower compartment but exposed toalgal cells in upper seawater medium. After Dorantes-Aranda *et al.* (2011).

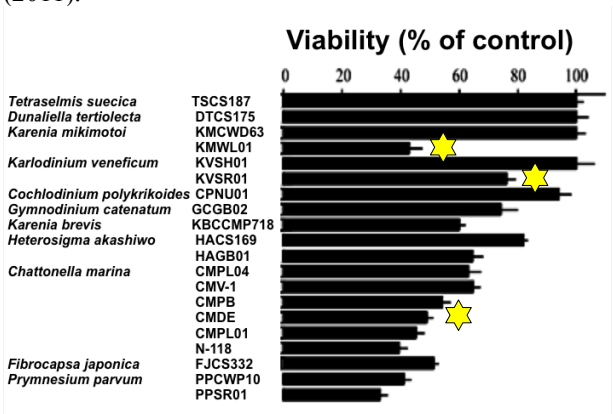

Fig.4. Loss of viability of RTgill-W1 cells after 48h exposure to a range of algal cultures, arranged from harmless (top) to most ichthyotoxic (bottom). Note strain variation for *Karenia mikimotoi, Karlodinium veneficum,* and *Chattonella marina*(asterisks).

Polyunsaturated fatty acids (PUFAs) are lipids in which the constituent hydrocarbon chain possesses two or more carbon–carbon double bonds (Fig.5). Paradoxically these compounds have a beneficial effect for human health, notably

heart disease, when consumed in moderation and used to replace saturated fat. These compounds are prone to oxidative degradation, called lipid peroxidation, in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage.

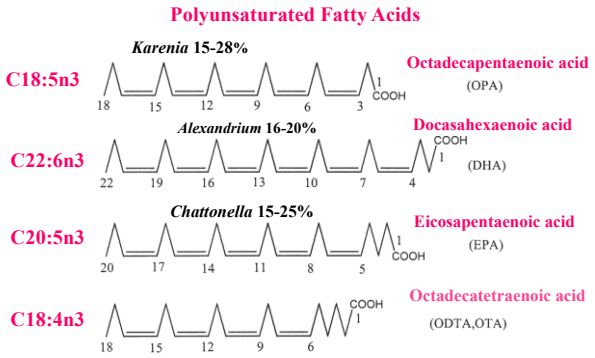

Fig.5. Molecular structures of dominant PUFAs in fish-killing algae. The dominant fatty acid in *Karenia* is OPA (15-28% of total fatty acids), but in *Alexandrium* this is replaced by DHA (16-20%) and in *Chattonella* EPA is dominant (15-25%).

Shilo (1967) working with fish-killing *Prymnesium parvum* blooms in Israel *Tilapia* ponds first pointed out how "lipid micels" released by the alga impact gill lamellae and interfere with osmoregulation. Okaichi (1983) investigating *Chattonella marina/antiqua* blooms killing yellowtail in the Seto Inland Sea focused on free fatty acids damaging fish gills. This was pursued by Arzul *et al.* (1995) trying to understand fish-killing *Karenia mikimotoi* blooms. The latter workers identified free fatty acids such as OPA (octadecapentaenoic acid) and EPA (eicosapentaenoic acid) as having the highest ichthyotoxic potency. More recently, Mardones *et al.* (2016) also confirmed the ichthyotoxicity by DHA (docasahexaenoic acid) from *Alexandrium catenella.* In whole fish experiments both Sola *et al.* (1999) and Marshall *et al.* (2003) demonstrated that exposure to OPA and EPA, respectively, did cause fish gill damage at concentrations in seawater of approximately 3ppm. This is short of what dense algal blooms would generate (1.5-2ppm).

Reactive Oxygen Species. The role of Reactive Oxygen Species (ROS) in ichthyotoxicity has long been suggested from whole fish experiments by Yang *et al.* (1995) and Tang & Gobler (2000) with *Heterosigma* and *Cochlodinium*, respectively, where application of ROS mopping enzymes such as catalase and peroxidase significantly improved fish survival. Oxygen radicals are unstable molecules (half-life in the order of seconds) that readily react with other molecules to cause damage to DNA, RNA, proteins, and thereby cell death. Fish-killing raphidophytes, notably *Chattonella,* are potent producers of ROS (Oda *et al.* 1997). Ruptured algal cells consistently produced more ROS (Fig.6). Several other fish-killing algae such as

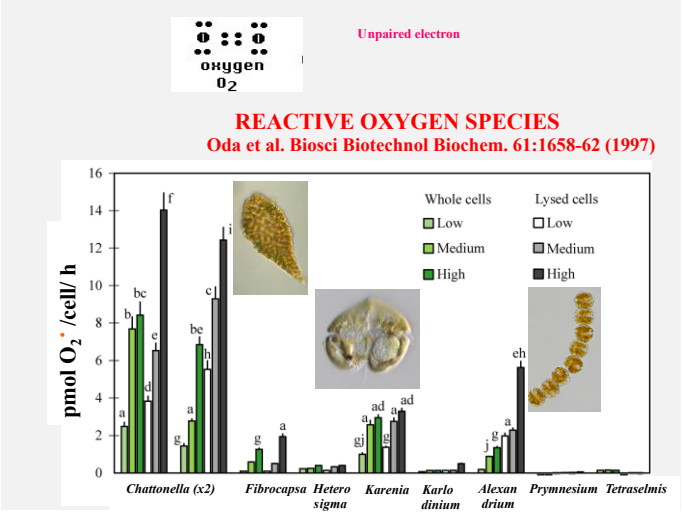

Fig.6. Production of superoxide radicals by fish-killing algal species as whole (green) and lysed cells (grey) at three algal concentrations. Australian (left) and Japanese (right) *Chattonella marina* strains are compared. After Dorantes-Aranda *et al.* (2015).

Using a xanthine-xanthine oxidase (X-XO) chemical reaction to generate superoxide at concentrations equivalent to fish-killing *Chattonella*, Marshall *et al.* (2003) demonstrated that superoxide on its own does not kill fish. Similarly, Twiner & Trick (2000) showed that hydrogen peroxide produced by *Heterosigma* did not explain ichthyotoxicity. Furthermore, more damaging hydroxyl radicals (invoked by Oda *et al.* 1997) could not be detected in *Chattonella* (Miller *et al.* 2011). ROS also exhibited negligible impact in our RT fish gill assay.

Synergism between ROS and free fatty acids

Pursuing the role of ichthyotoxicity by EPA, Marshall *et al.* (2003) demonstrated that when damselfish were challenged with EPA in the presence of ROS, this increased the potency of EPA by up to 15 fold (Fig.7). Similarly, DHA in synergism with ROS became 9 x more ichthyotoxic (Mardones *et al.* 2015). Synergisms between OPA and ROS, or OTA and ROS were less pronounced (Mooney *et al.* 2011). The

precise nature of the lipid peroxidation products generated remains poorly known, but may include

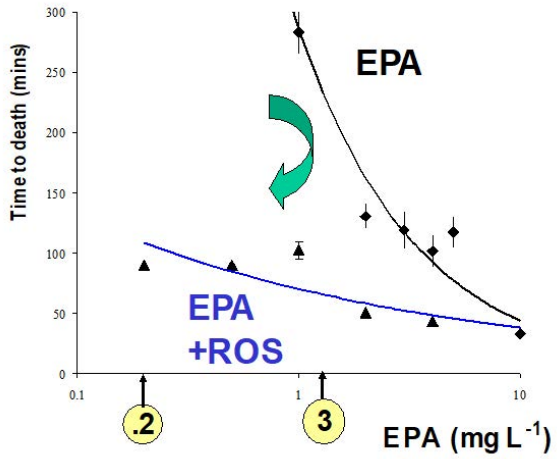

Fig.7. Toxicity of free fatty acid form of eicosapentaenoic acid (EPA) to damselfish: (top) EPA on its own, requiring 3mg/L to kill fish in 300min; (bottom) EPA in presence of X-XO generated superoxide, where 0.2 mg/L EPA could kill fish in 100 min. Modified after Marshall *et al.* (2003).

Role for true phycotoxins?

The dinoflagellate *Karlodinium veneficum* is known to produce chemically well-defined linear polyketide karlotoxins, which at ecologically realistic concentrations can account for their fish-killing potency (Place *et al*. 2012). While prymnesins from the haptophyte *Prymnesium parvum* have attracted considerable interest (Igarashi *et al*. 1999), it remains to be shown whether these compounds can account for the high ichthyotoxic potency. Similarly, cytotoxic chemical compounds, such as *gymnocin* from *Karenia mikimotoi,* remain to be conclusively linked to ichthyotoxic events (Satake *et al.* 2002). Polyether ladder brevetoxins from the Florida *Karenia brevis* are well known as the cause of Neurotoxic Shellfish Poisoning in humans (Baden 1989), but have also been widely claimed to be responsible for fish kills. In our work, purified brevetoxin PbTx2,3 exhibited limited ichthyotoxicity against RTgill cells, suggesting that peroxidation pathways may need be invoked in fish kills. The production of brevetoxins by raphidophytes (Onoue *et al.* 1990) is disputed. In our original work on Australian *Chattonella marina,*sodium channel radioreceptor assays for PbTx3 were weakly positive (Hallegraeff *et al*. 1998), but subsequent LC-MS analyses on the same material by two different analytical labs failed to detect the distinctive brevetoxin mass ions (Marshall et al. 2003; McNabb et al. 2006). Neuroactive compounds (but not brevetoxin) have recently been claimed also for the raphidophyte Heterosigma (Astuya et al. 2015)

A role for saxitoxin and analogues has often been implied in fish kill events by Alexandrium catenella/tamarense. Natural fish kill events however consistently revealed very low STX accumulation in fish gills, muscle, brain and inner organs (<4 gSTX eq./100g wet wt) , but at the same time noticeable edema, hyperplasia and necrosis of secondary gill lamellae. Mardones et al. (2015) found no evidence for a role of STX in fish gill damage, which instead was explained by DHA and ROS synergism. Similarly, Tasmanian salmon farms operate in dense Gymnodinium catenatum bloom areas, where net pen mussels can contain >15mg/kg PST but no fish kills nor tainting of salmon with PST has ever been detected.

Using the fish-gill ichthyotoxicity assay system and a criterion of 50% loss of cell viability in 48h at ecologically realistic concentrations, karlotoxin and DHA could account for fish kills in their own right, but not EPA, STX, PbTX, OPA nor OTA. Synergisms between DHA x ROS and EPA x ROS could explain fish kills, and so could putative fatty acid aldehydes. New analytical methods for the detection of lipid peroxidation products in seawater during fish kills are much needed.

Implications for Mitigation of Fish-Kill Events

Several different strategies are currently practised to mitigate the ichthyotoxic effects of HABs. These include cessation of fish feeding, towing away of cages from affected areas, perimeter skirts to protect against algal surface slicks, aeration or airlift upwelling to dilute harmful algal concentrations, or clay flocculation to reduce numbers of harmful algal cells (Rensel & Whyte, 2003). Mass fish mortalities lead to tonnes of dead fish being dumped in land fills (Port Lincoln, Australia, 1996) or dumped offshore (Chilean red tide 2016), and rarely used for fish meal. As argued here, with perhaps a single exception (Florida *Karenia brevis*), none of these "ichthyotoxins" are of human health significance, meaning that recently killed fish are still fit for human consumption. Once fish gills have been

compromised by HABs, fish rarely recover their normal growth rates (J. Rensel, pers.comm). Triggered by autonomous HAB monitoring systems, once fish start to die, a decision could thus be made to instigate emergency harvest operations. To prevent the build-up of histamines, fish should be kept alive as long as possible during harvesting. This can be achieved by diluting algal concentrations via airlift upwelling, but more effectively by targeted in-pen application of clays that mop up ichthyotoxins as opposed to using clays for cell flocculation (Seger *et al.* 2017). Our work explored different clays tested against different fish-killing algaewith ichthyotoxicity assessed with the gill cell line assay. Bentonite clay could eliminate *Prymnesium, Karenia* and *Karlodinium* ichthytoxicity at clay loadings of 0.05-0.25 g/L, significantly lower than those considered to be harmful to benthic marine invertebrates (1-10 g/L; Shumway *et al*. 2003). While extracellular *Chattonella, Heterosigma* and *Alexandrium* ichthyotoxins could only partially be removed, early application of these clays during the process of cell lysis eliminated ichthyotoxicity. These findings highlight the potential of HAB specific targeted applications of clay for ichthyotoxin adsorption (Seger *et al.* this volume).

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Mitigating fish-killing algal blooms with PAC modified clays: efficacy for cell flocculation and ichthyotoxin adsorption

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Abstract
Flocculation of harmful algal cells through treatment with modified clay minerals offers great promise in reducing clay loadings required for effective cell removal, yet the ichthyotoxin adsorptive properties of such modified clays have received little attention. We here compare *Chattonella marina* (raphidophyte) cell removal by conventional (kaolin, bentonite) and polyaluminium chloride (PAC) modified clays (bentonite and Korean loess), while newly exploring the capacity of PAC and PAC modified clays to mitigate *C. marina* and *Alexandrium catenella* (dinoflagellate) ichthyotoxicity (quantified with the RTgill-W1 cell line assay). Conventional clays proved ineffective in reducing *C. marina* cell numbers (14±5% removal at 0.5 g L^{-1}), but treatment efficacy could be much improved through modification of bentonite and Korean loess with PAC (85±5% removal at 0.1 g L^{-1}). Ichthyotoxin adsorption by PAC and PAC modified clays proved species specific. While both PAC (0.1 g L^{-1}) and PAC modified clays (0.25 g L^{-1}) could completely eliminate *C. marina* cytotoxicity, only bentonite clay proved effective in mitigating the negative impact of lysed *A. catenella* preparations on the gill cell line (69±10% gill cell viability). This work highlights how application of modified clays extends beyond improved cell flocculating properties to also include adsorption of selected ichthyotoxins.

Keywords: Mitigation, modified clay, ichthyotoxin adsorption, flocculation, fish-killing algae

Introduction

The threat that fish-killing algal blooms pose to an ever expanding global aquaculture industry has been well documented (Hallegraeff et al. 1993, this volume) and the requirement for effective mitigation strategies has never been greater. A physical control method, already practised in South Korea and China, is the flocculation of harmful algal cells with negatively buoyant clay particles (Park et al. 2013, Shirota 1989). Clay dispersed as an aqueous slurry on the water surface rapidly (minutes to hours) sweeps through the water column, entraining and ultimately sedimenting harmful algal cells. The factors influencing cell removal efficiency have been well studied (Sengco and Anderson, 2004) and the success of clay application proven to be algal species specific. Physical parameters, such as turbulence, clay type, clay loading and particle size, as well as algal cell concentration and size, have all been documented to influence cell removal efficiency (Beaulieu et al. 2005, Seger et al. 2017a,b, Sengco et al. 2004, Yu et al. 1994). Recent work has documented how adsorption of dissolved ichthyotoxins to clay minerals presents an important additional benefit to clay application (Pierce et al. 2004, Seger et al. 2015). However, concerns have been raised regarding the impact that high clay concentrations could potentially have on the benthic environment (Shumway et al. 2003). Numerous investigations have therefore sought to increase treatment efficiency and cost-effectiveness by reducing clay loadings (Sengco and Anderson, 2004). One strategy to increase application efficacy is the use of so-called modified clays, demonstrated to reduce concentrations required for successful flocculation by up to an order of magnitude (Sengco et al. 2005, Wu et al. 2010). Numerous organic and inorganic clay modifiers have been proposed to improve the adherence of clay particles to algal cells, yet their impact on the environment remains largely unknown. Perhaps the most promising modifier to date is polyaluminium chloride (PAC). Its ecotoxicology has been thoroughly investigated due to its wide use in drinking water treatment Srinivasan et al. 1999). Polyaluminium chloride has been declared safe for human health and application in perspirants up to 25% (FDA

Proença, L. A. O. and Hallegraeff , G.M. (eds). Marine and Fresh-Water Harmful Algae. Proceedings of the 17th International Conference on Harmful Algae. International Society for the Study of Harmful Algae 2017.

UNII 407PSC3OC7). Excellent removal efficiencies (100%) were achieved with PAC modified bentonite against *Prymnesium parvum* and *Karenia brevis* (Pierce et al. 2004 and Sengco et al. 2005). Particularly for algal species against which conventional clays can only achieve limited cell removal, such as the large-celled *Chattonella marina* (Wu et al. 2010), modified clays present an attractive option to reduce cell concentrations below levels considered harmful to aquaculture. However, the ichthyotoxin adsorptive properties of modified clays have not previously been investigated for species other than *Karenia brevis* (Pierce et al. 2004) and the potential of PAC itself to remove dissolved ichthyotoxins remains unknown.

For the highly potent fish-killers *C. marina* and *Alexandrium catenella*, reactive oxygen species (ROS) mediated lipid peroxidation of polyunsaturated fatty acids (PUFAs) has been shown to play an important role in observed high ichthyotoxicity (Mardones et al. 2015, Marshall et al. 2003). Lysis of algal cells can greatly amplify ichthyotoxic effects, as high concentrations of simultaneously released ROS and PUFAs act synergistically on the fish gill (Dorantes-Aranda et al. 2015). To avoid inadvertent amplification of ichthyotoxic effects, physical control strategies should aim to either prevent cell lysis (near impossible for highly fragile Raphidophytes) or rapidly scavenge extracellular ichthyotoxins. We here explore the capacity of PAC and PAC modified clays to adsorb *C. marina* and *Alexandrium catenella* ichthyotoxins quantified with the RTgill-W1 cell line assay. Additionally, minimal effective dosages for cell flocculation with conventional (kaolin, bentonite) and modified clays (bentonite, Korean loess) are compared for *Chattonella marina*.

Material and Methods

Algal cultures

Chattonella marina (CMN118; Japan) and *Alexandrium catenella* (DAVIS10; Aysén, Chile; group 1) were grown in GSe and L1 medium, respectively. Cultures were maintained at 20°C (17°C for DAVIS10), a salinity of 30 and light supplied at 120 µmol photons m^{-2} s^{-1}.

Cell removal

For *C. marina* flocculation experiments, cell removal efficiency of conventional kaolin (”Snobrite C”) and bentonite (”Ed”) clays was compared to that of polyaluminium chloride (PAC, Telford Industries) modified bentonite (”Ed”) and Korean loess (type A). Clay minerals are described in detail in Seger et al. (2015). Pilot experiments revealed a PAC:clay ratio of 0.4 to be most effective (data not shown). This ratio was employed in all subsequent experiments. *Chattonella marina* was harvested during the late exponential/early stationary phase (20,000 cells mL^{-1}) and transferred to 40 mL flat bottom, borosilicate test-tubes. Clays were suspended in deionised water, such that the dropwise addition of 2 mL to 30 mL of *C. marina* culture yielded final concentrations of 0-0.5 g L^{-1} (3 replicates per concentration). Control treatments received 2 mL of deionised water. After a settlling period of 3 h, cell concentrations in the overlying 25 mL were determined with a Coulter Counter (Beckman Z4) and removal effciencies calculated.

Ichthyotoxin removal

To determine the ability of PAC modified clays to adsorb dissolved *C. marina* (20,000 cells mL^{-1}) and *A. catenella* (12,000 cells mL^{-1}) ichthyotoxin(s), 60 µL of either conventional bentonite, modified bentonite or modified Korean loess (0.4 PAC:clay at 0.25 g L^{-1}) were added to 2.94 mL of algal culture in 15 mL centrifuge tubes immediately prior to sonication (30 and 45 s at 7 µm peak to peak for *C. marina* and *A. catenella*, respectively). To test for ichthyotoxin adsorption by PAC alone, the identical concentration of PAC used to modify clays was also tested (0.1 g L^{-1} PAC). Toxic controls received 60 µL of deionised water and nontoxic controls consisted of culture medium with added treatments (clays or deionised water). Resulting clay-algal lysate suspensions were centrifuged at 1400 x g (Hettich Universal 16A) and cytotoxicity of the supernatant tested on the gill cell line RTgill-W1 in specialised microplates with inserts (Corning 3381; Dorantes-Aranda et al. 2011). As an indicator of gill cell viability, cellular metabolic reduction of the dye Resazurin to fluorescent Resorufin was quantified in a plate reader (BMG Labtech) and reported as % viability compared to a non-toxic control (seawater + treatment).

Results and Discussion

Cell removal

Treatment of conventional bentonite and Korean loess modified with PAC significantly improved *C. marina* cell removal when compared to conventional kaolin and bentonite clays (Fig. 1 and 2). While conventional clays only removed 14±5% of cells at a relatively high clay loading of 0.5 g L^{-1}, an improved 85±5% removal was achieved with just 0.25 g L^{-1} of PAC modified clay.

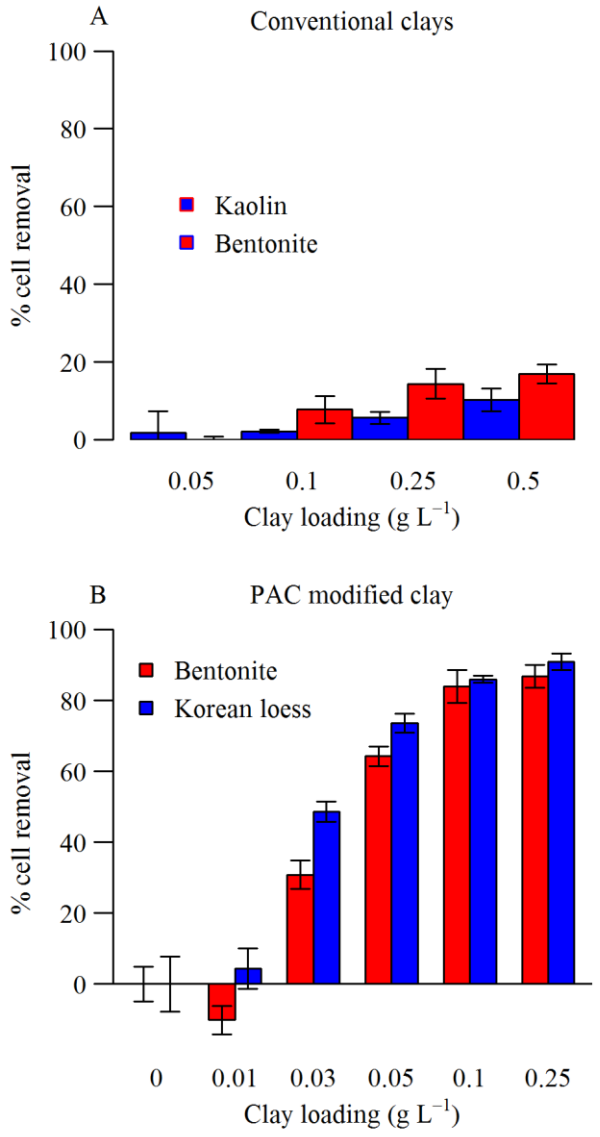

Figure 1 Flocculation efficiency (%) of *C. marina* cells treated with (A) conventional kaolin (blue) and bentonite (red) clay, as well as (B) PAC modified bentonite (red) and Korean loess (blue).

Similarly, Sengco et al. (2005) demonstrated that poor removal of *Prymnesium parvum* cells by conventional bentonite clay (10% removal) could be much improved through the addition of PAC (64% at 0.05 g L^{-1}). Modifying vermiculite clay with a surfactant (Gemini), Wu et al. (2010) reported much higher *C. marina* cell removal (100% removal with only 6.5 mg L^{-1} Gemini modified clay) than observed here with PAC.

Wu et al. (2010) attributed the high cell removal efficiency of this treatment to the algicidal properties of the Gemini surfactant. However, promoting cell lysis at the same time risks the release of intra-cellular ichthyotoxins (Dorantes-Aranda et al. 2013; Seger et al. 2017 a,b), a fact often overlooked in clay mitigation studies focusing on cell removal. Cell lysis in response to clay treatment has been reported by several authors for a range of fish-killing algal species (Archambault et al. 2003, Shirota 1989). Ichthyotoxins released in this way can only be removed by certain clay types with lower grain sizes and higher purities (Pierce et al. 2004, Sengco et al. 2005, Seger et al. 2015, 2017a,b).

Ichthyotoxin removal

Lysed preparations of *C. marina* and *A. catenella* proved highly toxic towards the gill cell line (only 36±6 and 2±1% viable gill cells remained after 2 h exposures, respectively; Fig. 3). For *C. marina*, treatment with PAC (0.1 g L^{-1}), conventional bentonite and PAC modified clays (0.25 g L^{-1}) completely eliminated cytotoxicity, while only PAC modified bentonite and conventional bentonite proved effective against *A. catenella* (69±10% gill cell viability). Pierce et al. (2004) previously reported that brevetoxin removal by bentonite clay could be improved through use of PAC (75 vs. 100% removal) and we here newly demonstrate that PAC on its own can effectively remove *C. marina* toxicity, as well as increase ichthyotoxin adsorption of otherwise poor adsorbents, such as coarse Korean loess (Seger et al. 2017b). While negatively buoyant clay particles are required in addition to PAC for effective flocculation to occur (Lürling and van Oosterhoot, 2013), our results suggest that other inexpensive clay minerals/sediments could replace high purity bentonites previously recommended for effective ichthyotoxin removal (Seger et al.

2015). However, we note ichthyotoxin adsorption by PAC to be algal species specific. Only bentonite type clay could significantly increase viability of gill cells exposed to lysed *A. catenella* preparations. To achieve maximum treatment efficacy, the type of clay to be modified with PAC therefore needs to be carefully selected based on the target algal species.

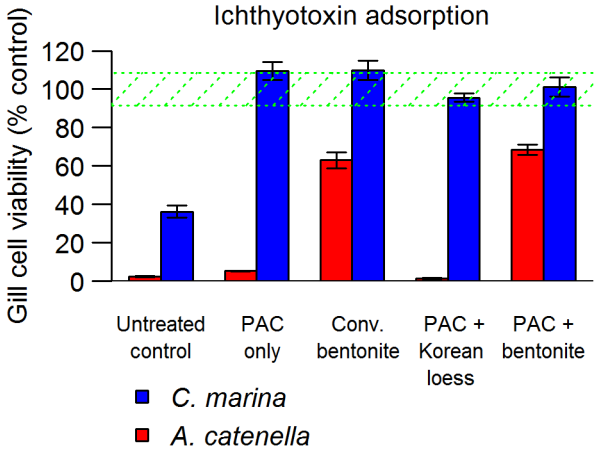

Figure 2. Gill cell viability after 2 h exposure to lysed *C. marina* (blue) and *A. catenella* (red) preparations treated with deionised water (toxic control), PAC (0.1 g L^{-1}) or PAC modified Korean loess or bentonite (0.25 g L^{-1}).

The application of modified clays to mitigate fish-killing algal blooms extends beyond their improved cell flocculation properties to include the adsorption of select ichthyotoxins. While we only focused on ichthyotoxin removal by PAC modified clays, future work screening a range of clay modifiers against various harmful algal species will add to our arsenal to mitigate the harmful impacts of fish-killing algae on aquaculture operations. No negative human health impacts of ichthyotoxins (with the exception of brevetoxins) have to date been reported and the direct adsorption of ichthyotoxin to modified clay particles offers great potential as emergency response to fish-farm threatening algal blooms.

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Environmentally friendly strategies for the prevention of harmful algal blooms using algicidal bacteria associated with seagrass beds

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Abstract

There is an urgent need for bloom mitigation strategies in aquatic areas subject to fishery damage by harmful algal blooms. Algicidal bacteria (AB) are promising agents which have been isolated and tested from coastal waters. ABs contributed to the rapid termination of HABs in the coastal waters such as the Seto Inland Sea. Large numbers of ABs were found to attach onto the surface of the seagrass *Zostera marina* (commonly 10^7 algicidal bacteria per gram wet weight) but without occurrences of any algal blooms. The algicidal bacteria *Alteromonas* strains S and K were detected as more numerous in seawater in seagrass beds than in offshore seawater, strongly suggesting that their origin is from seagrass beds in coastal environments. The presence of large number of ABs indicates the potential for preventing HAB occurrences. When we develop and restore natural seaweed- and seagrass-beds as a part of the Sato-Umi initiative, we postulate that this serves the ecological function to prevent the occurrences of HABs. Furthermore, restored seaweed and seagrass beds also serve as nursery grounds for important fisheries resources, and hence produce dual benefits.

Keywords: algicidal bacteria, harmful algal blooms, seagrass, prevention, *Zostera marina*

Introduction

In freshwater ecosystems such as lakes and marshes, various microbes such as viruses, bacteria, fungi, amoebae, heterotrophic flagellates, etc. play an important role in the extermination and/or mitigation processes of blooms caused by microalgae such as cyanobacteria, green algae, dinoflagellates, etc (Yamamoto 1987). It has been pointed out that algicidal bacteria that effectively kill microalgae are numerous and are involved in the termination of blooms (Daft *et al*. 1975; Mitsutani *et al*. 1987). In the studies of algicidal microorganisms in lakes and ponds, a soft-agar overlayer technique has been utilized for a long time and studies are well advanced. On the other hand, studies on marine algicidal microorganisms have been limited to a few diatom species, and the studies on toxic and red tide flagellates are very limited because most flagellates cannot grow in lawns on agar (Nagasaki and Imai 1994). Therefore, research on algicidal microorganisms for toxic and noxious flagellates has no established research methods.The MPN (Most Probable Number) method was applied to the enumeration of algicidal microorganisms against marine fragile red tide flagellates (Imai *et al*. 1998a). It was found that algicidal bacteria can be detected and isolated from the coastal waters of Japan. Just at or after the peak of the red tides, these algicidal microorganisms often increased and the red tides disappeared (Imai *et al*. 1998b; Kim *et al*. 1998). Algicidal bacteria therefore are thought to play an important role in the termination of red tides.

Examples of the activity of algicidal bacteria and growth inhibiting bacteria are shown in Fig. 1. Because the target species of *Chattonella antiqua* (Raphidophyceae) has no cell wall, their cells are easily ruptured by algicidal activities. In the case of growth inhibition, various patterns were observed as abnormal morphologies such as spherical form, stick type, amorphous shape, etc (Inaba *et al*. 2014). Many of the algicidal bacteria belong to gram-negative α-proteobacteria, γ-proteobacteria and Flavobacteriaceae (Mayali and Azam 2004; Imai *et al*. 2006).

Abundant existence of algicidal bacteria in seagrass beds

In seaweed beds, it was found that a large number of algicidal bacteria inhabited the biofilm on the surface of seaweeds (Imai *et al*. 2002, 2006, 2012). High densities of about 10^5 ~ 10^6 cells g^{-1} (wet weight) were detected for bacteria lethal to the dinoflagellate *Karenia mikimotoi* and the raphidophytes *Fibrocapsa japonica* and *Heterosigma akashiwo*. Those algicidal bacteria also showed high abundances in the seawater near the seaweed beds. Therefore, seaweed beds are probably supplying algicidal bacteria to the surrounding seawater.

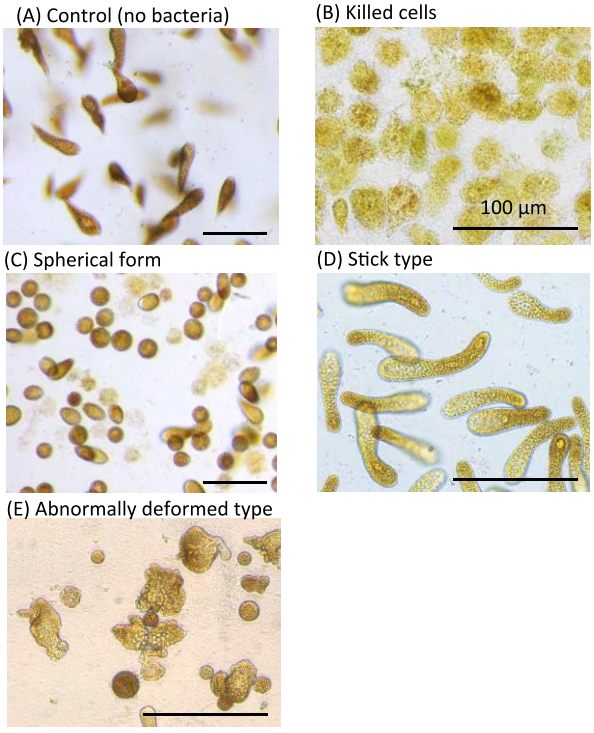

Fig. 1. Activities of algicidal bacteria and growth inhibiting bacteria against *Chattonella antiqua*. Scale bar, 100µm. After Inaba *et al*. (2014)

According to the high abundances of algicidal bacteria in seaweed beds, it is clear that seagrasses harbors many abundant algicidal bacteria in the biofilm on seagrass blades. We investigated the existence and densities of algicidal bacteria against five harmful algae (two raphidophytes *Chattonella antiqua* and *Heterosigma akashiwo*, and three dinoflagellates *Heterocapsa circularisquama*, *K. mikimotoi* and *Cochlodinium polykrikoides*) in a seagrass (*Zostera marina*) bed (0.2 ha) in Osaka Bay (Fig. 2) of the Seto Inland Sea (Imai *et al*. 2009, 2016).

The density of bacteria from the seagrass blades, which were algicidal to each red tide species showed a range of undetectable levels to as high as 6.4 x 10^7 g^{-1} (wet weight) from the seagrass blades (Fig. 3). The algicidal bacrteria against *K. mikimotoi* were the most numerous and followed by those against the dinoflagellate *C. polykrikoides*. The numbers of total bacteria that

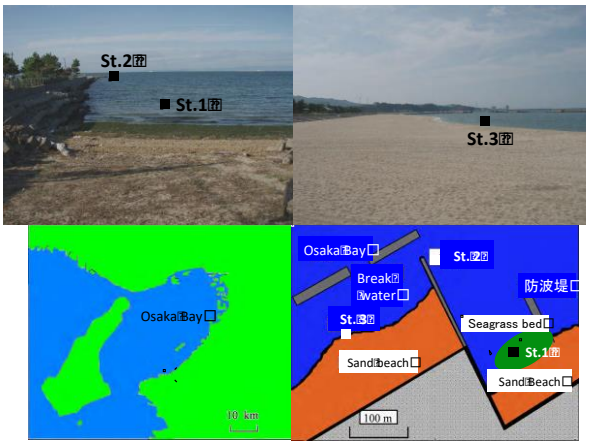

Fig. 2. Sampling stations of seagrass bed (St.1), short distant point (St.2) and sandy beach (St. 3) in Sennan, Osaka Bay, the Seto Inland Sea (Imai *et al*. 2016).

killed at least one algal species were approximately 1 x 10^7 to 9 x 10^7 g^{-1} (wet weight). Data obtained in July were interesting in that the total viable bacteria were 3.03 x 10^8 g^{-1} and algicidal bacteria were 9.12 x 10^7 g^{-1}, surprisingly indicating that a high percentage 30% of isolated bacteria from seagrass leaves possesed algicidal activity. In the seawater of a seagrass bed (St. 1 in Fig. 2), algicidal bacteria against each red tide species were detected in the range of about 10^3 to 10^4 cells mL^{-1} (Fig. 4). Most algicidal bacteria were present in the particle-associated fraction (> 3µm) at St.1 in seagrass bed. The densities of algicidal bacteria were less abundant in the fraction of free-living bacteria although some exceptions were noted. In the seawater of a sandy beach, algicidal bacteria showed lower densities than those in the *Zostera* bed. Phytoplankton densities were scarce in the seagrass bed than offshore (Imai *et al*. 2016). Hence, it is thought

that algicidal bacteria in seagrass beds are effectively killing red tide alga and play a potentially important role in preventing the growth of red tide alga in coastal environments

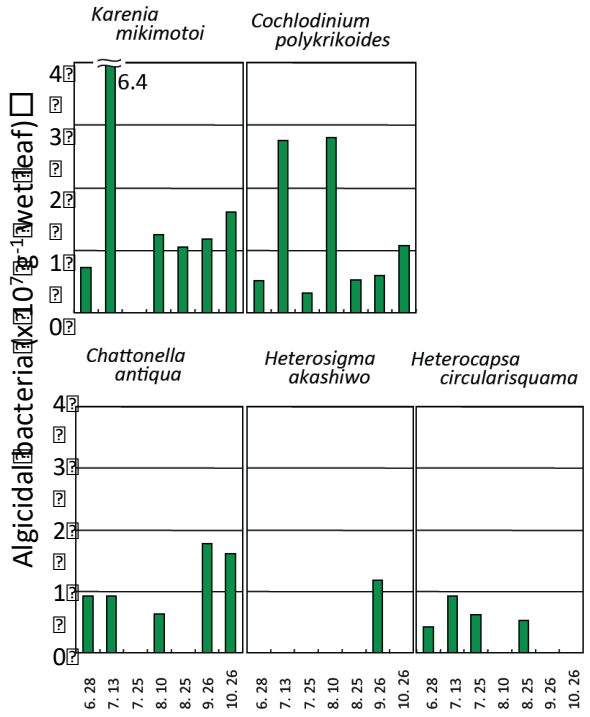

Fig. 3. Changes in algicidal bacteria detected from the biofilm on the surface of the seagrass (*Zostera marina*) leaves collected from Osaka Bay in the Seto Inland Sea in 2006 (Imai *et al*. 2016).

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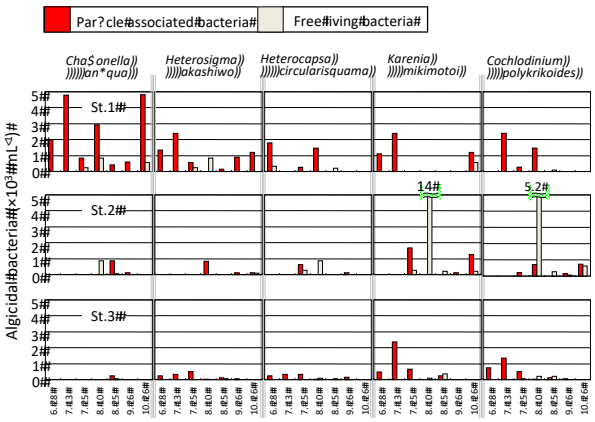

Fig. 4. Changes in algicidal bacteria in seawater of three sites (see Fig. 1). Nuclepore filters with pore size of 3µm were employed to fractionate free living bacteria and particle associated bacteria (Imai *et al*. 2016).

The seagrass *Zostera marina* was found to harbor growth-inhibiting bacteria against the toxic dinoflagellate *Alexandrium tamarense* from Usujiri Fishing Port, Hokkaido, Japan (Onishi *et al*. 2014). In the Puget Sound, WA, USA, algicidal bacteria and growth-inhibiting bacrteria against the red tide raphidophyte *Heterosigma akashiwo* and the dinoflagellate *A. tamarense* were investigated and also detected from seagrasses and seaweeds (Inaba *et al*. 2017). Findings of these bacteria suggested that seagrass and seaweeds provide an environment that probably influence the abundance of harmful algae in those regions. Thus, algicidal bacteria and growth-inhibiting bacteria against not only red tide phytoplankton but also toxic species seem to be ubiquitously inhabiting on the surface of seagrasses in the world.

Figures 5 and 6 show the map of sampling sites in the Seto Inland Sea and the distribution of three strains (S, K and D) of algicidal bacteria belonging to the genus *Alteromonas* in seawater at Genji Bay within a large seagrass bed and those in outer areas of Genji Bay, the Seto Inland Sea (Sakami *et al*. 2017). These algicidal bacteria were originally isolated for targetting *C. antiqua* from Hiroshima Bay (Imai *et al*. 1995). *Alteromonas* sp. strains S and K revealed significantly higher densities in the seawater in

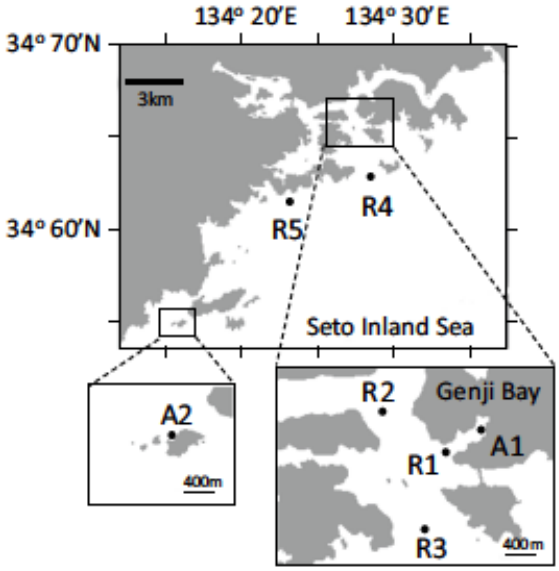

Fig. 5. Map of the sampling sites in the Seto Inland Sea.

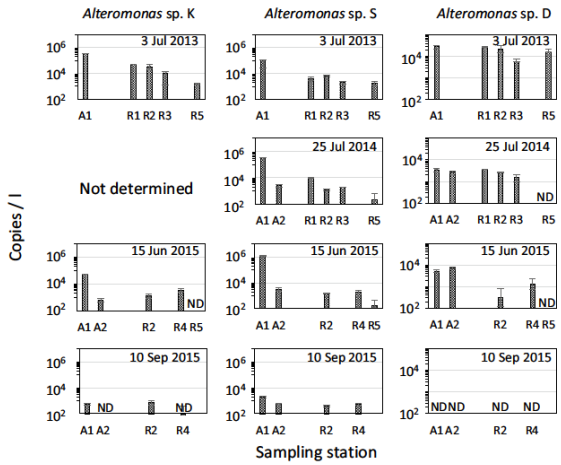

Fig. 6. Abundance of the three algicidal *Alteromonas* sp. Strains detected in seawater at seagrass beds (A1 and A2) and surrounding areas (R1 ~ R5) on different dates (Sakami *et al*. 2017)

the seagrass bed in Genji Bay than in the seawater in outer Genji Bay. And furthermore, the densities of strains S and K at Genji Bay significantly decreased after the seasonal loss of the seagrass bed there in autumn, indicating that the surface seagrass is an important habitat for these algicidal bacteria.

As described above, it was found that algicidal bacteria and growth-inhibiting bacteria against harmful algae were abundant in seagrass beds. It is thought that algicidal bacteria are continuously supplied from the seagrass beds to the surrounding water areas as shown in Fig 7. The potential function of seagrass beds is therefore considered to be ideal for red tide prevention from the view point of bioremediation. In other words, seagrass (*Z. marina*) provides the habitats for algicidal bacteria (biostimulation) and algicidal bacteria are continuously supplied into the seawater from the surface of seagrass blades (bioaugmentation). Moreover, the greatest benefit is that the cost for maintaining seagrass beds is not needed once healthy seagrass beds were established, and they will work for a long time under an appropriate management by human using such as the Sato-Umi concept (Yanagi, 2008).

The Sato-Umi is a new concept for sustainable fisheries and is identified as “High productivity and biodiversity in the coastal sea area with human interaction”. The process of establishing Sato-Umi started in the Seto Inland Sea. The establishment of Sato-Umi requires a correct regulation of the nutrient loading from the land to prevent red tide occurrences and to prevent oxygen-deficient water masses due to too much primary production in a short time promoted by the status of high nutrient levels of eutrophication.

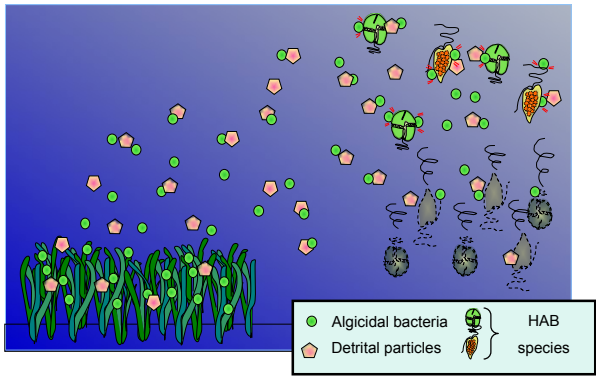

Fig. 7. Schematic representation of the role of algicidal bacteria on the surface of seagrass (*Zostera marina*) in coastal seas.

Overview

In the Seto Inland Sea, eutrophication had progressed during the era of high economic growth, and seaweed beds, seagrass beds, and tidal flats of shallow water areas largely had disappeared through large scale reclamations for the industrial developments. Considering that large numbers of algicidal bacteria inhabited the seagrass beds and seaweed beds, the disappearance of these areas resulted in the loss of the habitats for algicidal bacteria. This has direct implications for to the loss of the power of the sea to control and/or prevent the occurrences of red tides.

Restoration and creation of seaweed beds and seagrass beds in the coastal areas is proposed in oeder to prevent the occurrences of harmful algal blooms as part of the Sato-Umi Initiative (Fig. 8). Algicidal bacteria originating from seaweed beds and seagrass beds are continuously supplied to the waters, spreading, and thereby preventing red-tide occurrences in coastal areas in general (Imai 2015).

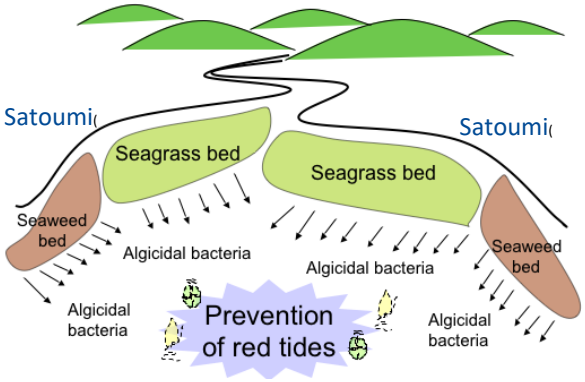

Fig. 8. Strategies for preventing the occurrences of harmful algal blooms by restoration and/or development of seaweed- and seagrass beds in coastal areas (Imai et al. 2009; Imai 2015). Algicidal bacteria would be provided to seawater and thereby prevent red tide occurrences by virtue of controlling phytoplankton populations to moderate levels.

Ecosystems of shallow coastal areas include seaweed beds, seagrass beds, and tidal flats, and these systems have high biodiversities. Seagrass beds are especially important for purifying water quality and promoting larval developments of useful fishery resources. Loaded inorganic nutrients into the shallow coastal areas would be quickly absorbed by seagrasses and epiphytic microalgae on seagrasses, and transferred through food webs to diverse and relatively long-lived higher organisms. When inorganic nutrients are loaded into the areas with no seagrass beds, phytoplankton communities directly take up those nutrients and immediately form dense blooms (Fig. 9). The surplus production could generate anoxia. Therefore, seagrass beds are considered to serve the ecological functions of preventing occurrences of phytoplankton blooms from the view point of nutrient cycling. Seagrass beds and seaweed beds also provide a positive and healthy image to the public. Hence, establishments of seagrass beds and seaweed beds in the coastal areas are expected to really produce environmentally-friendly prevention measures against harmful algal blooms, that can be easily understood by the public.

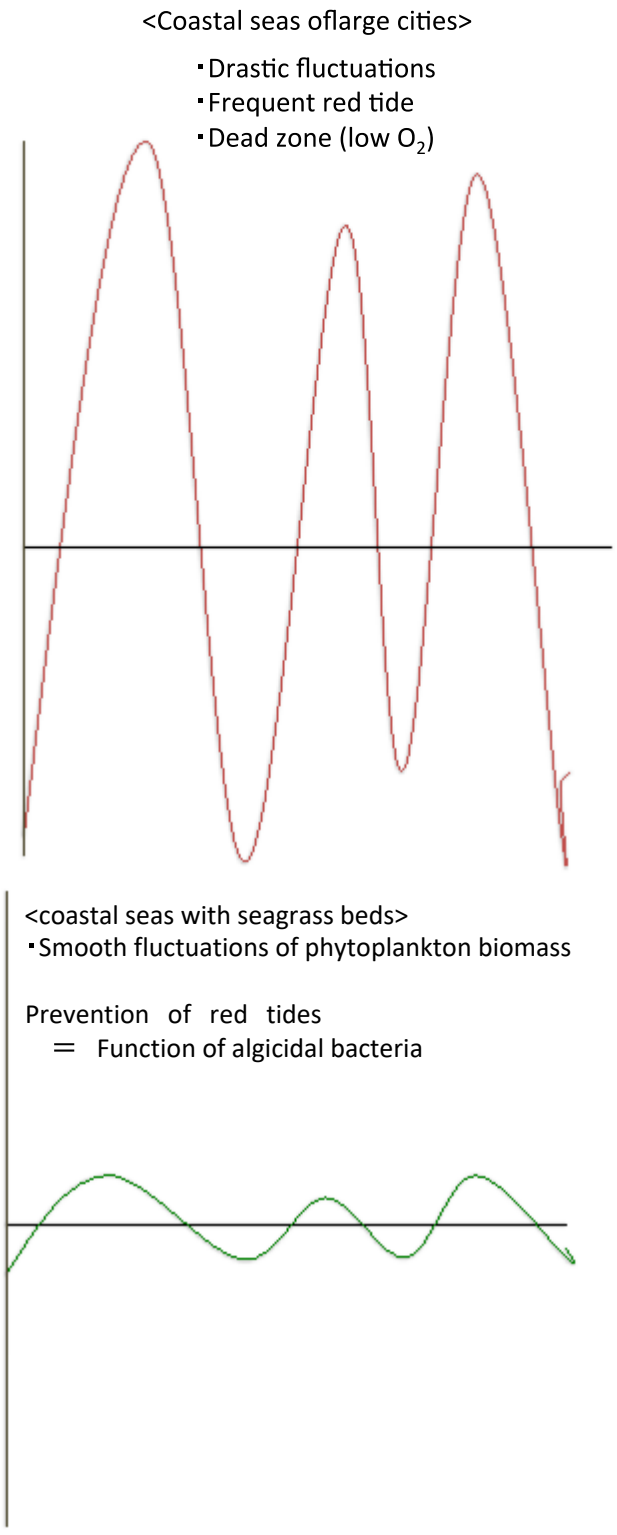

Fig. 9. Schematic representation on the responses of phytoplankton in seawaters of areas with and without seagrass beds.

Acknowledgements

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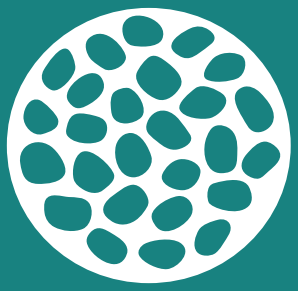

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