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HERSONISSOS-CRETE, GREECE, 1-5 NOVEMBER 2010

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PREFACE

The 14th International Conference on Harmful Algal Blooms (ICHA14) was held in Hersonissos (Crete island, Greece) from 1 to 5 November 2010. The conference was organised by the Hellenic Centre for Marine Research and the conference venue was the Creta & Terra Maris Convention Centre & Golf Resort, located in a beautiful peninsula of Northern Crete. ICHA14 brought together almost 500 participants from 66 countries. This was the first time the conference was scheduled in the Mediterranean. The historical island of Crete is the birthplace of the Minoan civilization – the first European civilization (3000-1450 BC) - the first link to the "European chain". Several thousand years later, in the same cradle, scientists from all over the world, gathered to participate in this international conference, including participants ranging from the Cook Islands, the Sultanate of Oman, to Europe, USA, and 40 Chinese scientists and graduate students.

The official opening of the conference was addressed by Dr Evangelos Papathanassiou, deputy director of Institute of Oceanography, Hellenic Center for Marine Research and by Dr Beatriz Reguera, president of ISSHA; welcoming letters sent by the deputy Minister of Foreign Affairs Mr Spyros Kouvelis and the governor of the local prefecture of Herakleion Mrs Evangellia Schinaraki were read.

More than 570 abstracts were submitted out of which 154 were selected for oral presentation and 380 for posters. A total of 534 abstracts, organised in a pdf in the format of the published Book of Abstracts were made available at www.issha.org after the conference. Parallel sessions were scheduled and the 5 plenary introductory sessions featured reviews covering the full range of HAB topics. We gratefully thank all key-note speakers for their valuable contributions. Our thanks go also to the able chairpersons of the various conference sessions: D. Anderson, L. Backer, M. Bricelj, A. Cembella, E. van Donk, G. Doucette, J. Engstrom-Ost, M. Estrada, Y. Fukuyo, E. Garcés, P. Glibert, G. Hallegraeff, R. Horner, A. Ishikawa, C. Legrand, J. Lewis, L. Medlin, J. Padisak, A. Penna, G. Pitcher, J. Ramsdell, B. Reguera, K. Rengefors, K. Sellner, S. Shumway, U. Tilmann, C. Wiegang and M.J. Zhou.

The scientific programme of ICHA14 focused on population dynamics of HABs, time series of bloom events focusing on climatic and anthropogenic induced impacts, impact of HABs on marine food webs and ecosystem structure and function, biological interactions such as allelopathy, mixotrophy, parasitism, symbiosis, bacteria and viruses, new regional bloom events, alien species, cyanobacterial ecology, physiology and bioactive compounds, genomics and genetic diversity of HABs, toxins: chemical structure and synthesis, detection and analytical methods and mechanisms of toxicity, novel sensor technologies for bio-sensing applications in HAB research and monitoring, management, mitigation and public outreach, and health aspects of HABs. For the first time, this conference included an expanded section on the genetics and genomics of HAB organisms, and a solid body of 50 papers and posters on climate change. The main points and discussions from the scientific sessions of ICHA14 were reported in Harmful Algal News (IOC of UNESCO) No 43, January 2011. The tradition of 3 page conference papers for the Proceedings format was upheld. In making our selection out of some 92 manuscript submissions, we have been guided by the comments of the International and National Scientific committees and other specialists whose opinions we sought. Our thanks go to all.

The conference period in mid autumn was blessed with warm and pleasant days and the participants enjoyed the mid-conference excursion to Knossos and the city of Heraklion. The Knossos Palace is the largest Bronze Age archaeological site on Crete and probably the ceremonial and political center of the Minoan civilization, the most visited tourist destination, near the main city of Heraklion.

At the 13th International Conference in Hong Kong, the Korean offer to host the 15th Conference in this series was accepted by consensus, and this offer was confirmed in Crete by Prof. H.G. Kim, accompanied by local authorities for scheduling in November 2012, in Changwon, Korea. Conference participants voted in favour of an offer from New Zealand to host ICHA16 in 2014. During ICHA14 the 7th General Assembly of the International Society for the Study of Harmful Algae was held which reviewed past and future activities. Thanks to ISSHA and the generous conference sponsors (FAO, NOAA, IAEA, SCOR, UNEP-MAP, Black Sea Commission) for their contributions which allowed 56 predoctoral students and experts from developing countries and economies in transition to receive financial support to attend the conference.

Preparation of the Proceedings was greatly facilitated and supported by the skills and dedication of Henrik Enevoldsen and Pia Haecky from the Intergovernmental Oceanographic Commission of UNESCO and Karin Rengefors as webmaster of the International Society for the Study of Harmful Algae.

The logistics of organising this meeting were facilitated by the staff of MINDWORK BUSINESS SOLUTIONS, of which Martha Natsoulidou and Popi Paraschaki deserve mentioning and especially Lina Nikolopoulou. We also thank

Konstantina Ballomenou, Mary Maniopoulou and Martha Papathanassiou from the Hellenic Centre for Marine Research for the large amount of administrative work they had to cope with in relation to the conference.

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PROGRESS ON *OSTREOPSIS* PHYSIOLOGICAL ECOLOGY, PHYLOGENY & TOXICOLOGY

Studies on cultures of *Ostreopsis* cf *ovata*: life cycle observations

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Abstract

Asexual and sexual reproduction were studied in cultures of *Ostreopsis* cf *ovata* isolated from several locations in the Mediterranean Sea. Asexual division took place in the motile stage by sharing of the theca (desmoschisis). High cell-size variability was observed in the cultures and differences in division capability were detected. Fusing gamete pairs were the only sexual state confirmed. Most of the gamete pairs isolated divided before the fusion was completed. Pellicle and thecate cysts were the only cyst-like cells which showed germination.

Introduction

Over the last few years it has been reported that blooms of the epiphytic palytoxin-producing dinoflagellates *Ostreopsis spp* have become established at some Mediterranean coastal sites (Aligizaki and Nikolaidis 2006; Mangialajo *et al.* 2007; Penna *et al.* 2010). One of these sites is Sant Andreu de Llavaneres beach (thereafter Llavaneres) (Catalonia, NE Spain), where in summer the bloom of *Ostreopsis* form a conspicuous, thick, brownish mucilage layer covering benthic macroalgae. The *Ostreopsis* bloom in Llavaneres occurred during the summer months following a clear seasonality, although no correlation was found between maximum cell concentrations and water temperature (Vila *et al.* 2010). The same authors also reported very low concentrations between December and April. Palytoxin has been detected in epiphytic samples from Llavaneres by haemolysis assay (Riobó *et al.* 2008) and high-performance liquid chromatography with postcolumn fluorescence derivatization (Riobó *et al.* 2006). These blooms are increasingly being associated with respiratory distress of people from the local area near the beach (Vila *et al.* 2010).

Material and methods

Llavaneres is located on the coast of Catalonia (Spain, western Mediterranean). Experiments were carried out with two strains (VGO820 and VGO1049) isolated from water samples taken from Llavaneres beach during summer blooms in 2004 and 2009. Isolations were carried out in L1 medium and when they reached sufficient abundance the growth experiment was performed in four enriched mediums: K, K/2, L1 and Schreiber. Cultures were performed in Erlenmeyer flasks of 50 or 100 mL at 20ºC, 10 h:14 h L:D photoperiod and 174.4 µmol m^{-2} s^{-1} light intensity. Two replicates were done and cell counts were performed on days 1, 5 and 9 after inoculation. Cell features of asexual and sexual reproduction were studied in tissue culture well plates with 2.5 mL K2+L1 (3:2) medium and in the same conditions of temperature and light as those mentioned above.

Results

The strains were characterized as *O. ovata* by molecular analyses based on internal transcribed spacer (ITS) and partial LSUrRNA sequencing. Table I shows the growth rates obtained for the four different media.

Table I. Growth rate (div/day) of *Ostreopsis ovata* cultures plotted in Fig 1.

	From days 1 to 5 (mean±SD)	From days 5 to 9 (mean±SD)
K/2	0.74±0,02	0.06±0,01
Schr.	0.53±0,19	0.23±0,12
K	0.49±0,06	0.06±0,03
L/1	0.50±0,04	0.18±0,06

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Maximum growth rate was 0.74±0.02 div/day obtained in K/2 during the first 4 days of culture. The growth pattern of K and K/2 was different to that of Schreiber and L1 (Fig 1).

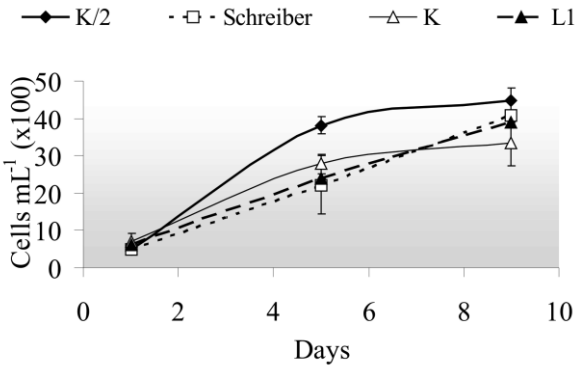

Fig. 1. Growth of *Ostreopsis* cf *ovata* in four different media.

During the first few days of culture, cells undergoing vegetative division were frequently observed. Cytokinesis was monitored. Examination of the theca from recently separated cells revealed that the fission plane was longitudinal, following the same pattern as that described by Besada *et al.* (1982) (Fig. 2a, b). The period from the start of observation of the partition wall until the cells split completely was 15–30 min. Daughter cells split very quickly but remained near to each other and connected by threads of mucus for hours or even days. By means of this process, the culture gradually became rich in small clumps of cells joined by threads of mucus. After 10 days the cultures contained an abundant amount of mucus, to which cells were attached and in which they were entwined. A high size variability of cells was observed in the cultures: 46±13 µm (length, mean±SD) (Figs 2c,d). Significant differences in division capability were observed between cells of different sizes (Fig. 3). Small cells (20–30 µm length) were not able to divide when they were isolated to individual plates. Medium-size cells (30-50 µm) displayed 0 to 1.5 divisions in 24 hours. Large cells (>50 µm) showed the highest division rate after being isolated to a new medium (from 1 to 2 divisions in 24 hours). The large cells were full of grains which appeared to be lipidic in the transmission electron microscopy (TEM). Ventrally located aggregation of spirally coiled fibres was also revealed by TEM. These structures were similar to those reported by Besada *et al.* (1982) and could be related to the mucus production, as these authors suggest. In the late exponential phase and in the early stationary phase, while the cultures still appeared healthy, the largest cells were awkwardly swollen towards the apical pore area. In the late stationary phase anomalous, probably aberrant cells started to be present in the cultures, and were very abundant during the definitively declining phase.

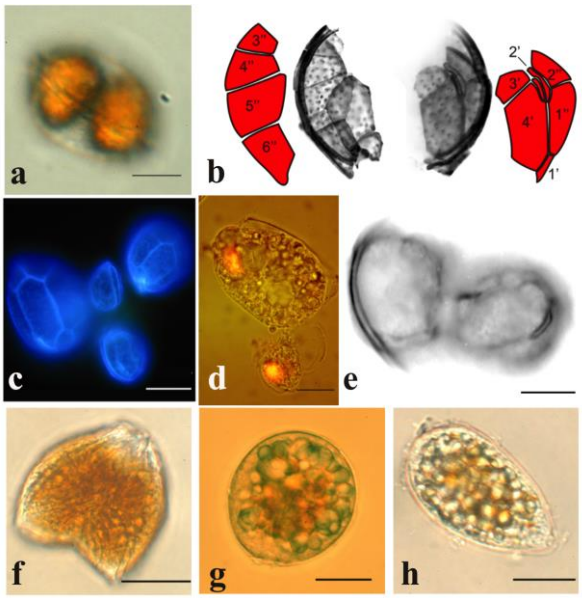

Fig. 2. *Ostreopsis* cf *ovata*. Dividing cell (a). Thecal fission pattern of the epitheca (b). Calcofluor-stained cells (c). Nuclei-stained cells (d). Calcofluor-stained gamete pair (e). Fusing gamete pair (f). Pellicle cyst (g). Thecate cyst (h). Scale=20µm

Gamete pairs were observed in nutrient-repleted cultures. Gametes formed an angle when fused and had the whole theca (Fig. 2e,f). These features clearly distinguished gamete pairs from dividing cells, because during division the cells were in the same plane and shared plates (compare Figs. 2a and 2b with Figs. 2e and f). When gamete pairs were isolated to individual plates to follow the cyst formation, the fusion was never completed. The fusing pairs continued moving for more than 24 hours without separating and did not definitely fuse. Finally, in most cases one of the gametes divided and formed one vegetative-like cell.

Resting cysts were not formed from isolated fusing gamete pairs. A very few single, double-

wall, cyst-like cells were observed on the culture plates on which strains are routinely maintained. No germination took place when they were isolated to a new medium, but they always degraded in a few days. On the other hand, pellicle cysts (Fig. 2g) – formed by ecdysis – were observed in cultures associated with stressful conditions. On some occasions they were thecate cysts because they were immobile and kept the theca without suffering ecdysis (Fig. 2h). Both pellicle and thecate cysts germinated in a variable range when isolated to a new medium.

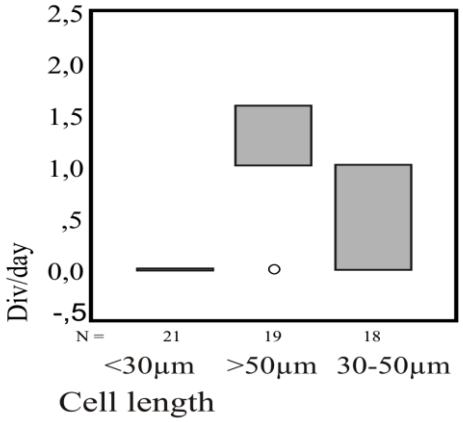

Fig. 3. Division rates of *Ostreopsis* cf *ovata* of different sizes

Discussion

The present paper describes some processes involved in asexual and sexual reproduction of *Ostreopsis* cf *ovata*. However, it was not possible to describe planozygotes and cysts, as reported for many other dinoflagellates. The gamete pairs kept fusing for a sufficient period of time for recombination to occur. However, planozygotes were not formed and the gametes divided while they were still joined. This process has also been described for *Gymnodinium catenatum* (Figueroa *et al.* 2006). Attempts were made to recognize cells that might be planozygotes because of their large size and dark colour. However, the fact that the longitudinal flagella of this species is short and not visible to light microscopy made it impossible to use the biflagellate feature to verify them. In addition, no resting cysts were formed during the experiments or when the fusing gametes or putative planozygotes were isolated to a new medium. The very few double-wall, cyst-like cells observed in routine maintenance of the cultures showed no germination and rapid degradation. Therefore, lacking more evidence about these cells, for the time being we think that they must be culture artefacts because of their rapid degradation. One question that is still open for understanding the blooms of *Ostreopsis ovata* is that of the overwintering population. Unsuccessful surveys were performed in Llavaneres in order to determine whether resting cysts in the substrates could explain the seasonality reported for this species in *Ostreopsis*'s Mediterranean blooms. No cysts have been described from any other region in the world for any of the species included in the *Ostreopsis* genus. The results showed in the present paper suggest that sexual reproduction does not lead to the formation of a resting state, as has been reported for many other dinoflagellates. Further studies must be carried out to confirm this fact, and whether the recombination process occurs in the gamete pairs without the need to complete the cytoplasmic fusion process

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Genetic and morphological diversity of the dinoflagellate genus *Ostreopsis* in Okinawajima Island, Japan

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Abstract

Genetic and morphological diversity of the genus *Osteopsis* in Okinawajima and Ishigakijima Islands in Ryukyu Islands was investigated. Benthic *Ostreopsis* dinoflagellates were found and isolated from various localities of Okinawajima and Ishigakijima. *Ostreopsis* spp were found everywhere and in all samples but the number of cells per sample was usually less than 100. Many cells were found on geniculate coralline algae such as *Actinotrichia fragilis, Galaxaura rugosa* and *Jania adhaerens.* In such samples diatoms were dominant and *Ostreopsis* cells were in small numbers. Morphologically, *O. siamensis, O. ovata, O. labens* and *O. lenticularis* were identified. From 12 isolated strains, part of the 28S rRNA D1D3 regions were sequenced. Phylogenetic analyses revealed that three different types were present. One type was closely related to *O. ovata* (GQ380659 and GQ380660) while another type was distantly related to *O. lenticularis* (AF244941) and the remaining type was distantly related to *O. siamensis* (FN256430 and FN256431). These data suggest that there exists a high genetic diversity of *Ostreopsis* species in the Ryukyu Islands. Recent worldwide outbreaks of *O. ovata* blooms are a serious problem and since the type locality of *O. ovata* is the Ryukyu Islands, a specific genetic entity should be assigned to *O. ovata.*

Introduction

Okinawajima Island and Ishigakijima Island are located in the middle and southern Ryukyu Islands, respectively. The Ryukyu Islands are a chain of islands approximately 1,000 km long in the western Pacific and eastern border of the East China Sea, stretching between southern Kyushu, Japan to east of Taiwan. The islands are strongly influenced by the warm Kuroshio Current and surrounded by well-developed coral reefs that have undergone recent deterioration. Since *O. siamensis* was established by Schmidt, 9 species are currently distinguished based on morphology. In the Ryukyu Islands, Fukuyo described *O. siamensis* and *O. ovata* in 1981 (Fukuyo, 1981) and Hoiriguchi added *O. labens* (in Murray, 2009). Benthic dinoflagellates of the genus *Ostreopsis* are putative producers of toxic substances such as palytoxin, ostreocin and analogues (Taniyama et al., 2003). Recently *Ostreopsis* spp., and especially *O. ovata, O. siamensis* and close relatives have become serious problems. For example, in recent years, *O. ovata* has bloomed along Mediterranean coasts, and the resulting brown cell masses and mucous cover wide areas and have caused respiratory illnesses (Vila et al., 2001; Aligizaki and Nikolaides, 2006; Ciminiello et al., 2006; Tottii et al., 2010). Additionally, the mortality of benthic invertebrates such as sea urchins has been recorded in Brazil and New Zealand due to *O. ovata* and *O. siamensis* blooms (Shears and Ross, 2009). Although *Ostreopsis* is ecologically important, little is known regarding the genetic divergence of species and strains within this genus. It is not clear what ecological parameters trigger the outbreaks of blooms and variation in toxin production, and whether the different genetic strains present influence toxicity. The present research isolated *Ostreopsis* spp. from various locations of the Ryukyu Islands, particularly around Okinawajima and Ishigakijima Islands, and

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compared their genetic diversity based on molecular phylogeny.

Fig. 1. Sampling sites in Ryukyu Islands, Japan.

Materials and Methods

Macroalgae, seagrass, coral rubble, sand and water samples were collected from different locations in Ryukyu Islands, mainly in Okinawajima Island (Fig 1). Unialgal clonal cultures of the *Ostreopsis* spp. were established by isolating single cells using a Pasteur pipette drawn out to capillary dimensions while observing them with a inverted microscope from freshly collected and enriched cultures samples. Each cell was washed several times by transferring through several drops of sterile medium. At each transfer a new sterile pipette was used. Cultures were initiated in culture in 15 ml tubes and then in 300 ml with PES (Provasoli, 1968) or IMK/4 (four times dilute IMK medium, Nippon Pharmaceuticals, Osaka, Japan) media at 24 ± 1°C, under a 14:10 h light/dark cycle at approximately 40 µmol photons m^{-2} s^{-1} provided by white fluorescent lamps. We examined morphological variation based on light (LM) and fluorescence microscopy (FM). Genomic DNA was extracted using a DNeasy plant mini kit (Qiagen, MA, USA) and further purified by a Gene Clean Kit (MP Biomedicals, OH, USA) according to manufacture's instruction. Primers of the D1D3 regions of 28S ribosomal RNA were utilized, and conditions of PCR amplifications were according to Takano and Horiguchi (2006). Obtained sequence data and GenBank data were aligned using Clustal X and then further aligned manually. Phylogenetic analyses were made by PAUP*4.0b10 using MP and NJ methods.

Results and Discussion

Ostreopsis spp. were ubiquitously found at all localities and in almost all samples but the number of cells was usually less than 100 per sample. Many cells were found on geniculate coralline algae such as *Actinotrichia fragilis, Galaxaura rugosa* and *Jania adhaerens.* While diatoms were dominant, *Ostreopsis* cells were often present only in small numbers (<100). Sometimes cell numbers exceeded 1000 per sample but in these cases often very few cells were found from the same species of macroalga from close localities. The cell numbers in this study were smaller than previous reports from other locations (Shears and Ross, 2009; Totti et al. 2010). Based on morphology, *O. ovata, O. lenticularis* and *O. siamensis* were identified but *O. lavens* was not found. Morphological variations among strains and even within single strains were also present. Molecular phylogenetic analyses of the D1D3 regions of 28S rDNA sequences revealed that three different types were recognized genetically. One type was closely related to *O. ovata* (GQ380659 and GQ380660) and another type was distantly related to *O. lenticularis* (AF244941) with the final type distantly related to *O. siamensis* (FN256430 and FN256431) (Fig. 2). These data suggest that a high genetic diversity of *Ostreopsis* species is present in the Ryukyu Islands. The type locality of *O. ovata* is the Ryukyu Islands (Fukuyo, 1981). Therefore, a specific genetic entity should be assigned to *O. ovata.* Further detailed studies of molecular phylogeny and morphology are needed on *Ostreopsis* spp in the Ryukyu Islands.

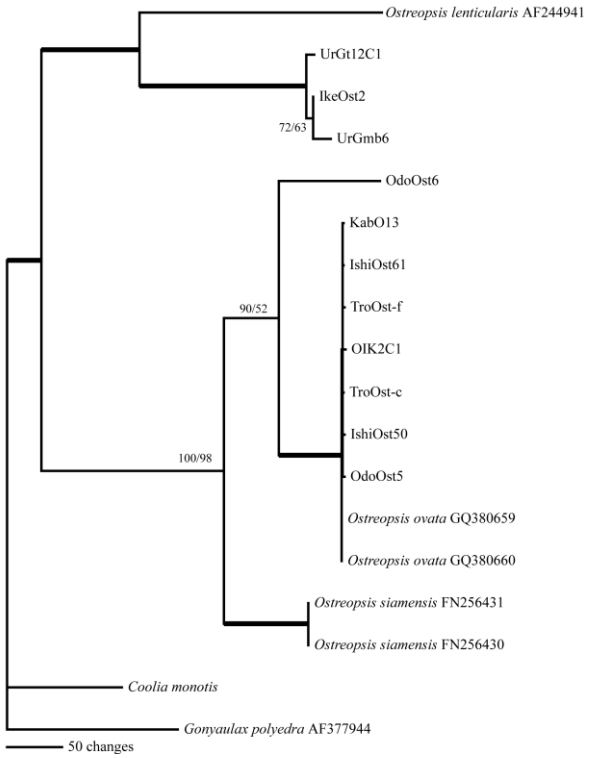

Fig. 2. Phylogenetic tree of *Ostreopsis* spp. inferred from the 28S rRNA gene D1D3 regions (708 bp) of MP tree. The bootstrap values (1000 replicates) are indicated at nodes (MP/NJ). The MP tree was generated by a heuristic search with TBR of the branch-swapping option and a random stepwise-addition option. The NJ tree was generated by Kimura's 2-parameter method. DDBJ accession numbers will be on the tree.

Acknowledgement

Miss Arisa Nakamura of "Program for creation of marine bio-industry in Okinawa" of MEXT, Japan for her strain maintenance. We thank also Dr. James Davis Reimer of the University of the Ryukyus for his valuable comments and discussion. This work was supported by the "Program for creation of marine bio-industry in Okinawa" of MEXT, Japan.

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Genetic diversity of the toxic genus *Ostreopsis* Schmidt and molecular method applications for species-specific and sensitive detection in natural samples

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Abstract

Ostreopsis is a toxic benthic dinoflagellate distributed worldwide, from tropical to temperate coastal waters, with nine described different species and responsible for the production of palytoxin-like compounds. In particular, two species as *O.* cf. *ovata* and *O.* cf. *siamensis* are being found with increasing frequency and abundance in temperate areas of the Mediterranean Sea, causing negative impacts on environment and human health. Species-specific identification, which is relevant for the complex of different toxins production, by traditional methods of microscopy is difficult due to high morphological variability, and thus different morphotypes can be easily misinterpreted. Molecular primers for the species-specific identification and quantification are designed and validated in cultured and natural samples using PCR based technologies. In monitoring activities of toxic *Ostreopsis* blooms, the PCR-based methods proved to be effective tools complementary or alternative to microscopy for rapid and species-specific estimation of *Ostreopsis* spp. in marine coastal environments.

Phylogenetic and Phylogeographical aspects

The phylogenetic position of the genus *Ostreopsis*, is clearly within the Gonyaulacales together with the other Ostreopsidaceae genus *Coolia* based on the SSU and LSU gene sequences (Saldarriaga *et al.* 2004). In the Mediterranean Sea, molecular phylogenetic and morphological investigations showed that all *Ostreopsis* spp. isolates grouped into two distinct species, *Ostreopsis* cf. *ovata* and *O.* cf. *siamensis*. Morphological observation under LM-epifluorescence and SEM microscopy of Mediterranean isolates fitted well with the original description of *O. ovata* Fukuyo and *O. siamensis* Schmidt. The phylogenetic analyses, which included isolates from SW Atlantic and Indo-Pacific areas, confirmed clustering of *Ostreopsis* isolates in two distinct species of *O.* cf. *ovata* and *O.* cf. *siamensis* (Penna *et al.* 2005) based on the ITS and 5.8S gene. In a recent study, several isolates of *O.* cf. *ovata* were collected in numerous localities throughout the world, but mainly in the Mediterranean Sea including the Atlantic and Indo-Pacific areas. The isolates were analysed by phylogenetic and phylogeographical analyses to test the hypothesis if this benthic microbial genus showed genetic diversity at macro-geographical scale. Numerous isolates of *Ostreopsis* spp. were sequenced and included in the molecular analyses. The phylogenetic analyses based on single and concatenated ribosomal genes of 5.8S, LSU (D1/D2) and ITS regions evidenced that different clades corresponded to different species within the *Ostreopsis* spp.: a clade represented by a single isolate of *Ostreopsis* sp. VGO881; a clade constituted by *O. lenticularis* and *O. labens*; a clade constituted by *O.* cf. *siamensis* and a clade comprising *O.* cf. *ovata* (Penna *et al.* 2010). *O.* cf. *ovata* was found widely dispersed throughout coastal waters of inter-tropical and temperate areas. Atlantic/Mediterranean regions *O.* cf. *ovata* seemed to constitute a panmictic population highly differentiated from Indo-Pacific populations. The other *Ostreopsis* species were restricted to just one of the two main warm-water oceanic basins of the Atlantic/Mediterranean and Indo-Pacific. The clade of *O.* cf. *siamensis* included isolates from the Mediterranean Sea without isolates from the Indo-Pacific region, where *O. siamensis* was originally described by Schmidt.

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Detection of *Ostreopsis* by molecular PCR assay

In the last few years, blooms of *Ostreopsis* in the Mediterranean Sea have dramatically increased associated with human health problems and negative impacts on benthic communities during the summer (Vila *et al.* this issue). These are emerging problems in warm temperate areas and there is urgency for new and innovative methodological approaches to be applied in monitoring plans and study of HAB emergent species such as *Ostreopsis* These programs include the rapid processes of identification and quantification of target species in huge amounts of samples. *Ostreopsis* species identification is very difficult due to high variability in morphological features (Aligizaki and Nikolaidis 2006).In this study, an efficient PCR-based assay was applied to natural samples in order to monitor the presence of *Ostreopsis* species in several Italian coastal areas.

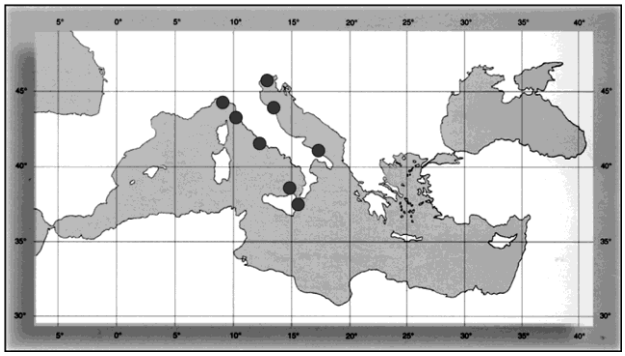

Fig. 1. Sampling stations along Italian coastline.

Materials and Methods

Samples were collected at several sites along the coasts of Italy in the summer 2009 (Fig. 1). Most localities sampled were commonly affected by blooms of *Ostreopsis* spp.. Samples of macrophytes were collected according to Totti *et al.* (2010) and in addition, surface seawater collected. All samples were processed for both molecular and microscopy analyses as described in Battocchi *et al.* (2010). The oligonucleotide primers used in the qualitative PCR based assay were designed in the high variable ribosomal regions of ITS and more conserved 5.8S sequence of *Ostreopsis* to satisfy the genus and species-specificity PCR reactions. The specificity and sensitivity of the PCR-based assay was assessed as described by Battocchi *et al.* (2010).

Results and Discussion

A total of 50 samples of macrophytes and seawater were analysed by both qualitative PCR assay and microscopy for the detection of *Ostreopsis*. All samples contained mixed microphytobenthic assemblages including target species. The abundance of *Ostreopsis* ranged from undetectable to 10^5 cells g^{-1} fw and from 10^2 to 10^5 cells l^{-1} in macrophytes and seawater, respectively. The higher abundance was registered during late summer (August-September). But microscopy determinations didn't allow to distinguish between the two species, *O.* cf. *ovata* or *O.* cf. *siamensis*, in the examined samples and determination was only at genus level. Whereas, the PCR-based assay confirmed the identification of the two *Osteopsis* species in the natural samples. The processed cells of the target species in the samples analyzed by PCR ranged from undetectable to $4.4 \times 10^5 \pm 1.3 \times 10^4$ cells g^{-1}fw and from undetectable to $4.0 \times 10^4 \pm 1.2 \times 10^3$ cells l^{-1} in macrophytes and seawater, respectively. Molecular taxonomical amplification signal for both genus and species derived by the amplification of 92 bp for the genus *Ostreopsis*, and 210 bp for *O.* cf *ovata*. This confirmed the amplificability of the target genomic DNA by PCR-based assay. The PCR detected the presence of *Ostreopsis* sp. in samples even when target cells were not observed by microscopy analysis. The positive detection of *Ostreopsis* sp. by PCR and microscopy was compared (Fig. 2). The positive detection by PCR assay was higher than microscopy by 16% for all field samples. The results obtained showed that the molecular PCR method has a higher efficiency of identification of the *Ostreopsis* compared with microscopy when applied to the same field samples. This is due to the exclusive species-specificity identification of *Ostreopsis*, as well as higher sensitivity of detection of *Ostreopsis* sp. in natural samples. It was found that only the *Ostreopsis* sp. is largely distributed along the Italian coasts only with the genotype *O.* cf. *ovata*.

Quantitative detection by Real Time PCR

To date *O.* cf. *ovata* species-specific identification by microscopy is difficult due to high morphological variability. A quantitative real time PCR (qrt-PCR) assay specific ,robust for the absolute quantification of the toxic dinoflagellate *O.* cf. *ovata* in environmental samples was developed. This approach considered alternative to traditional microscopy, may be applied for the monitoring of benthic HAB events in marine ecosystems.

Materials and Methods

Samples were collected from macrophytes and seawater during a bloom event of *O.* cf. *ovata* in the period of March-November 2009 at Conero Riviera (NW Adriatic Sea) and they were processed for both molecular and microscopy analyses. New species-

specific primers for *O.* cf. *ovata* were designed based on partial LSU gene sequences.

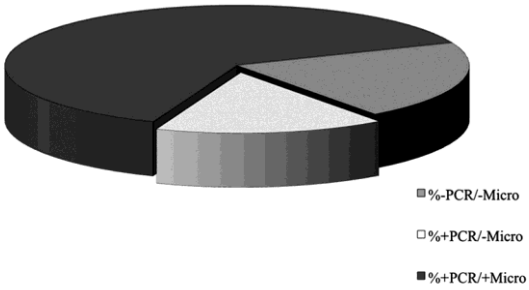

Fig. 2. Comparison of PCR and microscopy of field samples for Ostreopsis sp.. PCR amplifications compared with the corresponding positive and negative microscopy analyses of samples at different coastal sites in 2009 are shown. Data are expressed as % of total positive and negative values obtained with the two.

Environmental samples were lysed as described in Galluzzi *et al.* (2010) and total DNA was recovered for the qrt-PCR assay. The method is based on the SYBR Green real-time PCR technology and combines the use of a plasmid standard curve with a "gold standard" created with pooled crude extracts from field samples of the bloom. Based on similar PCR efficiencies of the two curves of plasmid and gold standards (96 and 98%, respectively), the exact rDNA copy number per cell was obtained in environmental samples. Analytical sensitivity of the PCR was set at two rDNA copy number and 8×10^{-4} cells per reaction for plasmid and gold standards, respectively; the sensitivity of the assay was of cells 1^{-1} based on lysis buffer volume. The reproducibility was determined on the total linear quantification range of both curves confirming the accuracy of the technical set-up in the complete ranges of quantification over time.

Results and Discussion

Species-specificity of the new designed primers was demonstrated: (a) *in silico* using BLAST; (b) by qrt-PCR carried out with purified DNA from *O.* cf. *ovata* and *O.* cf. *siamensis* cultures; (c) by qrt-PCR assay performed with macrophyte samples containing mixed microphytobenthos assemblages to ensure the absence of non-specific amplification products. Validation of the qrt-PCR assay was performed by quantification of 40 environmental samples from macrophytes and seawater during a bloom event of *O.* cf. *ovata*. The results obtained were compared with microscopy. For each environmental sample the exact no. of cells and rDNA copy number per cell was determined. There was a significant positive correlation (n=18, Spearman, r=0.97, $p<0.0001$) between cell densities on macrophytes and water column. A good correspondence was found between results obtained with microscopy and with molecular methods. This high correlation was particularly evident during the bloom event (Spearman, r=0.96, $p<0.0002$). In the range of low cell numbers, the PCR reaction of two macrophyte samples in which no *Ostreopsis* cells were found by microscopy resulted in a positive detection and/or quantification, showing better sensitivity of the qrt-PCR assay. The similar efficiencies of the pLSUO and gold standard curves allowed us to correctly quantify mean copy number of rDNA per cell in the *O.* cf. *ovata* bloom event. This is very important because for the first time a molecular assay has allowed us to quantify a toxic benthic microalgal species such as *O.* cf. *ovata* in surveys using only the pLSUO standard curve. Furthermore, the timely and specific detection of harmful algal species prior to bloom development is a crucial component of most HAB management programmes and is a necessary tool for researchers studying population dynamics and developing models to forecast HAB events.

Acknowledgements

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Occurrence of *Ostreopsis* cf. *siamensis* along the upwelling coast of Portugal (NE Atlantic)

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Abstract

The coast of Portugal is located in the warm temperate/sub-tropical transition of the North Atlantic. It is affected both by seasonal upwelling and water mass exchange with the Mediterranean basin. On October 2007 a sampling program aimed at the early detection of *Ostreopsis* species along the Portuguese coast was initiated. Here, we report the detection of *Ostreopsis* cf. *siamensis* on two sites on the Atlantic coast of Portugal. Cells were studied by LM and SEM and two cultures have been analyzed for species-specific genotyping. The final alignment of the ribosomal sequences of the analyzed culture with all ribosomal sequences of *Ostreopsis* available in the database used in this study revealed total identity match(100%) with *O.* cf. *siamensis* belonging to the Mediterranean clade. *O.* cf. *siamensis* was observed to colonize artificial sheltered habitats in upwelling exposed coasts suggesting that man-made microhabitats may play an important role on the establishment and geographical expansion of *Ostreopsis* species across natural barriers. Further studies will be needed to clarify the phylogenetic and phylogeographic position of *O.* cf. *siamensis* from Atlantic waters.

Introduction

The coast of Portugal is located in the warm temperate/sub-tropical biogeographical transition of the North Atlantic. It is part of the major discontinuity in the eastern boundary of the NE Atlantic, being affected both by seasonal upwelling and water mass exchange between the Mediterranean and Atlantic basins (Relvas et al. 2007). Shellfish production along the coast of Portugal is regularly affected by harmful algal blooms. The first episode of human poisoning attributed to the consumption of contaminated shellfish in Portugal dates back to mid-1900s. In 1986, a national monitoring program for toxic phytoplankton and marine biotoxins in shellfish was initiated. Since then, the major toxin groups and the causative species have been identified, namely, DSP associated with *Dinophysis acuta* and *D. acuminata*, PSP associated with *Gymnodinium catenatum* and ASP associated with species of *Pseudo-niztschia* (see Vale et al. 2008 for a review). Despite the sporadic detection of toxic and potentially toxic benthic dinoflagellate species, such as *Prorocentrum lima* and *Coolia monotis* in plankton surveys (Moita and Vilarinho, 1999), monitoring programs targeting this group have so far never been considered a priority. On October 2007, following reports of nuisance blooms associated with *Ostreopsis* species in the neighbouring Mediterranean Sea, a sampling program aimed at the early detection of *Ostreopsis* species along the Portuguese coast was initiated. Here, we report the occurrence of *Ostreopsis* cf. *siamensis* in two sheltered man-built marinas along the upwelling coast of Portugal.

Material and Methods

On October 2007 and July 2008 samples were collected in major shellfish production areas in the South coast of Portugal (Fig. 1). Samples were collected by harvesting macroalgae and mixed microalgae mats attached to pontoons and other surfaces, eel grass and floating organic debris. Plankton net tows (20 µm) were also collected. From 2008 onwards, two recreational marinas on the west coast, Cascais (38°41'36"N; 9°24'53"W)

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and Sines (37°57'2"N; 8°51'53"W), were sampled in summer and autumn (Fig.1). These marinas were built in 1999 and 1994 respectively. Epiphytic communities were collected from floating pontoons, at depths of 20-50cm, by detaching macroalgae and other sessile organisms, such as bryozoans, from pontoon walls and buoys. Occasionally, field samples were collected by scuba diving. Plankton samples were collected by 20 µm and 10 µm net tows. The net sample was pre-sieved before collection with a 100 µm sieve.

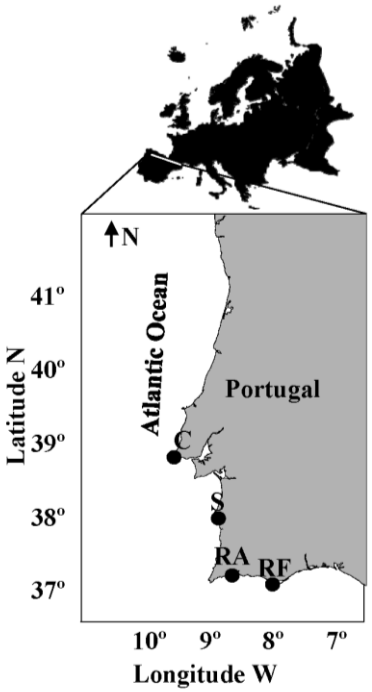

Fig. 1. Location of sampling stations. RF and RA, natural coastal lagoons; C (Cascais) and S (Sines), recreational marinas where *O*.cf. *siamensis* was recorded.

The epiphytic community was studied by vigorously shaking the substrate inside the collection bottles. The water was then poured into plastic Petri-dishes to be checked under the inverted microscope (Olympus IX70). Net samples were checked the same way. When detected, cells of *Ostreopsis* sp. were isolated by micropipette and transferred to culturing cell wells with filter-sterilized seawater from the sampling site. More than one cell were kept in the same well. Thus, resulting cultures are mono-specific but non-clonal. Cultures were routinely maintained in f/20-Si medium (salinity 35) at 19º C±1ºC with overhead illumination of 40 µmol.m^{-2}.s^{-1} and a L:D cycle of 14:10. Cell morphology was studied by SEM (JEOL JSM-5200LV) and light microscopy (LM). For tabulation studies by LM cells were stained with acid Lugol or Calcofluor White for epifluorescence microscopy (Fritz and Triemer, 1985). For molecular analyses, approximately 10 mL of exponentially growing culture were harvested by centrifugation (4000 rpm, 5 min). The pelleted cells were rinsed twice with filter sterilized (0.22 µm) artificial seawater. Total genomic DNA was extracted as described in Penna *et al.* (2005). PCR amplification of the 5.8S rDNA and ITS regions and direct sequence reactions were conducted as described in Penna et al. (2008). The 5.8S rDNA-ITS sequences obtained in this study were aligned in BLAST *silico* platform and using the confidential ribosomal sequence database at the Department of Biomolecular Sciences, University of Urbino.

Results and Discussion

Ostreopsis cf. *siamensis* was first detected in June 2008 along the SW coast of Portugal from macroalgae samples collected in the marina of Sines (Fig.1). Despite the sampling effort at shallow natural coastal lagoons in the South coast, in autumn 2007 and summer 2008, species of *Ostreopsis* were never detected in these coastal sites. *O.* cf. *siamensis* was detected again at Sines in October 2008, September 2009 and November 2010. On September 2010, *O.* cf. *siamensis* was detected further North in Cascais marina (Fig. 1). Initially, specimens of *Ostreopsis* were only found as epiphytes growing on mixed filamentous mats of unidentified macroalgae and the phaeophyte *Colpomenia peregrina* (June 2008). In 2010, contrasting with previous records, *O*.cf. *siamensis* in Sines and Cascais was only detected in net samples. In Sines *O*.cf. *siamensis* always occured within a benthic dinoflagellate community which included, *C. monotis*, *P. lima*, *P. emarginatum* and *Amphidinium* spp.. while in Cascais *Amphidinium* spp. were the only other benthic dinoflagellates present. Five cultures of *O.* cf. *siamensis* were established and are now kept in the culture collection of the University of Lisbon (ALISU). The morphological characteristics agree well with the description of *Ostreopsis siamensis* by Fukuyo (1981) and *Ostreopsis* cf. *siamensis* by Penna et al. (2005) (Fig. 2).

The wall is smooth covered with scattered pores of only one type. In side view the cells do not show any evident undulation. Field specimens have a dorso-ventral (DV) axis between 60.3-

61.7 µm and width range of 45.9-49.8 µm (n=9). In culture the DV axis varied between 36.1-72.5 µm and the width between 22.8-54.9 µm (n=43). Two size populations could be identified based on the DV axis. One population of small cells with a DV between 36-49 µm and a population of larger cells between 51-72.5 µm. Small cells were more abundant in aged cultures.

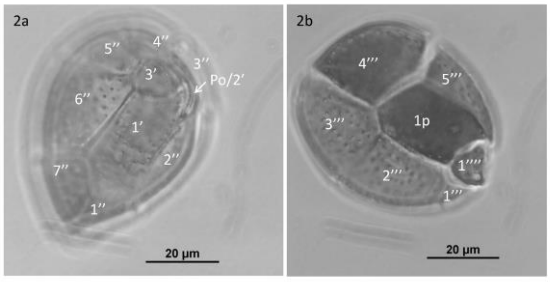

Fig 2. *O.* cf. *siamensis* theca from culture stained with acid lugol. (a) Epitheca and (b) hypotheca with scattered trichocyst pores. Diferential staining of plates in the hypotheca reveals the fission line of vegetative cell division.

The final alignment of the ribosomal sequence of the tested *Ostreopsis* culture with all ribosomal sequences of *Ostreopsis* species available in the database used in this study revealed total identity match (100%) with *O.* cf. *siamensis* sequences belonging to the Mediterranean clade (Penna et al. 2005). The geographical distribution of *Ostreopsis* spp. is generally associated with low energy systems and described as inter-tropical and from warm waters, as in the Mediterranean Sea (Tindal and Morton, 1998; Penna et al. 2010). Recent reports of *O. siamensis* from the southeastern part of the Bay of Biscay (Laza-Martinez et al. 2011) and the present work extend the geographical distribution of this species to more temperate regions in the Atlantic basin. Detection of *O.* cf. *siamensis* along the upwelling coast of Portugal, so far with a known distribution restricted to artificial recreational marinas, suggests that man-made microhabitats may play an important role in the establishment and geographical expansion of *Ostreopsis* species across natural environmental bariers. Currently it is not clear if the detection of *O.* cf. *siamensis* in the NE Atlantic is a result of increased sampling effort or a result of a recent introduction. Further studies on the phylogeography of this species based on more regional strains and other global populations will be needed to clarify the phylogenetic and phylogeographic position of *O.* cf. *siamensis* in Atlantic waters.

So far, populations of *O.* cf. *siamensis* have not been detected in high numbers along the Portuguese coast, nor have any toxic events been reported. However, the recent report of planktonic blooms of *O.* cf. *siamensis* along the upwelling coast of Morocco (Bennouna et al. 2010) should be considered as an early warning.

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Gulf of Trieste, northern Adriatic Sea: first record of *Ostreopsis ovata* bloom

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Abstract

The Regional Environmental Protection Agency of Friuli Venezia Giulia (ARPA-FVG) monitored the coastline of the Gulf of Trieste during summer 2009. At the end of September 2009 a bloom of *O. ovata* was revealed in a tidal pool of the coastline of the Gulf. During sampling hydrological parameters were recorded. The composition and abundance of the epiphytic community and nutrient concentrations were analyzed. During the bloom the dinoflagellate abundance reached 3.10^6 cells L^{-1}. The geomorphological characteristic of the tidal pool, its sheltered position from the wind and wave action, together with the good weather condition and nutrient concentrations, supported the development of *O. ovata* bloom.

Introduction

In the last decade *O. ovata* blooms have become more frequent along Italian coasts. Italian government funded a national "Monitoring Project" to check the presence of *O. ovata*. Monti et al. (2007) reported the first record of *O.* cf. *ovata* in the Gulf of Trieste in 2006, but up to summer 2009 in the gulf we have not been aware of any bloom. From June to August 2009 ARPA-FVG investigated four sampling areas. At the end of September 2009 a bloom of *O. ovata* was revealed. The aim of this study is to report the results of the "Monitoring Project" and the occurrence of the first *O. ovata* bloom in the Gulf of Trieste.

Material and method

The Gulf of Trieste is located at the north-eastern end of the Adriatic Sea. The eastern and south-eastern parts of the Gulf are characterized by cliffs with overhanging rocks, bays and pebbly beaches, while the north-western part is characterized by the Marano and Grado lagoonal system with low lying sandy coasts. Four sampling stations were chosen taking into consideration the most suitable geomorphological characteristics of the coast for the growth of epiphytic dinoflagellates and the areas where *O. ovata* has been formerly found. St. AP, CP and DP are located in rocky littoral areas with pebbly beaches and overhanging rocks while st. GP and HP are placed in sandy beaches near breakwaters (Fig.1). Samplings were carried out twice a month from June to August 2009. Hydrological parameters were measured using a multiparametric probe. The "Monitoring Project" checked the presence of eight benthic toxic dinoflagellates: *Coolia monotis*, *Gambierdiscus toxicus*, *Ostreopsis lenticularis*, *O. mascarenensis*, *O. ovata*, *O. siamensis* and *Prorocentrum lima*. Since these dinoflagellates are epiphytic species, the composition of the epiphytic community of different macroalgae was analysed. In each sampling station 3 macroalgal thalli were collected in 3 different places along the seashore, at a depth ranging from 0.5 to 2.0m, by means of a plastic bag in order to collect the macroalgae together with surrounding water. In the laboratory, for each sampling station, the 3 macroalgae were washed three times with filtered seawater (0.2 µm filter). The water of the three washings was added to the surrounding water collected with the macroalgae. The solution was fixed with acid Lugol's solution (0.2% final) and analyzed by inverted light microscope (Nikon TE-2000). Cell abundance was expressed as cells per gram of wet weight of macroalgae (cell g^{-1} ww). During sampling, additional sea water aliquots were collected, to check the presence of dinoflagellates in the water column (cell L^{-1}) following the Utermöhl (1958) method, and for nutrient analyses. Nutrient analyses were performed colorimetrically for am monium, silicate, phosphate, nitrate and nitrite using standard autoanalyzer techniques (µM) (Parsons et al., 1984).

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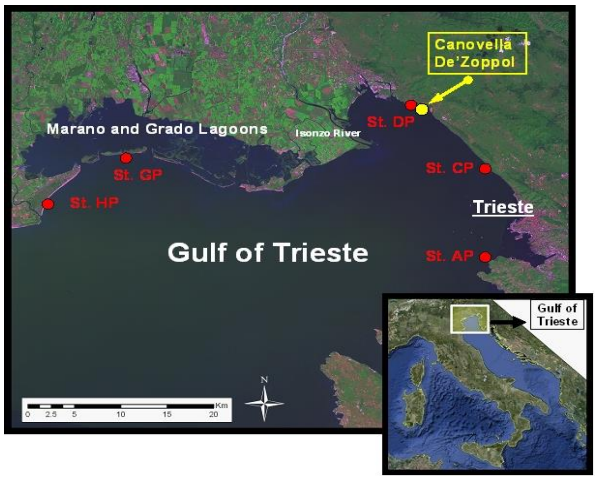

Fig.1. Map of sampling stations.

Results

Table 1	06/06/09	06/25/09	07/03/09	07/28/09	08/11/09	08/25/09
cell gr^{-1} ww	St. AP					
C. monotis	46	0	923	460	271	123
O. cf. *ovata*	0	0	0	0	54	31
P. lima	550.5	425.5	692	153	163	277
	St. CP					
C. monotis	59.5	2514.5	1136	593	274	109
O. cf. *ovata*	0	0	0	85	0	763
P. lima	224	785.5	142	339	91	218
	St. DP					
C. monotis	180	332	1639	462	278	33
O. cf. *ovata*	0	0	22	0	0	167
P. lima	1350	44	0	252	0	0
	St. GP	St. HP				
C. monotis	1500	775	0	242	0	0
O. cf. *ovata*	0	55	0	0	0	0
P. lima	0	55	0	0	0	0

Benthic communities never showed distress signs. Several macroalgal species were found: at st. AP *Cladophora prolifera, Cystoseira compressa*, *Padina pavonia* and *Stypocaulon scoparium*; at st. CP *Dictyota dichotoma* var. *intricata*, *Pterocladiella capillacea* and *S. scoparium*; at st. DP *Calosiphonia dalmatica*, *Ceramium* spp., *Cladostephus spongiosum* and *D. dichotoma*; while at st. HP *Ceramium* spp., and *Ulva lactuca* were collected. During the monitoring in all investigated sites *C. monotis*, *O.* cf. *ovata* and *P. lima* were found (Table. 1). In general, abundances never exceeded $2.5.10^3$ cell g^{-1}ww. In June and July *C. monotis* and *P. lima* prevailed while their abundances decreased in August with increasing *O.* cf. *ovata.* In the water column these species were never found with the exception for 20 cells L^{-1} of *O.* cf. *ovata* observed end of August at st. CP.

In 2009 hydrological data and nutrients were comparable to those observed in previous years (Table 2).On 29th September we discovered the presence of an *O.* cf. *ovata* bloom in coastal resort Canovella De'Zoppoli, characterized by a tidal pool demarcated from the rest of the beach and in contact with the open sea by means of a rocky reef. The tidal pool has a pebbly bottom and is sheltered from wind and wave actions by the rocky reef. The maximum depth is 1.5m and macroalgae are almost absent. During the bloom the water showed a mucilaginous brown pellet coating the pebbly bottom like a thick web rich in gas bubbles. Gas bubbles were lifting from the bottom to the surface where brown macro-aggregates were floating (Fig. 2).

Table 2	Temperature (°C)	Salinity (psu)	Oxygen (% sat.)	Chl *a* (µgL^{-1})
AP	23.09±2.73	35.72±1.02	108.7±7.9	0.6±0.2
CP	23.06±2.70	35.18±1.55	106.4±10.2	0.4±0.1
DP	23.42±2.86	32.25±2.03	104.8±5.7	0.7±0.1
GP-HP	25.68±2.14	29.60±2.01	104.1±8.6	0.8±0.3

Microscopic analyses of water and macro-aggregates samples (UNI EN 15204, 2006) revealed the prevalence of *O.* cf. *ovata*. Its highest abundance was found in the water in contact with the pebbles (Table 3). On 1st October water temperature was 22.27°C, salinity 37.29 psu and dissolved oxygen was 142 % saturation indicating high metabolic activity. Phosphate and nitrate concentrations were 0.24 and 2.1 µM, respectively.

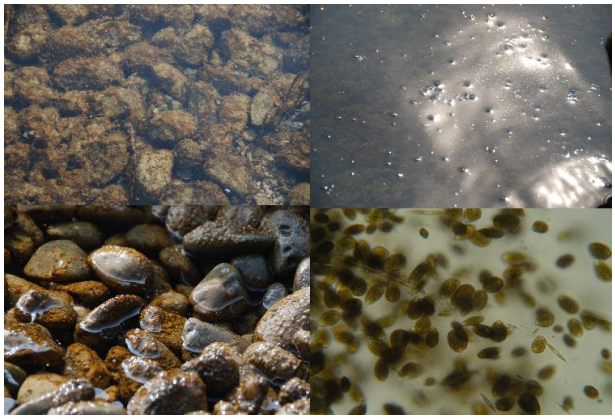

Fig.2. Floating brown macro-aggregates associated with Ostreopsis bloom.

Discussion and conclusions

During **"**The Monitoring Project" *O.* cf. *ovata, C. monotis* and *P. lima* were principally found at sites characterized by rocky shoreline. Their abundances were generally low throughout the monitoring period. St. CP was the most affected site. *C. monotis* showed its maximum abundance in June at st. CP and in July at st. AP and DP, moreover its abundance decreased with the increasing of *O.* cf. *ovata.* The maximum abundance of *O. ovata* was recorded at st. CP in water mass characterized by relative high values of temperature, salinity, dissolved oxygen and nitrate concentration as already observed by other authors in different places. Moreover st. CP was the only one sampling site where a few cells of *O.* cf. *ovata* were found in water column. St. CP and DP were characterized by the presence of brown and red macroalgae while at st. AP brown macroalgae prevailed. On the other hand at st. HP only a few green and red macroalgae were found. Probably the coastline geomorphology, the sediment texture and the diverse macroalgal community influenced the development of different epiphytic communities. The geomorphological characteristic of the tidal pool, its sheltered position from the wind and wave action, together with good weather conditions and nutrient concentrations, supported the development of *O.* cf. *ovata* bloom. It is worth to highlight that the lack of macroalgae inside the tidal pool did not affect *O.* cf. *ovata* growth and its bloom development.

Table 3.

	Sample description:	**Cells L^{-1}**
09/29/09	tidal pool: pellet scraped from the pebbles with water surrounding the pebbles	3.076.416
09/29/09	tidal pool: surface water surrounding the pebbles	2.636.928
09/29/09	dock near the tidal pool: surface water	4.6800
10/01/09	tidal pool: surface water	5.020
10/07/09	tidal pool: surface water	400

Acknowledgments

We wish to thank the technical and scientific staff of the Agency ARPA-FVG. Thanks go to Dr. Daniela Fornasaro of the OGS-Biological Oceanography Department, of the National Institute of Oceanography and Experimental Geophysics of Trieste, for the light microscope photograph.

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Ecology of a bloom of *Ostreopsis* cf. *ovata* in the northern Adriatic Sea in the summer of 2009

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Abstract

Since 2006, blooms of the benthic dinoflagellate *Ostreopsis* cf. *ovata* have occurred on the rocky coasts of the northern Adriatic Sea. Such blooms are associated with noxious effects on human health and the mortality of benthic organisms, due to the production of palytoxin-like compounds. We investigated a bloom of *O.* cf. *ovata* from March through to November 2009, which included assessment of the role of environmental parameters on the bloom dynamics at two stations on the Conero Riviera (NW Adriatic Sea). *O.* cf. *ovata* developed from August to November, with the highest densities in mid-September ($6.4x10^4$ cells cm^{-2}, i.e. $1.3x10^6$ cells $g^{-1}fw$). Cell densities were significantly higher on rocks than on seaweeds and at sheltered sites compared to exposed sites. The presence of a single *O.* cf. *ovata* genotype was confirmed by PCR assay. In contrast other Mediterranean sites, the bloom developed with decreasing temperatures. Nutrient concentrations did not appear to affect bloom dynamics. Toxin analysis performed by liquid LC/MS revealed high concentrations of ovatoxin-a (up to 14.8 pg $cell^{-1}$).

Introduction

Benthic dinoflagellates belonging to the family Ostreopsidaceae are common members of benthic microalgal communities in both tropical and temperate areas (Rhodes 2011). Two *Ostreopsis* species, *O.* cf. *ovata* and *O.* cf. *siamensis*, are found with increasing frequency in a number of Mediterranean coastal areas (Vila *et al*. 2001; Aligizaki and Nikolaidis 2006; Mangialajo *et al*. 2008; Totti *et al*. 2010). *Ostreopsis* includes benthic species associated with a variety of substrata. *Ostreopsis* blooms have often been associated with noxious effects on human health (Gallitelli *et al*. 2005) and with mortality of benthic marine organisms (Shears and Ross 2009), due to production of palytoxin-like compounds, represented primarily by ovatoxin-a (Ciminiello *et al*. 2010). Although the influences of temperature and hydrodynamic conditions on bloom development have been addressed in previous studies (Totti *et al*. 2010), the role of nutrients in bloom dynamics has not been examined until now. In this study, we investigated a bloom of *O.* cf. *ovata* along the Conero Riviera (northern Adriatic Sea) during the summer of 2009 in relation to nutrient concentrations. Furthermore, we analyzed the toxin content and composition of the bloom.

Materials and methods

Sampling was carried out at two stations (st. 1 and st. 2) along the Conero Riviera (Ancona, NW Adriatic Sea), both characterized by shallow depth (1 m) and a rocky bottom: at st. 1 two sites were sampled, representing a sheltered (st. 1/L) and exposed (st. 1/H) site. Sampling was carried out from 25 March to 28 October 2009 with a frequency of 7-15 days. Surface temperature (CTD) and marine meteorological conditions (Douglas scale) were recorded. Water samples for nutrient analysis (in triplicate) were collected at each site, filtered through 0.45 µm filters and stored in polyethylene bottles at -22°C. At each station, seawater samples were collected to determine the abundance of dinoflagellates in the water column. Samples were preserved by adding 0.8% neutralized formaldehyde. Two seaweed species (*Ulva rigida* and *Dictyota dichotoma*) and pebbles were also

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sampled for benthic dinoflagellates (3 replicates). All benthic substrata were collected (seaweeds, molluscs shells, rocks) in order to avoid loss of epiphytic cells and treated to obtain complete removal of *Ostreopsis* cells following Totti *et al.* (2010). *Ostreopsis* cells were identified and counted according to the Utermöhl method. Final data were expressed as cells g^{-1} fw/dw (macroalgae), cells cm^{-2} (macroalgae, rocks) and cells l^{-1} (planktonic cells). The analysis of dissolved inorganic nitrogen (DIN), phosphate and silicate was carried out following Strickland and Parsons (1968). Molecular analysis by PCR for *Ostreopsis* species identification was carried out on formalin-fixed macrophyte and seawater samples (Battocchi *et al*. 2010). Toxin content was analyzed in epiphytic populations of *O.* cf. *ovata* collected at both stations on 18 and 19 September and 21 October, by LC/MS (Ciminiello *et al*., 2010).

Results

PCR amplifications revealed only the presence of the genotype *O.* cf. *ovata* in all samples examined. A time series of *O.* cf. *ovata* abundance on benthic substrata (macroalgae and rocks) and as a component of the phytoplankton is shown in Fig. 1B. The first occurrence of *O.* cf. *ovata* was recorded at the end of July. At st. 1/L, maximum abundances were recorded on macroalgae on 9 September (64×10^3 cells cm^{-2} corresponding to 1313×10^3 cells g^{-1} fw and 16416×10^3 cells g^{-1} dw) while at st. 2 the maximum abundance was recorded on rock substrata on 23 September (26×10^3 cells cm^{-2}). High densities of *O.* cf. *ovata* persisted at both stations, until the end of September, before decreasing until bloom termination.Cell densities of *O.* cf. *ovata* in the water column paralleled that of the benthic substrata (max 92×10^3 cells l^{-1}, at st. 2 on 3 September); with densities increasing in the water column after moderate hydrodynamic events Comparison of cell abundances at the sheltered (st. 1/L) and exposed (st. 1/H) stations showed significantly higher concentrations of *O.* cf. *ovata* at the sheltered site ($p < 0.01$). During the sampling period, the surface temperature ranged from 10.4 to 27.9 °C, reaching a maximum in August. The highest abundances of *O.* cf. *ovata* were observed within the temperature range of 22.7 - 22.9 °C. The time series of DIN and PO_4 was similar at both stations. DIN ranged from 2.391 to 17.338 µmol l^{-1} during the *O.* cf. *ovata* bloom, while PO_4 ranged from 0.008 to 0.324 µmol l^{-1}. Although the bloom peak coincided with a general decrease in PO_4 concentrations, no significant correlation was found between *O.* cf. *ovata* abundances on benthic substrata and DIN or PO_4. During the bloom, deleterious impacts on humans were reported at both stations. Moreover, at the sheltered site, mortalities of benthic invertebrates (limpets, sea urchins, mussels) and macroalgae were observed. The analysis of the toxins of *O.* cf. *ovata* from macrophyte samples, showed a prevalence of ovatoxin-a (OVTX-a) with maximum value of 14.3 pg $cell^{-1}$ (and 0.93 pg $cell^{-1}$ for pPLTX at st. 1/L on 18 September).

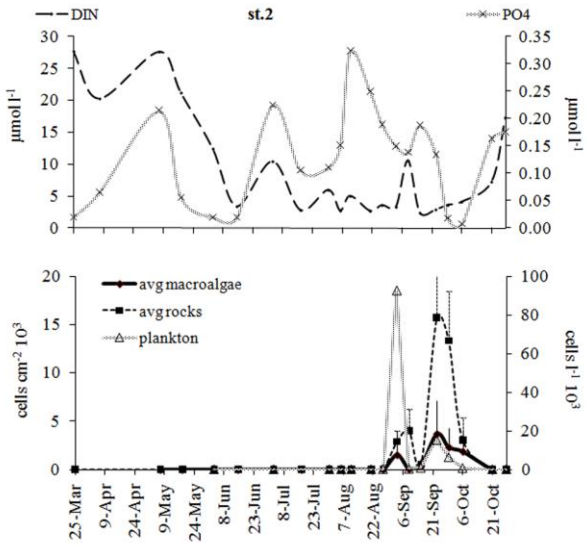

Fig.1. Nutrient concentrations and *Ostreopsis* cf. *ovata* cell densities at st.2. (A) Dissolved inorganic nitrogen (left y-axis) and phosphate (right y-axis). (B) *O.* cf. *ovata* abundance on macroalgae and rocks (left y-axis), and in the water column (right y-axis).

Discussion

Ostreopsis cf. *ovata* blooms along the Conero Riviera seem to be among the most intense of 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. The

highest densities of *O.* cf. *ovata* were recorded at the sheltered site, highlighting the important role of hydrodynamics in bloom development (Totti *et al*. 2010; Shears and Ross 2009). The highest cell densities were recorded in association with decreasing temperatures in contrast to previous studies in the N Adriatic Sea (Monti *et al.* 2007; Totti *et al*. 2010). These authors suggest that *Ostreopsis* spp. needs relatively high temperatures to proliferate, suggesting that global warming may influence *Ostreopsis* expansion in the Mediterranean (Granéli *et al*. 2011; Hallegraeff 2010). However, the relationship with seawater temperature is not the same in all geographic areas (Mangialajo *et al*. 2008; Selina and Orlova 2010), and it has been suggested that the influence of temperature could be strain specific (Pistocchi *et al*. in press).Recent studies have provided increasing evidence of a link between nutrient enrichment and harmful algal events (Hallegraeff 2010). However, in our study, although peak abundances coincided with a decrease in nutrient concentration, we did not observe a clear relationship between the bloom and nutrient concentrations;an observation supported by other studies (Vila *et al*. 2001; Shears and Ross 2009).The toxin profile showed the presence of ovatoxin-a (OVTX-a) and of putative palytoxin (pPLTX). OVTX-a values (2.25–14.3 pg cell^{-1}) were higher than those reported from other parts of the Italian coast (Ciminiello *et al.* 2008, Guerrini *et al*. 2010). This is reflected in the increasing intoxication of humans and in the mortalities of benthic marine organisms in this area.

Acknowledgements

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Ostreopsis cf. *siamensis* blooms in Moroccan Atlantic Upwelling waters (2004-2009)

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Abstract

In 2004, *Ostreopsis siamensis* Schmidt blooms were detected for the first time on the central upwelling coast of Morocco by the HAB and phycotoxins monitoring program. The species identification was confirmed by genetic studies. In October 2004, *O. siamensis* blooms reached $3.7x10^3$ cells L^{-1} in seawater samples of the cape Ghir. In the following years, with a sea surface temperatures of 20 - 24°C, the blooms became recurrent, longer and increased in abundance reaching 9.8 x 10^3 to 12x 10^3 cells.l^1 in 2008, with a maximum of 10^5 cells.l^{-1} observed in August 2009. The detection of toxins in mussels collected from the same area, by mouse bioassay, evidenced the presence of lipophylic toxins. At Cape Ghir, which is an important upwelling center on the Morrocan coast, the cell maxima occurred in rocky areas, well exposed to winds and waves, and not in wind sheltered areas south of the cape, highlighting the importance of hydrodynamism on the re-suspension of this epiphytic species in the water column.

Introduction

In the last decade, the occurrence of the tropical benthic dinoflagellate *Ostreopsis* has increased in many temperate regions (Dale *et al.* 2006). The geographic expansion of *Ostreopsis* species has been related to spreading by ship ballast water (Lilly et al., 2002), and to changes in ocean climate and circulation patterns (Riobo *et al.*, 2008). On the Moroccan coast, the HAB monitoring program carried out from January 2004 to December 2009, observed the occurrence of *Ostreopsis* outbreaks on the Cape Ghir area, providing an opportunity to identify the species involved and to investigate some oceanographic features of bloom dynamics. In particular, it allowed the study of the bloom relationship with prevalent winds, sea surface temperature and surface circulation patterns. In parallel, the effect of *Ostreopsis siamensis* concentration on shellfish toxification was evaluated.

Materials and methods

Water and mussels were collected from 4 stations in the coast of Agadir during the HAB and phycotoxins Moroccan national monitoring program (Fig.1). The results of mussels' toxicity were obtained by the Mouse Bioassay (Yasumoto et al. 1984, modified). The species were identified by inverted contrast and epifluorescence microscopy and confirmed by genetic based on PCR amplification of 5.8S–ITS and LSU ribosomal genes (Penna *et al*., 2005).

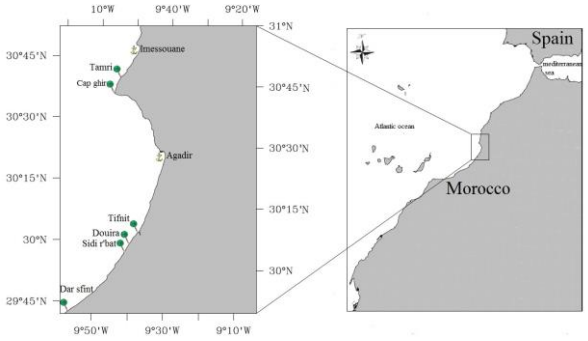

Fig. 1. Sampling area.

Sea surface temperature (SST) averages were computed from EUMETSTAT´s Ocean and Sea Ice Satellite Application Facility (O&SI SAF) "Regional SST" product, for the "CANA" region. These SST estimates are computed using data from the AVHRR (Advanced Very High Resolution Radiomenter) (Brisson *et al.*, 2001). NCEP re-analysis North/Soputh wind stress data for the

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location 11.25W, 31.43N were used to characterize the upwelling forcing winds. To assess the circulation patterns in the vicinities of Agadir, the solutions from the HYCOM Model - HYCOM + NCODA - Navy Coupled Ocean Data Assimilation - Global 1/12° Analysis) were analysed.

Results and discussion

This study represents the first quantification of Atlantic Moroccan coastal waters *Ostreopsis* proliferation during 2004 - 2009. *Ostreopsis* blooms were first observed in Cape Ghir seawater during 2004. Blooms reappeared in the following years, and increased in concentration and time. In 2007, the species involved was *Ostreopsis siamensis* identified through PCR amplification. During 2007, cells were observed from late summer to early autumn, in 2008 from early summer to late autumn, and in 2009 from spring to late autumn (Fig.2). As in the Mediterranean coast (Mangialajo *et al.* 2010), the occurrence of *Ostreopsis* in Agadir area showed the most important first bloom in summer and a second one, less important, in autumn. In the north Aegean Sea, the presence of *O. cf. siamensis* exhibited a clear seasonal pattern dominating the period from mid summer to late autumn (Aligizaki and Nikolaidis, 2006). During the blooms, sea surface temperature ranged from 20 to 24°C, with maxima recorded in August and September. In the Mediterranean, *Ostreopsis* was found between 11.5°C and 29.7°C (Vila et al., 2001; Aligizaki and Nikolaidis, 2006). Although the seawater temperature may play a major role in a specific site, it does not seem to affect the genus distribution and to be a primary driver to control the outbreaks (Carlson and Tindall, 1985; Mangialajo *et al.* 2010).

Highest *Ostreopsis* concentrations were always observed in Cape Ghir, and at a station further north, at Tamri. Both sites are located on a rocky coastline highly exposed to northerly (upwelling favourable) winds and waves. In contrast, other dinoflagellates species, like *Karenia* sp. and *Gymnodinium* spp., were favoured at Douira - Sidi R'bat and Dar sfint, in the shadow of the Cape, on a sandy coast with calmer waters (Fig.1). This suggests that turbulent conditions north of the cape favoured resuspension of this epiphytic species in the

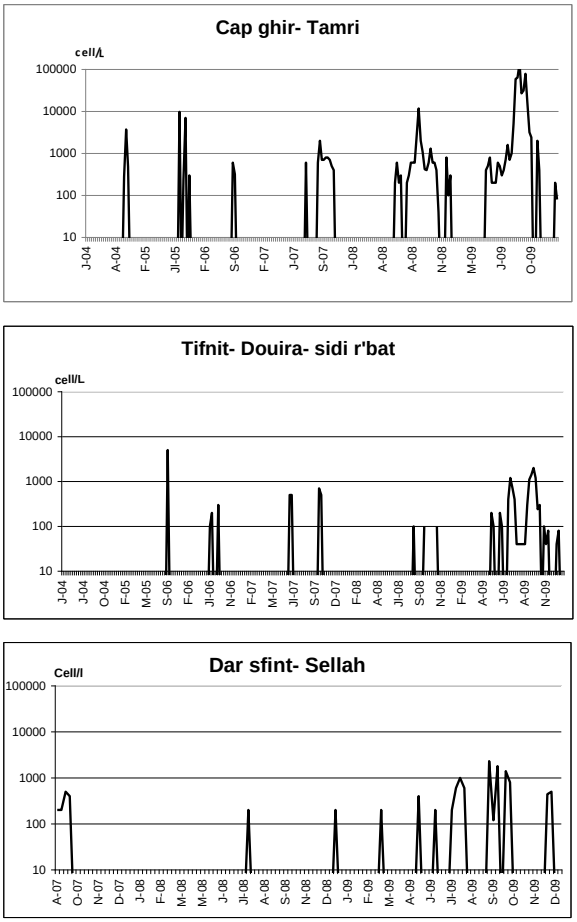

Fig. 2. Time distribution of *Ostreopsis* on the waters monitoring stations

water column although not dispersing the cells. The maximum concentration of *Ostreopsis* detected in seawater samples was 10^5 cells.$\mathbf{l^{-1}}$ and was observed at Cap Ghir site, in August 2009. However this value cannot fully represent the stock of epiphytic *Ostreopsis* cells on the macroalgae, rocks or sand from the same site since advection of cells from other areas must be taken into account, despite the good correlation between epiphytic and planktonic cell abundance observed in the Mediterranean. (Mangialajo *et al.* 2010). According to these authors, *Ostreopsis* monitoring shall be focused on benthic material since the concentration of cells is more conservative and represent the stock of available biomass.

Satellite-derived SST and N/S wind stress from atmospheric re-analysis data, for the periods preceding the bloom events at Cape Ghir, show that in the first years of *Ostreopsis* blooms, as in 2005, the blooms occurred during periods of

upwelling relaxation, in agreement with Shears and Ross (2009) who suggested that blooms of *Ostreopsis* will be more intense after periods of calm sea conditions that induce stratification and excessive warming of surface waters. After 2005, the species seems to be established in the region and its concentration is not directly related to upwelling intermittency / intensity. This seems to agree with Mangialajo *et al.* (2010) who highlighted that maximal abundance periods were local and year specific and that seawater temperature relationship with *Ostreopsis* cell concentration is not detectable when increasing the study scale. During the bloom events, the hydrodynamic model solutions for the surface circulation also suggest that the cape Ghir area was affected by waters from the Safi coast flowing southward and turning westwards at the cape. Unfortunately monitoring sampling did not cover that area, in order to evaluate the species presence on the Safi coast.

Simultaneously to the high concentrations of *Ostreopsis* and low concentration of other common lipophylic producer species in surface waters, lipohylic toxins were detected by mouse bioassay (MBA) in mussels from the same areas. Since positive tests are not indicators of the type of toxins involved, there is a need of further development of other methods that shall be optimised concerning the selectivity and sensitivity of the PTX-group toxins in shellfish tissues. The risks of PTX exposure through shellfish consumption are still unknown (Deeds and Schwartz, 2009) but high PTX-like toxins were found in Caribbean mussels as well as in mussels and clams of the North Aegean Sea where toxic *Ostreopsis siamensis* are now known to occur (Aligizaki et al., 2008). During the above different toxic episodes, bans in shellfish-harvesting activity limited the exposure of Moroccan consumers to possible problems of PTX like phycotoxins, which might be present in shellfish. So, no human intoxications were reported. The commercialisation of the molluscs was allowed after a total purge of the marine environment of this region.

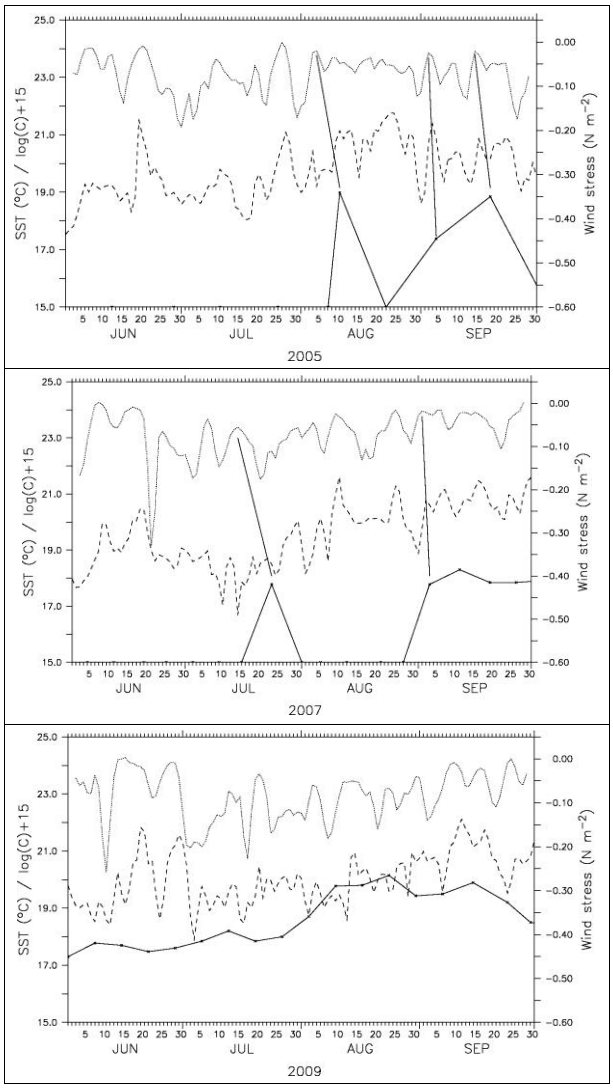

Fig. 3. Environmental conditions off Cape Ghir: satellite-derived sea surface temperature (°C, left axis, dashed line), N/S wind stress (N m^{-2}, right axis, dotted line) and cell concentration (log(C)+15, left axis, solid line) for 2005, 2007 and 2009 during periods of *Ostreopsis* proliferation.

Conclusion

It is not yet clear whether the apparent biogeographical expansion of *O. cf. siamensis* is real since the HAB monitoring in seawater only became regular in the Agadir coast after 1999 and there is still no monitoring program of microphytobenthic communities along the Moroccan coast. After the first detection of *Ostreopsis* blooms, and due to their re-occurrence in the following years, the monitoring program of HABs has included this

genus among the potentially harmful species to be monitored along Atlantic and Mediterranean coasts of Morocco. *O. siamensis* was the first toxic species to be identified and confirmed for the Cape Ghir area. Future research shall include a better knowledge of bloom dynamics, the species ecology and toxicity through the isolation and maintenance of the species in cultures as a first step.

Acknowledgements

This work was developed under the frame and cooperation of Portuguese and Moroccan National Science Foundations. The authors would like to thank A. Lahnin and all the staff of the Agadir's Regional Center of INRH for their collaboration. Wind data are from the NCEP/NCAR Reanalysis Project at the NOAA/ESRL, SST maps were obtained from the EUMETSAT Ocean & Sea Ice SAF). The HYCOM model results were obtained from the consortium web page at http://www.hycom.org.

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Impact of *Ostreopsis ovata* on marine benthic communities: accumulation of palytoxins in mussels, sea urchins and octopuses from Italy

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Abstract

Since 1998 sheltered marine areas along the coast of Tuscany (Italy) have been affected by blooms of the potentially toxic benthic dinoflagellate *Ostreopsis ovata*. These phenomena caused stress signals in the benthic community, affecting both sessile and free-moving organisms. Sea urchins were observed to lose their spines, whilst sea stars had their arms folded over. In July-August 2008, the area was impacted by a new massive bloom of *O. ovata*, which concentration reached two maximum peaks: 88,760 cell/L on July 24th and 95,200 cell/L on August 26th. Marine organisms (mussels, sea urchins and octopuses) suffering from the event, were taken and tested for the presence of palytoxin-group toxins (PlTXs) using both LC-MS/MS and the hemolysis assay. Both techniques confirmed that all samples were contaminated by PlTXs. Ovatoxin-a was the dominant compound according to highly specific LC-MS/MS analyses. The contamination of edible fauna by PlTXs poses a serious risk for the consumers and should be further investigated.

Introduction

The first bloom of the potentially toxic benthic dinoflagellate *Ostreopsis ovata* along the coast of Tuscany (Italy) occurred in summer 1998 (Sansoni *et al.* 2003). During the episode the Regional Agency for Environmental Protection and the Local Health Unit were alerted after a few dozen bathers showed respiratory problems. Since then research on *Ostreopsis ovata* has focused primarily on its potential risk to human health through inhalation of aerosolized toxins. As reported also for other areas worldwide (Granéli *et al.* 2002; Shears and Ross 2009), these phenomena also cause stress signals in the benthic community, affecting both sessile and free-moving organisms. Here we report on the impact of these blooms on a marine benthic community of the Tyrrhenian Sea (Marina di Massa, Tuscany, Italy) during summer 2008. The episode was characterized by toxin accumulation in the food web (mussels, sea urchins and octopuses) implying a potential threat to coastal ecosystem functionalites, fisheries and, again, human health.

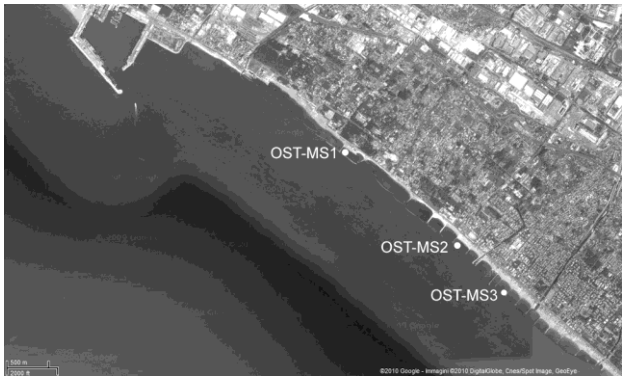

Fig 1. Sampling stations along the coast of Tuscany where blooms of *Ostreopsis ovata* are recurrent. Breakwater barriers parallel with the shoreline and artificial jetties are clearly visible.

Materials and methods

Sampling activities were organized following specific protocols (APAT 2007) which required, besides the collection of seawater and marine organisms, the use of a form with predefined fields for accurate description of monitoring area and its

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environmental status. Observations from tourists or tourist operators were also recorded.For *Ostreopsis ovata* cell counts, the coastal waters of Marina di Massa were sampled at 3 stations: OST-MS1, OST-MS2 and OST-MS3 (Fig. 1). Sampling frequency was monthly from April to October, but during the high risk period (July-August) it was weekly.Sea urchins were collected at the same sites as water samples when *O. ovata* reached its maximum. Observations from tourists and tourist operators, indicating extensive suffering of marine organisms, led to sampling of mussels from the pier of Marina di Massa and of stranded octopuses. The benthic microalga *Ostreopsis ovata* was identified and counted in samples of seawater using an inverted microscope (Utermöhl 1958). Whole tissue of mussels, sea urchins and octopuses was homogenised and 2 g extracted with 18 mL MeOH 90% using Ultraturrax®. The extract was centrifuged and an aliquot (1 mL) of the supernatant was further purified using SPE. Cartridges (Oasis® HLB 3 cc 60 mg) were conditioned with 2 mL MeOH and equilibrated with 2 mL water. Subsequently 1 mL sample dissolved in water was loaded and washed with 2 mL MeOH 50%. PlTXs were eluted with 2mL MeOH 80%. The purified extract, after filtration, was used for determination of palytoxins by both hemolysis assay and LC-MS/MS. Quantification of palytoxins was made by external calibration using a palytoxin standard solution (WAKO Pure Chemical Industries, Ltd, Japan). The LC-MS/MS method is based on Ciminiello *et al.* (2008) with modifications. A 1200L triple quadrupole mass spectrometer (Varian Inc., Walnut Creek, CA, USA) was used. Chromatographic separation was achieved by using a Luna C18 (2) 5µm 150x2.00mm (Phenomenex, Torrance, California, USA) (Varian Inc., Walnut Creek, CA, USA) column and gradient elution with (A) water and (B) acetonitrile, both containing 0.1% acetic acid. Before injection into the analytical column, extracts of marine organisms were further concentrated using an online SPE cartridge (Phenomenex Strata-X 25µm online extraction cartridge 20x2.0mm). Multiple reaction monitoring (MRM) experiments were carried out in positive ion mode in order to identify and quantify palytoxin-like compounds. The following transitions were used: m/z 1314>327 for putative PlTX e m/z 1298>327 for OVTX-a. The hemolysis neutralization assay (HNA) is based on Riobò *et al.* (2008) with minor modifications. Phosphate Buffer Saline sheep blood solutions with and without ouabain were prepared to the same final erythrocytes concentration. The mixtures were incubated at 25°C for 1 hr and mixed with one volume of the appropriate palytoxin dilution or samples extracted and incubated over 20 hrs at the same temperature. Erythrocytes were separated by centrifugation and a portion of each supernatant was further transferred to a microwell plate to measure absorbance at 405 nm into a microplate reader.

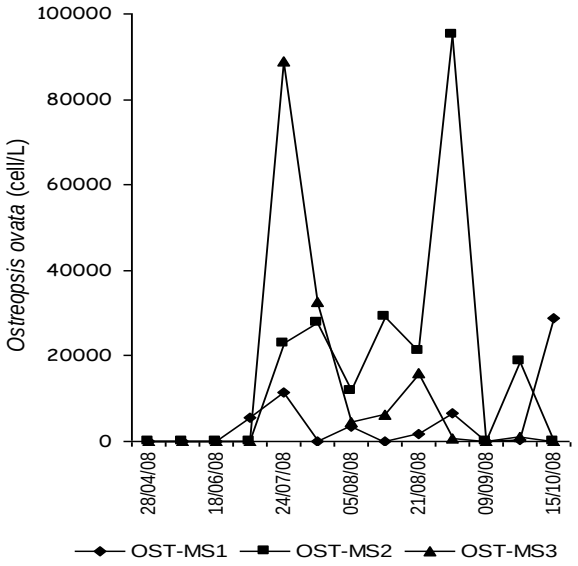

Fig 2. Densities (cell/L) of *O. ovata* along the coast of Marina di Massa (Tuscany).

Results and Discussion

In July-August 2008, the coast of Marina di Massa (Tuscany) was impacted by a new massive bloom of *Ostreopsis ovata*. The concentration reached two maximum peaks: 88,760 cell/L on July 24th and 95,200 cell/L on August 26th (Fig. 2). The most affected sites were sheltered, shallow rocky reefs, moderately exposed to wind action. Breakwater barriers built parallel with the shoreline and artificial jetties protecting these areas from erosion also favoured scarce hydro-dynamism and warming of the seawater, where temperature in July-August 2008 was above 26 °C. Blooms of *O. ovata* appeared as a rusty-brown mucilaginous biofilm covering rocks, macroalgae and other sessile organisms. Most likely due to the anoxic conditions, shells of *Patella* spp. were laying on the sea bottom, together with dead, blackened sea urchins. Tourist operators found stranded octopuses and informed the competent authorities. LC-MS/MS chemical analyses and hemolysis neutralization assays carried out on suffering mussels, sea urchins and octopuses confirmed that all samples were contaminated by PlTXs. Ovatoxin-a was the dominant compound

Table 1. LC-MS/MS and HNA results on marine organisms. *occasional sampling; LOD = limit of detection; LOQ = limit of quantification; /// = not done

Sample code	Date	Sampling station	Marine invertebrates	LC-MS/MS		HNA
				pPlTX µg/kg	OVTX-a µg/kg	PlTXs µg/kg
1	30/07/08	OST-MS2	Sea urchins	<LOQ	164	///
2	30/07/08	OST-MS3	Sea urchins	<LOD	<LOQ	5
3	30/07/08	Pier*	Mussels	<LOQ	131	///
4	30/07/08	Stranded*	Octopuses	115	971	466
5	26/08/08	OST-MS1	Sea urchins	<LOQ	114	99
6	26/08/08	OST-MS3	Sea urchins	<LOQ	87	69
7	28/08/08	Pier*	Mussels	<LOQ	228	103

according to highly specific LC-MS/MS analyses, with maximum concentrations of 164µg/kg in sea urchins and 238 µg/kg in mussels whole flesh (Tab. 1). The highest contamination was determined in stranded octopuses (971 µg/kg OVTX-a and 115 µg/kg pPlTX), suggesting biomagnification along the food chain.

Conclusions

Although it is recognized that blooms of *O. ovata* typically occur in shallow sheltered marine areas, where high temperatures and anoxia represent adverse conditions for the benthic community, and that some marine organisms can accumulate very high levels of palytoxins in their tissues without apparent harm (Gleibs and Mebs 1999), biomagnification of toxins along the food web might have played a role in the observed ecosystem suffering (e.g. octopuses found stranded). Some recent papers have attempted to improve our knowledge about the direct toxicity of PlTXs on vertebrates and invertebrates. The few existing data are mainly related to ecological studies on the impact of *Ostreopsis* spp. in sea urchin communities and ecotoxicological effects in bivalves. Other reported effects of PlTX in invertebrates are retrieved from standard bioassays (Ramos and Vasconcelos 2010). However, observed toxicity effects are usually related to the concentration tested/cells density and final toxin uptake by the test organism has been rarely investigated. This prevents us from assessing unambiguously that toxin content determined in animal tissues during this study is sufficient to be regarded as the primary cause of animal suffering/death. During the 2008 episode no cases of human intoxication were reported in Tuscany. Nevertheless the contamination of edible fauna by PlTXs poses a serious risk for the consumers and should be further investigated.

Acknowledgements

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Complex palytoxin-like profile of *Ostreopsis ovata*: Identification of four new ovatoxins by high resolution LC-MS

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Abstract

High resolution (HR) LC-MS investigation of an Adriatic *Ostreopsis ovata* culture is reported. It highlighted the presence of putative palytoxin and ovatoxin-a in combination with four new ovatoxins. Elemental formulae and information about their structural features were gained.

Introduction

Over the past decade, Italian coastlines have been plagued by the recurring presence of the benthic dinoflagellate *Ostreopsis ovata*, that has caused severe sanitary emergencies due to production of toxic aerosols (Ciminiello *et al.* 2009a) and seafood contamination. The most alarming phenomenon occurred in 2005, along the Ligurian coasts (Italy), when, concurrently with an unusual proliferation of *O. ovata*, hundreds of people required medical attention after exposure to marine aerosols. Liquid chromatography tandem mass spectrometry (LC-MS) investigation of toxin profile of the plankton collected during toxic outbreaks allowed us to disclose the presence of putative palytoxin (PLTX) and a new palytoxin-like molecule, ovatoxin-a (OVTX-a), in the plankton; this latter presents 2 oxygen atoms less than palytoxin and the same A moiety (Figure 1). A new LC-MS method for their detection was developed (Ciminiello *et al.* 2006, 2008). LC-MS analyses of cultured *O. ovata*, demonstrated that both PLTX and OVTX-a contained in the natural plankton sample were produced by *O. ovata* itself (Guerrini *et al.* 2010). In-depth investigation of an *O. ovata* culture by HR LC-MS and MS^2 is reported here. It confirmed the presence of putative PLTX and OVTX-a, and highlighted the occurrence of four new ovatoxins, OVTX-b, OVTX-c, OVTX-d, and OVTX-e. Elemental formulae were assigned to the new ovatoxins and information was gained about their structural features.

Material and Methods

Adriatic *O. ovata* (Fukuyo, 1981) cultures were established in natural seawater, at salinity of 32 psu, temperature of 20°C and a 16:8 h L:D cycle (ca. 90 μmol m^{-2} s^{-1} from cool white lamp). Cell pellets (4,336,578 cells) collected on the 21th day of growth were extracted thrice by sonicating with methanol/water (1:1, v/v) for 6 min. The crude extract (V=30 mL) was analyzed directly by LC-MS.-HR LC-MS experiments were carried out on an Agilent 1100 LC binary system coupled to a Thermo-Fisher LTQ Orbitrap XL™ FTMS equipped with an ESI ION MAX™ source. A 3 μm gemini C18 (150 × 2.00 mm) column eluted at 0.2 mL/min with water (A) and 95% acetonitrile/water (B), both containing 30 mM acetic acid, was used. Gradient elution: 20-50% B over 20 min, 50-80% B over 10 min, 80-100% B in 1 min, and hold 5 min. HR full MS experiments (positive ions) were acquired at a resolving power of 100,000. $HRMS^2$ data were acquired at a 60,000 resolving power (collision energy = 25%),by selecting as precursor the $[M+2H+K]^{3+}$ ion at *m/z* 906.8 (PLTX), *m/z* 896.2 (OVTX-a), *m/z* 910.8 (OVTX-b), *m/z* 916.1 (OVTX-c), and *m/z* 901.4 (OVTX-d and OVTX-e). Elemental formulae were calculated by using the mono-isotopic ion peak of each ion cluster and a mass tolerance of 5 ppm. Calibration curve of PLTX (25, 12.5, 6.25, 3.13, and 1.6 ng/mL) was used in quantitative analyses (triplicate injection).

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Results and Discussion

HR LC-MS experiments in positive full MS mode were carried out on the crude extract of an Adriatic *O. ovata* culture by using a slow gradient elution that provided sufficient chromatographic separation of the major components of the extract. HR full MS spectra were acquired both in the *m/z* 2000-3000 and *m/z* 800-1400 ranges. In the former range, mono-charged ions due to $[M+H]^+$ of each palytoxin-like compound appeared together with $[M+H-nH_2O]^+$(n = 1-3) ions and fragment ions $[M+H\text{-A moiety-}nH_2O]^+$ (n = 0-6), diagnostic of B moiety. In the range *m/z* 800-1400, each palytoxin-like compound presented bi-charged ions due to $[M+H+K]^{2+}$, $[M+H+Na]^{2+}$, and $[M+2H]^{2+}$, tri-charged ions due to $[M+2H+K]^{3+}$ and $[M+2H+Na]^{3+}$, as well as ions due to multiple water losses from the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions. Such a complex ion profile combined to the high value of exact masses of palytoxin-like compounds made difficult unambiguous elemental composition assignment: a number of possible elemental formulae were ascribable to each compound, even using a mass tolerance of 5 ppm. A cross-checked interpretation of elemental formulae of all mono-, bi, and tri-charged ions contained in full MS spectra of each compound helped us to dispell doubts on elemental composition of ovatoxins. Some preliminary information on the structure of new ovatoxins was gained by interpretation of their fragmentation patterns in the light of those of PLTX and OVTX-a. Cleavage between carbons 8 and 9 of PLTX is highly favoured (Figure 1), and divides the molecule in two moieties, A and B (Uemura *et al.*, 1985). Ions associated to both moieties are formed in the full MS spectrum of PLTX as well as in its MS^2 spectra whatever precursor ion (mono-, bi-, or tri-charged ions) be used. Many palytoxin-like compounds, such as mascarenotoxins (Lenoir *et al.*, 2004),[16] 42-OH-palytoxin, and OVTX-a, (Ciminiello *et al.* 2008, 2009b) present the above MS behaviour. Thus, elemental composition of A and B moiety of new ovatoxins was obtained through interpretation of $HRMS^2$ spectra of their $[M+2H+K]^{3+}$ ions, that paralleled that of PLTX

Figure 1

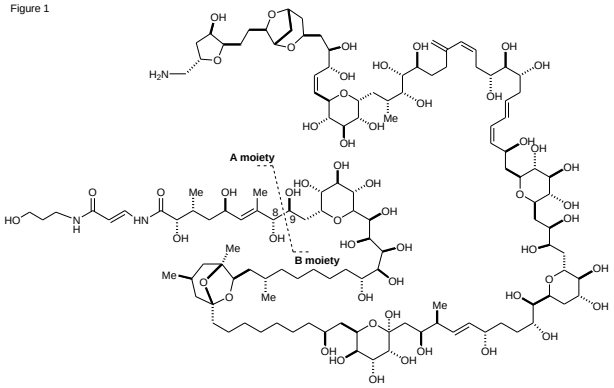

Fig.1. Molecular structure of ovatoxin-A

in containing: i) tri-charged ions due to subsequent losses of water molecules (2 to 7) from the relevant precursor ion, ii) a mono-charged $[M+H\text{-B moiety-}H_2O]^+$ ion, diagnostic of A moiety, in the region *m/z* 300-400, and iii) bi-charged $[M+H+K\text{-A moiety-}nH_2O]^{2+}$ (n = 0-5) ions, diagnostic of B moiety, among which the $[M+H+K\text{-A moiety-}2H_2O]^{2+}$ ion was the most abundant. Table 1 reports the principal mono-, bi-, and tri-charged ions of palytoxin and ovatoxins (mono-isotopic ion peaks) contained in the culture extract, together with elemental formulae assigned to each compound (M) and to the relevant A and B moiety. A comparison of elemental formulae of new ovatoxins with that of ovatoxin-a indicated that: i) OVTX-b presents C_2H_4O more than OVTX-a. The structural difference between the two molecules resides in the A moiety whereas structure B is identical. Based on structural features of palytoxin-like compounds isolated by Uemura et al.(1985), it could present the addition of a hydroxyl group and two methylene groups in the A moiety, thus being putative bishomo-hydroxyovatoxin-a; ii) OVTX-c presents $C_2H_4O_2$ more than OVTX-a. Compared to this latter, it presents additional C_2H_4O atoms (potentially a hydroxyl and two methylene groups) in the A moiety and an extra oxygen atom (potentially a hydroxyl group) in the B moiety; iii) OVTX-d and OVTX-e are isobaric compounds that present one oxygen atom more than OVTX-a. OVTX-d presents the same A moiety as OVTX-a and one additional oxygen atom (potentially a hydroxyl group) in the B moiety, while OVTX-e contains one additional oxygen atom in the A moiety and the same B moiety as OVTX-a.

Table 2. Principal ions (mono-isotopic ion peaks) contained in HR full MS spectra of palytoxin and ovatoxins, elemental formulae assigned to each compound (M) and to relevant A and B moiety.

			Principal ions (*m/z*)			Elemental formulae		
Toxin	**%**	**Rt (min)[a]**	**$[M+H]^+$**	**$[M+2H-H_2O]^{2+}$**	**$[M+2H+K]^{3+}$**	**M**	**A moiety**	**B moiety**
PLTX	0.6	10.78	2679.4893	1331.2417	906.4851	$C_{129}H_{223}N_3O_{54}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{48}$
OVTX-a	54	11.45	2647.4979	1315.2480	895.8255	$C_{129}H_{223}N_3O_{52}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{46}$
OVTX -b	27	11.28	2691.5233	1337.2595	910.4976	$C_{131}H_{227}N_3O_{53}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{46}$
OVTX -c	6	10.90	2707.5173	1345.2566	915.8286	$C_{131}H_{227}N_3O_{54}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{47}$
OVTX –d	12	11.07	2663.4905	1323.2439	901.1533	$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{47}$
OVTX –e							$C_{16}H_{28}N_2O_7$	$C_{113}H_{195}NO_{46}$

With the purpose of gaining information about the relative abundance of putative palytoxin and ovatoxins in the *O. ovata* culture, extracted ion chromatograms (XIC) were obtained from the HR full MS experiments by summing the most abundant peaks of both $[M+2H-H_2O]^{2+}$ and $[M+2H+K]^{3+}$ ion clusters for each compound. The resulting chromatographic peak areas were compared to that of PLTX standard injected under the same conditions. Percentage of individual compounds on the total toxin content of the *O. ovata* culture extract is reported in Table 1. The whole of the new ovatoxins represents about 46% of the total toxin content and, thus, their presence should be considered when LC-MS based monitoring programs are carried out. Discovery of new ovatoxins (Ciminiello *et al*. 2010) poses the need to update the LC-MS method for detection of PLTX recently developed (Ciminiello *et al.*, 2006); particularly, it should be taken into account that the $[M+H\text{-B moiety-}H_2O]^+$ ion monitored as product ion in multiple reaction monitoring (MRM) experiments is the same for PLTX, OVTX-a and -d (at *m/z* 327), as well as for OVTX-b and -c (at *m/z* 371); this, combined to mass vicinity of some precursor ions, could result in interference in MRM detection. In order to overcome such drawbacks, complete chromatographic separation among potentially interfering compounds should be achieved or HRMS detection considered.

Acknowledgements

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Implementation Of Analytical Methods For Detection Of Palytoxins In Shellfish

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Abstract

Blooms of *Ostreopsis ovata* have been recently reported in several areas of Italian coastline, including the Marche region and specifically the coast of Monte Conero. Different *Ostreopsis* species are proven to produce analogues of palytoxins (PlTXs). Since 2006, with co-occurrence of *Ostreopsis ovata* in seawater, wild mussels collected along the coast of Monte Conero have been found positive to the mouse bioassay for polar lipophilic toxins, with unusual rapid death of mice, even if no cases of toxicity in humans have been reported. In Europe there are neither regulatory limits for PlTXs in seafood nor official methods of analysis. Three different analytical methods for detecting palytoxins in shellfish were implemented and compared: a specific bioassay (MBA PlTXs), a hemolytic assay (HNA) and an instrumental one using QQQ-LC-MS. MBA PlTXs is specific but shows low sensitivity, HNA has good sensitivity and low susceptibility to interference from other toxic compounds, LC-MS is the most adequate for determining palytoxins, but requires high economic investment. At the moment a combination of different methods is used for monitoring samples.

Introduction

Blooms of *Ostreopsis ovata* have been recently reported in several areas of the Italian coastline including the Marche region, specifically along the coast of Monte Conero (Fig. 1). Different species of *Ostreopsis* are proven to produce analogues of palytoxins (PlTXs), which can represent a health hazard through the food chain (Usami et al., 1995, Taniyama et al., 2003). High concentrations of PlTXs have been found in shellfish in the Caribbean Sea (Gleibs and Mebs, 1999), and more recently in mussels and clams in the north Aegean area simultaneously affected by a massive bloom of *Ostreopsis* spp (Aligizaki et al. 2008). Since 2006, with the co-occurrence of *Ostreopsis ovata* in the seawater, wild mussels collected along the coast of Monte Conero were positive to the mouse bioassay for polar lipophilic toxins (MBA Step2), with unusual very rapid death of mice, even if no cases of toxicity in humans were reported (Bacchiocchi et al., 2007). In Europe there are neither regulatory limits for PlTXs in seafood nor official methods of analysis even though EFSA has recently published an opinion, suggesting a limit of 30 µg/kg of edible part of shellfish, considering only acute effects. The aim of this work was to implement and compare three different analytical methods for detecting palytoxins in shellfish: a specific bioassay (MBA PlTXs), a hemolysis neutralization assay (HNA) and an instrumental method which uses a QQQ-LC-MS/MS

Materials and Methods

Macrophyte and surface water samples were collected from sites along the rocky coasts of Conero Riviera (Fig. 1) by the Regional Agency for the Environment Protection (ARPAM). Seawater samples were fixed with formaldehyde 2-4%, transferred to laboratories of ARPAM and used to determine the qualitative and quantitative composition of phytoplankton. The phytoplankton were characterized after treatment with Lugol, sedimentation in Uthermöhl tubes and analysis with inverted microscope. The macroalgal samples were separately transferred to hermetically sealed vessels and the sampling bags rinsed with filtered (0.22 µm mesh) seawater collected at the sampling sites. The rinsing water was added to the vessels shaken for 2 min and the cell suspension was filtered through a plankton net (20 µm mesh). The procedure was repeated several times to obtain a plankton pellet that was suspended again in filtered seawater. An aliquot of each sample was fixed with

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Fig 1. Monte Conero

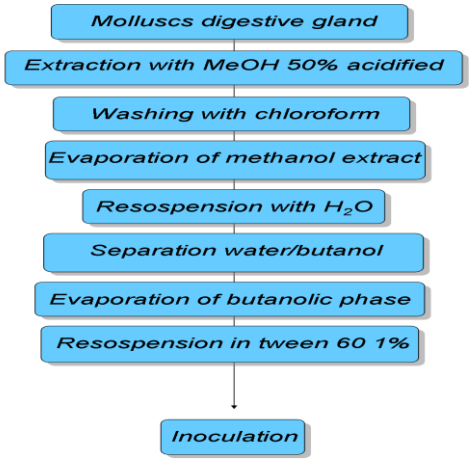

Fig 2. Specific bioassay (MBA PITXs)

formaldehyde 2-4%, treated with Lugol and used for cell identification and counting by inverted microscope. During periods when ARPAM reported the presence of *Ostreopsis ovata* in water and on macroalgae collected along the coast of Monte Conero, all samples of mussels collected in the same area, positive to the MBA Step 2, and some negative controls, were analysed with the MBA PlTXs. MBA PlTXs includes a first extraction of toxins from shellfish homogenate with 50% methanol acidified with acetic acid, removal of lipophilic components with chloroform, a final extraction with butanol, and inoculation in mice of the residues obtained (Fig. 2) (Taniyama et al., 2003, Bacchiocchi et al., 2007). 18 of them were analysed with the HNA, semiquantitative, based on hemolytic properties owned by PlTXs on sheep erythrocytes, characteristically inhibited by oubain (Fig.3) (Bignami, 1993, Habermann and Chhatwal,). Finally 12 samples were tested with LC-MS/MS tests based on Ciminiello et al., 2006 and Ciminiello et al., 2008. Samples were extracted with 90% methanol for 2 min with Ultraturrax and centrifuged for 10 min at 3000 rpm.

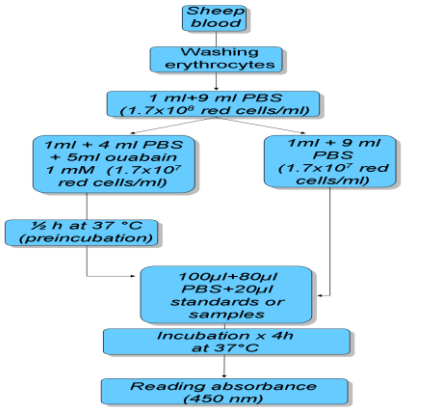

Fig 3. Hemolysis neutralization assay (HNA)

An aliquot of the extract, after a clean-up with HLB 3cc cartridge (60 mg, OASIS-Waters), was passed through a filter membrane of 0.20 microns. Palytoxin was separated chromatographically using a Varian XRs Ultra 2.8 C18 50 x 2.0 mm column (Varian Inc., Walnut Creek, CA, USA) with gradient elution with water and acetonitrile, both containing 1% acetic acid. The identification of putative Palytoxin (pPlTX) and Ovatoxin-a (OVTX-a) was performed considering more intense transitions (*m/z* 1314>327 for putative Palytoxin and *m/z* 1298>327 for Ovatoxin-a). Quantification was obtained by comparison with the peaks obtained from different dilutions of a Palytoxin standard solution (Wako Chemicals GmbH - Neuss, Germany) and assuming that Ovatoxin-a shows the same molar response. Overall 63 samples were examined.

Results and Discussion

Fig. 4 shows *Ostreopsis ovata* concentrations in the study area in the years 2006-2009. The most significant results for the evaluation of the methods tested are reported in Table. 1. MBA PlTXs showed a good correlation with MBA Step2 in presence of palytoxins, although more specific, but characterized by a significant loss of analyte in the preparative steps. The HNA has shown a good correlation with both mouse bioassays, the best sensitivity among the different methods examined and a lower susceptibility to interference from other toxic compounds, compared to mouse-test. The LC-MS/MS test identified in samples two isomers of palytoxin: ovatoxin-a (OVTX-a) and a putative palytoxin (pPlTX).

It also allowed us to explain many of the discrepancies between the two mouse bioassays, due to the presence in the samples not only of palytoxins but also yessotoxins. The

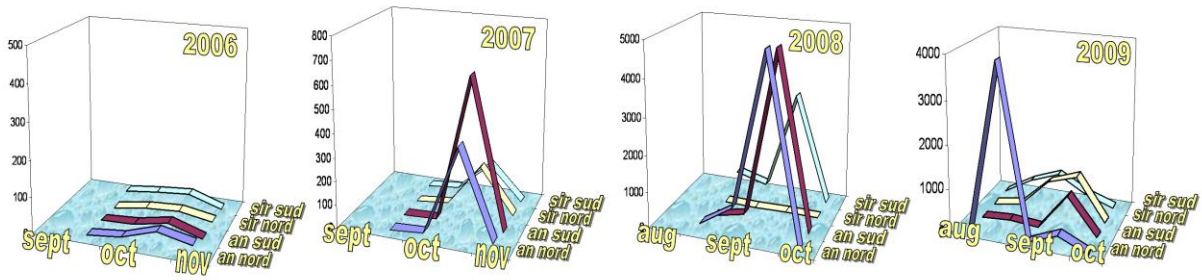

Fig 4. Ostreopsis ovata concentration along the coast of Monte Conero in the years 2006-2009 (cell/L 1000x)

Sample			MBA			HNA	LC-MS/MS	
n°	Sampling date	Sampling point	DSP	PITXs		µg/Kg p.e.**	µg/Kg p.e.***	
			Step 2	*1 ml/mouse**	*0.5 ml/mouse**		*OVTX-a*	*pPLTX*
1	13-10-06	An nord	+	20'	33'	31	13	<LOD
2	13-10-06	An sud	+	38'	39'	8	<LOD	<LOD
3	13-10-06	Sir Nord	+	34'	42'	NA	30	14
4	13-10-06	Sir Sud	+	15'	27'	NA	<LOD	<LOD
5	06-11-06	An Nord	-	NA	NA	NA	<LOD	<LOD
6	06-11-06	An sud	+	NA	NA	<LOQ	<LOD	<LOD
7	06-11-06	Sir Nord	+	410'	alive	NA	<LOD	<LOD
8	06-11-06	Sir Sud	+	NA	NA	NA	<LOD	<LOD
9	27-08-07	An nord	+	NA	NA	<LOD	13	<LOD
10	27-08-07	An sud	+	NA	NA	NA	<LOQ	<LOD
11	24-08-09	An Nord	+	40'	60'	17	<LOD	<LOD
12	24-08-09	Sir Nord	+	45'	60'	70	71	<LOD
13	24-08-09	Sir Sud	+	45'	60'	20	<LOQ	<LOD
14	01-09-09	An Nord	+	50'	100'	240	NA	NA
15	01-09-09	An Sud	-	alive	alive	<LOD	NA	NA
16	01-09-09	Sir Nord	+	70'	200'	100	NA	NA
17	21-09-09	An sud	+	NA	NA	200	NA	NA
18	21-09-09	An Nord	+	15'	20'	160	NA	NA
19	21-09-09	Sir Sud	+	10'	20'	320	NA	NA
20	06-10-09	An Nord	+	NA	NA	240	NA	NA
21	06-10-09	An Sud	+	NA	NA	160	NA	NA
22	06-10-09	Sir Nord	+	10'	20'	300	NA	NA
23	06-10-09	Sir Sud	+	NA	NA	200	NA	NA

Table 1. Most significant results of analysis performed with MBA Step 2, MBA PITXs, HNA and LC-MS/MS. On blue background positive tests. An=Ancona, Sir=Sirolo, NA=Not analyzed *Time of death (min). ** LOD=2 µg/Kg p.e. LOQ=6µm/Kg p.e. *** LOD=17 µg/Kg p.e. LOQ=50 µg/Kg p.e.

instrumental test had a better correlation with the MBA PlTXs and lower correlation with HNA. This could be explained by different sensitivity of the two methods (Fig. 5). The instrumental method is the most suitable for determining palytoxins, but requires a major economic investment by laboratories. Furthermore there are neither validated methods for LC-MS/MS nor certified reference materials. A combination of the other assays could be useful in monitoring samples, also with complex toxic profiles.

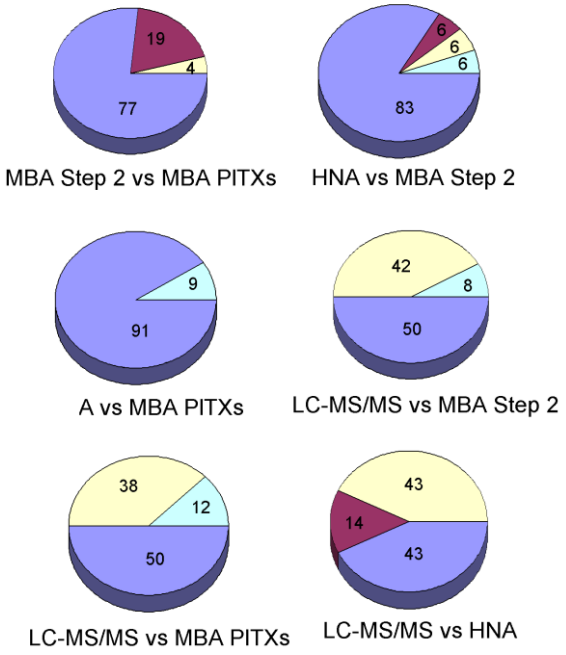

Fig 5. Correlations in percentage between methods tested: blue= +/+, red= +/-, yellow= -/+, light blue= -/-.

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Determination of Palytoxins in Samples from *Ostreopsis* outbreaks in Llavaneres (NW Mediterranean Coast)

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Abstract

The objective of the present work was to link *Ostreopsis* blooms with respiratory problems. To test this hypothesis, samples of seawater, macroalgae, benthic marine invertebrates and aerosols were collected during 2009 and 2010 in the framework of the EBITOX project. Different extraction procedures have been used depending on sample type. The extracts were analysed by haemolytic assay (Riobó et al 2008) and liquid chromatography with fluorescence detection (LC–FLD) (Riobó et al 2006) and the presence of palytoxin was confirmed by liquid chromatography coupled with mass spectrometry (LC-MS) (Riobó et al 2006). Strains isolated from Llavaneres were cultured in the laboratory and analysed by the same chemical and biological methods. Toxins from field samples and cultures were compared. Presence of palytoxins in macroalgae and cultures was confirmed but it has not yet been detected in aerosol filters or dissolved in seawater. Therefore, the hypothesis remains unresolved.

Introduction

Dinoflagellates of the genus *Ostreopsis* have been related to harmful episodes in many Mediterranean coastal areas since 1998. In August 2004 one important event occurred in Llavaneres beach (Catalan coast, Spain) affecting 74 people with rhinitis and breathing problems. Since then, many Mediterranean heavy blooms of *Ostreopsis* have coincided with respiratory problems in people staying near the beach, suggesting a possible link. Respiratory poisoning could be due to: i) presence in aerosol drops of *Ostreopsis* cells (breathing problems could be allergic or due to palytoxin) or ii) Presence of palytoxin in aerosol drops (toxin must be dissolved in sea water). Presence of palytoxin has been confirmed in cultures obtained from *Ostreopsis* cells isolated during blooms in both, cells and culture filtrate (Guerrini et al 2010). However, despite respiratory intoxications reported, the link between *Ostreopsis* blooms and respiratory problems has not been verified because the presence of palytoxin dissolved in seawater or aerosol samples has not yet been demonstrated. In the present work we investigated the presence of palytoxin in samples of seawater, macroalgae, sea urchins and aerosol collected in *Ostreopsis* outbreaks occurred in Llavaneres.

Materials and Methods

Palytoxin was analysed by haemolytic assay (Riobó *et al*. 2008) and LC–FLD and the presence of palytoxin was confirmed by LC-MS (Riobó *et al*. 2006). Different extraction procedures have been used depending of sample type. Seawater and macroalgae samples were collected monthly or biweekly in winter, and weekly or every 3-4 days in summer since 2007 to 2009.

Seawater sampling.

In order to extract palytoxins from seawater during *Ostreopsis* blooms, different approaches have been tried:

i) 8 to 10 L of seawater filtered in GF/C glass fiber filters and extraction of palytoxin from seawater filtrate has been tried by the following

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procedures: SPE with Sep pak C18 cartridges; Partition with Butanol (Ciminiello et al.2008); Adsorption on Diaion Column; C18 EMPORE Disks (De la Iglesia et al., 2009)
ii) palytoxin released in the seawater was tracked in situ with Diaion HP20 adsorbing resins previously activated in the laboratory following the solid phase adsorption toxin tracking procedure (SPATT) by Mackenzie (Mackenzie et al., 2004). This monitoring tool simulates the biotoxin contamination of filter feeding bivalves.

Cultures. *Ostreopsis* cells from the study area were isolated and cultured in laboratory. Morphologic and genetic analysis revealed that all the cultured strains were *Ostreopsis ovata*. Toxin extraction was performed with 100 % MeOH.

Aerosol sampling. Aerosol samples were taken once a week during the bloom season in 2009 and during the bloom peak in 2010. Samples were collected with two high volume air samplers fitted with 15 cm diameter quartz fibber filters (Whatman, Maidstone, UK) installed near the beach. In 2010 the air samplers were working continuously for 3 days. The air volume filtered by the samplers was 30 m^3/h. Filters were replaced every 6 or 7 hours. A total air volume of 1326 m^3 was filtered and then bubbled into a container with 6L of distilled water. Extraction from filters was performed with MeOH in a Soxhlet extractor with 10-12 hours cycles. Distilled water was evaporated to dryness and then dissolved in 30 mL of MeOH for toxin analyses.

Benthic marine invertebrates. Six pieces of sea urchins (*Paracentrotus lividus*) were collected in the study area. Extraction of the whole flesh was made with 100% MeOH. The intestinal content was observed under LM.

Results

Palytoxin, analyzed by haemolysis assay and HPLC-FLD, was detected in epiphytic samples taken during the bloom (Fig.1). Then cultures of *Ostreopsis* were established from cells isolated in the study area (Fig.1). A palytoxin analog with a molecular weight of 2647 Da was found. The toxin content in cells from cultures has been estimated to be 0.3 pg/cell. Seawater extracts obtained following SPATT procedure showed typical haemolytic activity due to palytoxin. Instead, filtered environmental seawater samples resulted negative. Aerosol samples resulted negative for toxins since palytoxin was not detected by LC-FLD in filters neither in distilled water. Filters and distilled water were found not to be toxic by haemolytic assay. Toxicity was not detected by HPLC-FLD neither by haemolytic assay in sea urchins taken in the study area; although *Ostreopsis* cells were observed by LM inside sea urchins.

Conclusions

The bloom was toxic. Presence of palytoxin has been detected and confirmed in the epiphytic community and in *Ostreopsis* cultures isolated from Llavaneres (Fig.1). However, palytoxin has not yet been detected in aerosol samples and neither dissolved in samples of 8 to 10L of filtered environmental seawater. The positive palytoxin haemolytic result of seawater was achieved following the SPATT procedure, but confirmation by analytical methods is required. Whereas there is clear coincidence in time between *Ostreopsis* blooms and human intoxications by inhalation, we are not yet able to demonstrate if the causative agent is palytoxin, the entire *Ostreopsis* cells, or another agent that causes an allergic reaction. Further studies are needed in order to solve the hypothesis.

Acknowledgements

This study was funded by the Spanish national project EBITOX (Study of the biological and toxinologic aspects of benthic dinoflagellates associated with risks to the human health) CTQ2008-06754-C04-04. We are in debt with A. Alastuey (Institut Jaume Almera, CSIC) who lent us the aerosol samplers. We acknowledge the support by the Water Catalan Agency (Generalitat de Catalunya), I. Manzano (CZ Veterinaria, S.A. Porriño, Pontevedra) for providing sheep blood for testing hemolytic activity, and facilities offered by

the family Aceña from Restaurant Pins Mar (Sant Andreu de Llavaneres).

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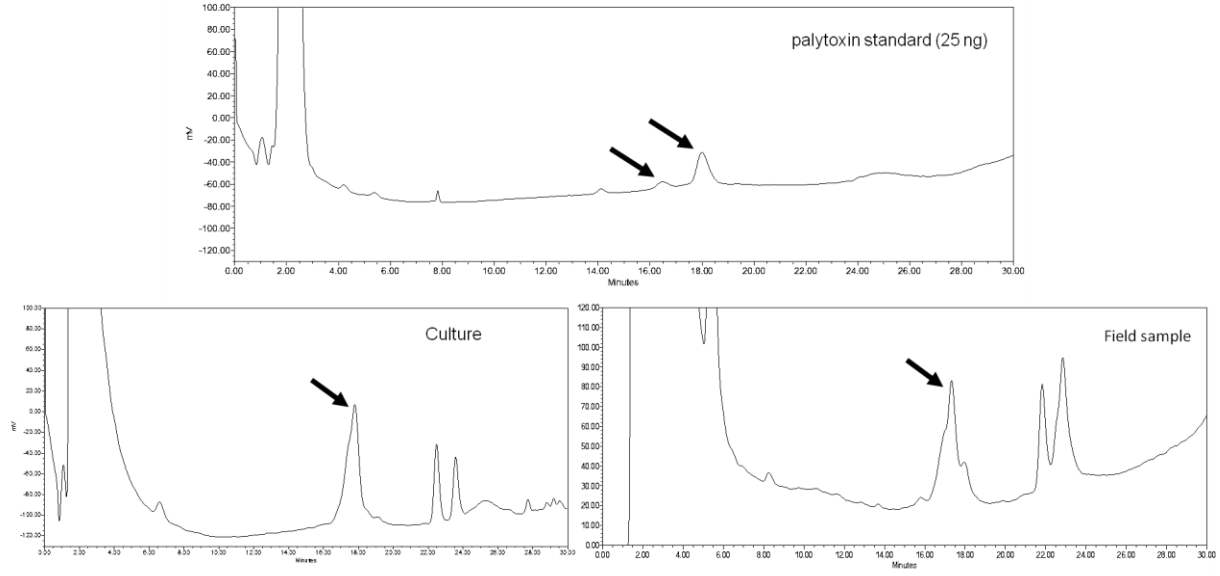

Fig.1 Chromatograms obtained by HPLC with fluorescence detection for palytoxin standard and samples derivatized with ACCQ reagent following the method described by Riobó et al (2006) with slight modifications.

Ostreopsis isolates from the Pacific region

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Abstract.

The genus *Ostreopsis* has been isolated from many parts of the world and *O. ovata* has formed blooms in the Mediterranean Sea in recent years, causing respiratory illnesses Epiphytic *O. ovata* cells were recently isolated from the south coast of Rarotonga, Cook Islands, attached to the calcareous green macroalga, *Halimeda* sp., which grows in the lagoonal reef environment. Extracts of cultures of this isolate were negative in a haemolysis neutralisation assay (HNA) for palytoxin-like compounds. Extracts were also analysed, using a novel LC-MS method for detection of palytoxin, and palytoxin analogues were detected. *O. siamensis* isolates from New Zealand were all positive by the HNA and palytoxin compounds were detected in these isolates by the novel LC-MS method. An *Ostreopsis* sp. isolate from Hawaii was negative by both HNA and LC-MS.

Introduction

The dinoflagellate genus *Ostreopsis* is known to occur globally, from temperate, and tropical Australia, to the Indian Ocean, Eastern Asian coastal waters, the Caribbean and, in the last decade, the Mediterranean and colder waters of the northern Sea of Japan (Rhodes 2010). In the Mediterranean, *O. ovata* has caused human illnesses due to the effects of aerosols from blooms, and uptake of palytoxin-like compounds by shellfish has been reported (Aligizaki *et al.* 2008). *Ostreopsis* is also found commonly in the Pacific region where it occurs as an epiphyte on many species of macroalgae, in particular the calcareous reds and greens. In northern New Zealand vast benthic/ epiphytic blooms of toxin-producing *O. siamensis* occur regularly in the summer months (Rhodes *et al.* 2000; Shears and Ross 2009). However, no illnesses have been associated with these blooms in New Zealand and uptake by shellfish, at least *in vitro*, is low (Rhodes *et al.* 2008). The DNA sequence data of Cook Island and Australian isolates were compared with known isolates from New Zealand. The toxicity of Cook Island and Hawaiian *Ostreopsis* isolates was determined to ascertain whether palytoxin analogues could be part of the ciguatera-poisoning complex occurring in the Pacific region. New Zealand and South Australian isolates were also examined for toxicity and all isolates were analysed for palytoxin-equivalents using a newly developed LCMS method (Selwood *et al.*, this proceedings).

Methods

Ostreopsis cells were isolated from *Halimeda* sp. in lagoonal reef areas along the southern coast of Rarotonga, Cook Islands between 2007 and 2009 and from surface sediment samples collected from eel grass beds in Franklin Harbour, South Australia in 2009. A Hawaiian isolate was obtained from seawater samples (collected by Chris Holland, NOAA, USA) from Waikiki Beach, Honolulu. New Zealand isolates, from the Northland region, were maintained in the Cawthron Institute Collection of Micro-algae (CICCM). Established cultures were grown in F/2 medium (Guillard 1975), diluted 1:1 with filtered seawater. Identification was by light microscopy (inverted Olympus CK2 and IX70 epifluorescence microscopes) and scanning electron microscopy (SEM; cells fixed in glutaraldehyde 3%, formaldehyde 2%, phosphate buffer 0.1 M, passed through an EtOH series, critical point dried and gold coated for SEM -FEI Quanta 200). DNA extractions were carried out as described (Rhodes *et al.* 2010), and the D8–D10 region of the LSU was PCR amplified using the primers FD8 and RB (Chinain *et al.* 1998). Bayesian phylogenetic analyses were carried as described previously (Rhodes *et al*. 2010). Toxicity was determined by the haemolysis neutralisation assay (HNA; Bignami 1993; Briggs *et al.* 1998) and by intraperitoneal injection of cell extracts into mice (Rhodes *et al.* 2002; Briggs 1998). Toxin detection was by LCMS as described by Selwood et al. (14th ICHA, Book of Abstracts).

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Results and Discussion

Identification of the *Ostreopsis* isolates to genus or species level was made on the basis of morphology and phylogenetic analysis of the D8-D10 LSU region of the rDNA (Figure 1). Isolated cultures are now maintained in the CICCM (Table 1).

Table 1. *Ostreopsis* cultures used in this study and held in the Cawthron Institute Collection of Microalgae (CICCM).

Species	CICCM code
O. ovata Cook Is.	CAWD174
O. siamensis NZ	CAWD 96, 147, 173
Ostreopsis sp. Australia	CAWD179
Ostreopsis sp.Cook Is.	CAWD184
Ostreopsis sp. Hawaii	CAWD185

Toxicity of *O. ovata* in the Mediterranean has been recorded in recent years, with *O. ovata* isolates being equally as toxic as *O.* cf *siamensis* (Penna *et al.* 2005; Riobo *et al.* 2006), and with blooms being associated with human illnesses in Italy (Brescianini et al. 2006; Ciminiello *et al.* 2006). *O. ovata* (CAWD174) and *O.* cf. *ovata* (CAWD184) from the Cook Islands and *Ostreopsis* spp. from Australia and Hawaii were all non-toxic by the HNA. In comparison, *O. siamensis* from New Zealand (isolates CAWD96, 147 and 173), tested in the same laboratory, produced concentrations of 0.11, 0.31 and 0.03 pg/cell respectively (Table 2).

The variability of toxin production within species has been noted previously and *O. ovata* from tropical regions has been considered of limited toxicity. Extracts (ethanol) of mass cultures of *O. ovata* from the Cook Islands were also tested in mice by intraperitoneal injection and, despite testing up to 144 mg/kg, were non-toxic. In comparison, New Zealand isolates of *O. siamensis* had an LD_{50} similar to palytoxin itself, i.e. 0.72 µg/kg (Rhodes *et al.* 2002).

Table 2. 'Palytoxin-equivalents' produced by *O. siamensis* (NZ) and *O. ovata** (Cook Is.) as determined by heamolysis assay (HNA) and LCMS (Selwood et al., abstract this conference).

	HNA	LCMS
	palytx. equiv. pg/cell	
CAWD96	0.11	0.74
CAWD147	0.31	1.23
CAWD173	0.03	0.31
CAWD174*	ND	1.18

Interestingly, *O. ovata* (CAWD174), extracts of which were negative for palytoxin-like compounds by HNA, was positive for a palytoxin-like compound by LCMS (Table 2; Selwood et al., 14th ICHA, Book of Abstracts). Palytoxin equivalents were detected by LC-MS in all *O. siamensis* isolates, although the estimated concentrations differed from the HNA concentrations, probably due to differences in the palytoxin standards used. This will be investigated further. Ciguatera fish poisoning (CFP) has become an increasing problem in the Cook Islands in recent years and the involvement of palytoxin in CFP continues to be raised as a possibility. *Gambierdiscus australes* co-occurred with *Ostreopsis* in the Cook Island samples, but no ciguatoxin was detected, either by LCMS or by radioligand binding assay. However, maitotoxin was detected (unpubl. data) and extracts were toxic to mice (Rhodes *et al.* 2010). *Amphidinium carterae* also occurred in high numbers in the same samples and will be investigated further, as extracts caused respiratory paralysis in mice at high doses by intraperitoneal injection and oral administration. In conclusion, six of the nine currently recognised *Ostreopsis* species have been reported as producing palytoxin-like compounds (Rhodes 2010), although there is considerable strain variability in toxin production. A tenth species, isolated from Hawaiian waters in 2001, is currently being described (Morton *et al*. 2010). Uptake of

palytoxin and its derivatives by invertebrates (Rhodes *et al.* 2008; Aligizaki et al. 2008) and fish has been recorded (Munday 2008) and, with the increasing detection of *Ostreopsis* species in temperate environments, issues arising from toxic blooms of this genus are likely to increase. For example, *Ostreopsis* has recently been reported in the Sea of Japan (Selina and Orlova 2010) and *O. siamensis* is known to occur in Tasmania, Australia (Pearce *et al.* 2001). It has also been observed in seawater samples from the cool coastal waters near Wellington, New Zealand (Rhodes 2010). The newly developed LCMS assay will allow rapid determination and an early warning of any palytoxin risk.

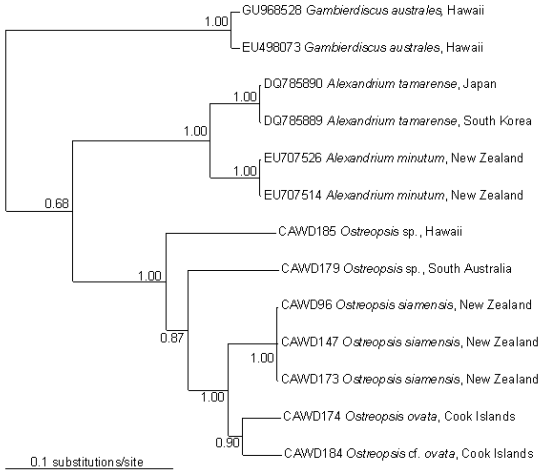

Fig. 1. Bayesian phylogenetic tree of the D8-D10 region of the LSU rDNA from *Ostreopsis* species isolated from the Pacific region with sequences from closely related species in GenBank.

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PROGRESS ON *DINOPHYSIS* PHYSIOLOGICAL ECOLOGY, PHYLOGENY & TOXICOLOGY

Pigment composition in the dinoflagellate genus *Dinophysis*, and associated cultures of *Myrionecta rubra* and cryptophyte species

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Abstract

Recently, Park *et al* (2006) succeeded in cultivating the dinoflagellate *Dinophysis acuminata* using a three-species approach including the ciliate *Myrionecta rubra*, and the cryptophyte *Teleaulax* sp.. Despite this major advance, there still exists controversy about the nature of the *Dinophysis* plastids, whether they are klepto-plastids from *M. rubra* or if it harbors some permanent plastids. Until to date, there have been no reports about the pigment composition of *Dinophysis* strains and associated organisms in culture. We characterised the pigment composition using HPLC analyses of *M. rubra*, *Teleaulax* sp, and for the first time, several *Dinophysis* species maintained in the laboratory.

Introduction

The achievement of culturing *Dinophysis* species has opened the possibility of new studies on the ecology and physiology of this genus.In particular, there exists a debate about the nature of the plastids in *Dinophysis*, whether they are "kleptoplastids" or if it harbours permanent plastids. This discussion has been only addressed from morphological and molecular perspectives. None of these studies have included characterization of the lipophilic pigment composition on *Dinophysis* species, even though Meyer-Harms and Pollehne (1998) related the "cryptophyte-like" pigment pattern (alloxanthin and Chl c_2) in field samples to *Dinophysis norvegica*. Pigments are chemotaxonomic markers which could show new light to this discussion. In the present study we analyzed the lipophilic pigments in three *Dinophysis* species and their associated cultures (their prey, the photosynthetic ciliate *Myrionecta rubra*, and the cryptophytes (*Teleaulax* sp). preyed on by the ciliate.

Methods

Cultures

Dinophysis tripos, *D. caudata* and *D. acuminata* isolated from the Galician Rías were cultured in L20 medium and maintained on a 12:12 L:D cycle, at 150 μEm^2s^{-1} light irradiance. The ciliate *Myrionecta rubra* fed with the cryptophytes *Teleaulax* sp. was added periodically as prey and cultured under the same conditions.

Pigment analyses

The HPLC method used was the method of Zapata *et al*. (2000). The chromatographic equipment was a Waters (Milford, MA, USA) Alliance HPLC system. The stationary phase was a C8 column (Waters Symmetry).

Results

HPLC pigment analyses (Fig. 1) showed that the three *Dinophysis* species studied shared a common pigment composition, with Chl c_2, alloxanthin and crocoxanthin as major accessory pigments, the same as in the ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax* sp.

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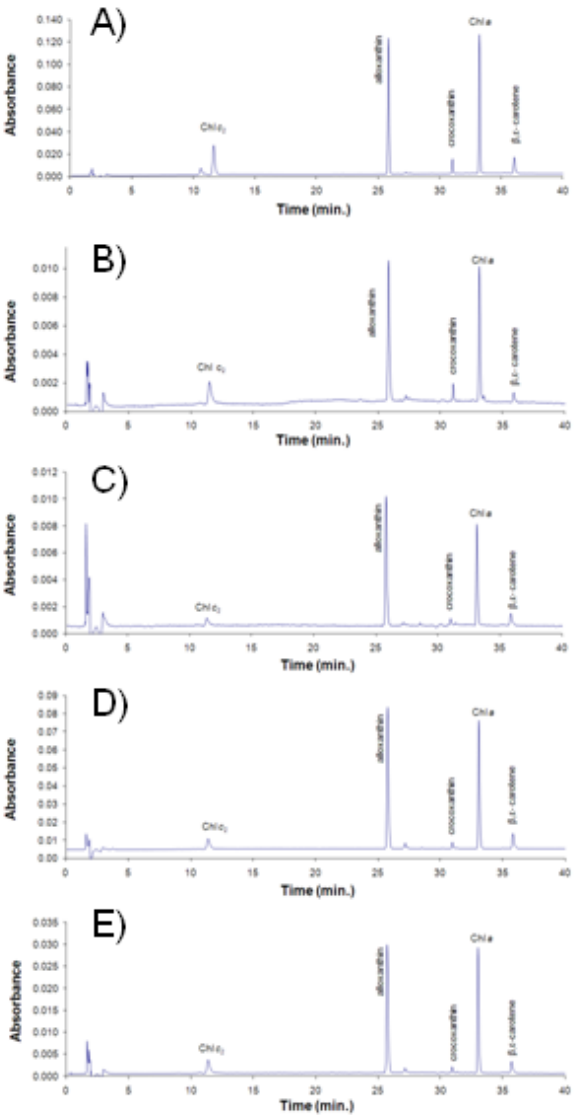

Fig 1. HPLC chromatograms of A) *Teleaulax* sp., B) *Myrionecta rubra*, C) *Dinophysis acuminata*, D) *Dinophysis caudata* and E) *Dinophysis tripos*.

However, *Dinophysis* cultures displayed different pigment ratios to Chl *a* (lower Chl c_2 and higher alloxanthin) in comparison with *M. rubra* and *Teleaulax* sp. Similar ratios have been found in some cryptophytes as *Hemiselmis* sp. (Table 1). Chromatograms of *Dinophysis*, *Myrionecta* and *Teleaulax* showed peaks at the same retention time of monadoxanthin, but the UV-VIS spectrum analysis showed that it corresponded to an unknown cis-isomer carotenoid.

Table 1. Pigment ratios to Chl *a* of different cryptophytes, the ciliate *M. rubra* and *Dinophysis* species. Presence/absence of the carotenoid monadoxanthin is also indicated.

Pigments / Species	Monadoxanthin	chl2/ chl *a*	Alloxanthin /chl *a*
Hemiselmis sp.	-	0.801	0.052
Rhodomonas baltica	+	0.454	0.105
Teleaulax sp.	-	0.503	0.111
Myrionecta rubra	-	0.658	0.065
Dinophysis acuminata	-	0.811	0.048
Dinophysis caudata	-	0.867	0.046
Dinophysis tripos	-	0.833	0.062

Conclusions

We did not find any pigment signatures which could reveal unambiguously a different (permanent) plastid in *Dinophysis*. Quantitative differences in Chl c_2 and alloxanthin in *Dinophysis* vs. *M. rubra* or *Teleaulax* sp. could be explained by some (or several) of the following hypotheses to be tested:

1) Photoadaptation in cryptophyte plastids after being engulfed by the new hosts (*Dinophysis* or *M. rubra*).
2) Changes in physiological state of the plastids during permanence into the new host cells.
3) Pigment contributions from other "cryptophyte-like" plastids with different pigment ratios to those in *Teleaulax* sp.

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Molecular analysis of chloroplasts of *Dinophysis acuta* from Huelva (Spain) fed with different cryptophytes

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Abstract

Two possible origins for *Dinophysis* plastids have been considered, one suggests they were taken from a cryptophyte at some point of the dinoflagellate evolutionary history and maintained permanently since then and the other claims that they are collected from their prey and used for some time before they get lost. This issue has been controversial up to now. In this paper we have tried to investigate the origin of *Dinophysis acuta* chloroplasts by molecular analysis of the *psb*A gene in dinoflagellate cells fed with *Myrionecta*, itself fed with several cryptophyte isolates or *Teleaulax* sp. The results obtained are in agreement with an ancient origin and permanent status of *D. acuta* chloroplasts.

Introduction

Dinophysis Ehrenberg is a genus of marine dinoflagellate producer of polyether toxins. For many years, the inability to maintain these organisms in laboratory cultures was the main obstacle in their study. Recently, predator-prey lines have been established for different *Dinophysis* species such as *D. acuminata, D. caudata*, *D. fortii* and *D. infundibulus* (Nagai *et al.* 2008, Nishitani et al. 2008, Park *et al*. 2008, Kamiyama and Suzuki 2009). All these cultures are based on a feeding line of three organisms: a cryptophyte, *Myrionecta* a ciliate, and *Dinophysis*. The first successful culture of *Dinophysis* was archieved by Park *et al.* (2006), when *D. acuminata* was found to grow when fed with *Myrionecta rubra*. This ciliate has an endosymbiont that resembles *Teleaulax,* a cryptophyte, but there is no agreement of whether the endosymbiosis is permanent or not. While it is known that photosynthetic species of *Dinophysis* have chloroplasts of cryptophyte origin (Schnepf and Elrächter 1999, Lucas and Vesk 1990, Takishita *et al.* 2002, Janson and Granéli 2003), during the last decade it has been controversial whether chloroplasts are permanent, or otherwise, are acquired periodically by a process called kleptoplastidy, a specific process by which *Dinophysis* steals chloroplasts from other organisms. This process could represent an early stage of plastid acquisition, where the organelle is not yet under the complete control of the host. Recently, Park *et al.* (2010) followed the fate of plastid *psbA* gene in cross-feeding/starvation experiment in established cultures of *Dinophysis caudata* fed with *M. rubra* grown on two different cryptophytes. He suggests that *D. caudata* treats in different ways plastids taken up from different cryptophytes by the ciliate. Furthermore, he states that *D. caudata* may posses its own permanent plastid, but can also obtain them from *M. rubra* and be maintained for a month. To try to understand the origin of the chloroplasts of *Dinophysis*, in this work various cultures of a ciliate, *Myrionecta* sp. from Huelva have been fed with different cryptophytes. In turn, these ciliates have been used to feed different cultures of *D. acuta* isolated from the same geographical region. In order to identify whether *Dinophysis* chloroplasts come from its prey or, on the contrary, have been acquired throughout its evolutionary history, we have examined the chloroplast *psbA* gene in these laboratory cultures in the different microorganisms involved in the feeding line.

Materials and methods

Cultures. Individual cells of *Dinophysis acuta*, *Myrionecta rubra* and *Teleaulax sp.* were isolated from weekly samples of the Andalusian Monitoring Programme in Huelva (Andalucía, Spain) in summer 2008. The cryptophytes CRY1V,

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CRY2V and CRY6V where isolated from Vigo seawater. All cultures were grown non-axenically in L1 medium without silicates (Guillard and withut prey for a week to assure no cryptophyte in the culture. Once *Myrionecta* culture was cryptophyte free, several cultures were established by feeding with *Teleaulax sp*. or either one of the cryptophytes CRY1V, CRY2V and CRY6V. These cultures where named MT, MCRY1V, MCRY2V and MCRY6V. All cultures were maintained with the corresponding cryptophytes over three months.

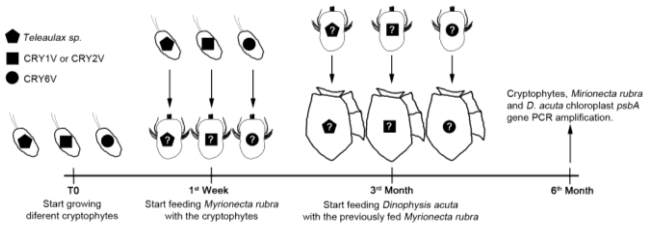

Fig. 1. Time course schematic representation of the established cultures and when PCR amplification were carried out.

In the establishment of *Dinophysis acuta* cultures, first *D. acuta* was maintained without addition of prey for 2 wk after isolation. Next, *D. acuta* was fed with the previously established *Myrionecta* cultures. These cultures were named DMT, DMCRY1V, DMCRY2V and DMCRY6V. In addition, control cultures were started by maintaining *Dinophysis acuta* without *Myrionecta* prey for a period over 3 months. These cultures were named DMTH, when *D. acuta* had been previously fed with MT, and DMCRY1VH2 when *D. acuta* had been previously fed with MCRY1V. Fig1 shows a diagram of the cultures.

PCR amplification, cloning and sequence analysis. The chloroplast *psbA* gene was amplified from cryptophytes, *Myrionecta sp.* and *Dinophysis acuta* using primers *psb*AF and *psb*AR2 (Hackett *et al*. 2003). All reactions were done with an initial denaturalization at 94°C for 10 min; followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; and ended with a 10 min extension at 72°C. For cryptophyte PCR amplifications 1 or 3 µl of culture were used. In the case of *Dinophysis acuta* and *Myrionecta rubra* cultures, cells picked up one by one under inverted microscope. All cells prior the PCR amplifications were treated as in Raho *et al*. (2008). Correct length PCR products were cloned using pGEM-T Easy Vector System (Promega, Madison, WI, USA). Positive clones were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) and an Applied Biosystem ABI 310 (PE Applied Biosystems, Foster City, CA, USA) automated sequencer. Selected sequences were aligned with CLUSTAL X (Thompson et al., 1997) and Neighbor Joining (NJ) Phylogenetic tree constructions were performed with PAUP* 4.0 (ver. 4.0b10) (Swofford, 1998) using the Kimura 3 parameter (Kimura, 1981). Support for the NJ branches was tested by Bootstrap (Felsenstein, 1985) analysis of 1000 repetitions.

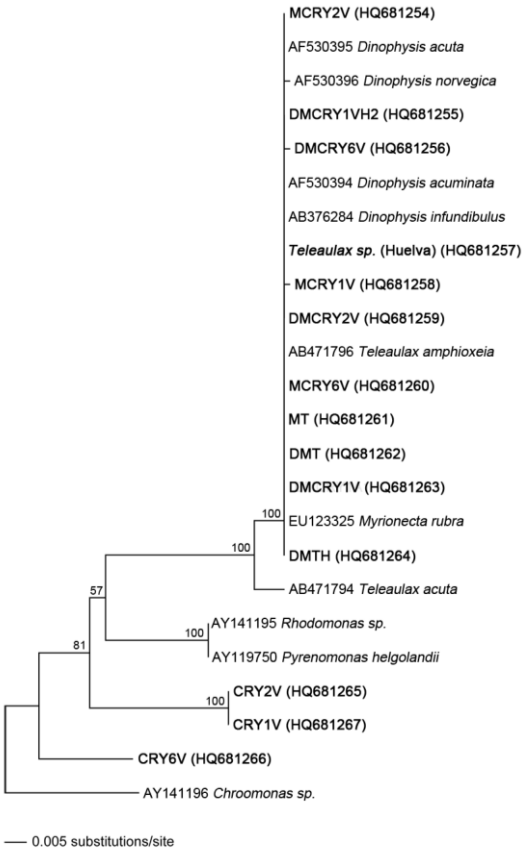

Figure 2: Phylogenetic tree of chloroplast *psbA* gene showing the position of *Teleaulax sp.*, cryptophytes CRY1V, CRY2V and CRY6V, the *Myrionecta rubra* fed with these cryptophytes (MT, MCRY1V, MCRY2V and MCRY6V), and the *Dinophysis acuta* fed with those *Myrionecta* cultures (DMT, DMCRY1V, DMCRY2V and DMCRY6V). Also, cultures named DMTH and DMCRY1VH2 represent control cultures where *Dinophysis acuta* were maintained without prey for a period over three months. Bootstrap values (1000 replicates) are shown at the internal branches. Sequences from this work are represented in bold.

Results

Four different cryptophyte strains were used as potential chloroplast donors for *D.acuta*: *Teleaulax sp.*, CRY1V, CRY2V and CRY6V.

Phylogenetic analysis of *psbA* sequences revealed that CRY1V and CRY2V have the same sequence and CRY6V has a sequence clearly divergent of those of other cryptophytes used as chloroplast donors (accession numbers: HQ681257, HQ681265, HQ681266, HQ681267). No matter what of the four cryptophytes is given to *Myrionecta sp*. as a prey, the *psbA* sequences from all cultures (MT, MCRY1V or 2V and MCRY6V; acc. numbers: HQ681261, HQHQ681258, HQ681254, HQ681260) appeared clustered as a monophyletic group together with the *Teleaulax* sequences (Figure 2). All *psbA* sequences from *D. acuta* fed with different *Myrionecta* cultures (*Dinophysis* cultures DMT, DMCRY1V or 2V, and DMCRY6V; acc. numbers: HQ681262, HQ681263, HQ681259, HQ681256) clustered with *Myrionecta* and *Teleaulax*. The sequences from *D. acuta* cultures maintained without prey (DMTH and DMCRY1VH2; acc. numbers: HQ126864, HQ681255) also clustered in this group (Figure 2). These results are sketched in Figure 3.

Conclusions

Until Park et al. (2006) established the first cultures of *Dinophysis*, the origin of their chloroplasts has been a difficult issue because the impossibility of performing controlled experiments. Here, three cryptophytes other than *Teleaulax* were given to the ciliate *Myrionecta rubra* as prey and, in turn, they were used as a prey for *D. acuta*. Cultures of *Dinophysis* starved for >3 months were used as control. From each culture *psbA* gene was amplified by PCR. The results indicate that even when *D. acuta* is fed with *Myrionecta* cultures fed non-*Teleaulax* cryptophytes, the dinoflagellate chloroplasts are of *Teleaulax* origin. Equally, sequences in control cultures of the dinoflagellate not fed with any prey for 3 months still show a *Teleaulax*-like *psbA* sequence, indicating permanence of chloroplasts independently of the prey. All clones obtained, either from *Myrionecta* or *Dinophysis*, showed the same *Teleaulax*-like *psbA* sequence, results that not support the theory of a mixed population of permanent or recently acquired chloroplast (Hacket et al. 2003, Minnhagen and Janson 2006), at least with a significant frequency. Our results suggest that *Dinophysis* and *Myrionecta* have permanent chloroplasts that may have been obtained from *Teleaulax* at some point of their evolutionary history. It is however surprising that the *Teleaulax*, *Myrionecta* and *Dinophysis psb*A sequences have not diverged up to now. Further studies using other genes or genomic approaches would help to elucidate this interesting biological mystery.

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Genetic variability and molecular of *Dinophysis* species (Dinophyceae) from single cell analysis of mitochondrial *cox1* gene

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Abstract

The identification of *Dinophysis* spp. is a crucial task in harmful algae monitoring programmes due to the occurrence of several toxin producer species responsible for diarrethic shellfish poisoning (DSP) events. The morphological variability exhibited by some *Dinophysis* species, as those in the *D. acuminata*-complex makes difficult its identification in field samples. Other taxonomic methods, such as molecular analyses based on ribosomal genes and intergenic regions (ITS), display limited resolution in the genus *Dinophysis* due to their low inter-specific variability. In the present study we explore the potential of *cox*1 gene as a marker to differentiate among *Dinophysis* species, based on previous findings of high resolution for two morphological similar species, *D. acuminata* and *D. ovum*.

Introduction

The genus *Dinophysis* comprises about 200 species (Sournia 1986) thirteen of which have been reported to contain potent lipophilic toxins (Moestrup 2004; Raho *et al.* 2008). Identification and quantification of toxigenic *Dinophysis* spp. is routine in monitoring programmes of harmful algae. The taxonomic identification of *Dinophysis* spp. is mainly based on the cell contour (Larsen and Moestrup, 1992), but intra-specific differences in morphology are frequently observed as a result of their polymorphic life cycle, feeding behaviour, active division and biogeography (Reguera and González-Gil, 2001; Reguera *et al.* 2007). Morphological variability adds uncertainty in identification and quantification of phytoplankton samples, especially when two close species of *Dinophysis*, such as the pair *D. acuminata* and *D. sacculus* Stein, both included in the ‘‘*D. acuminata* complex’’, co-occur (Lassus and Bardouil, 1991; Bravo *et al.* 1995; Koukaras and Nikolaidis, 2004; Raho *et al.* 2008). Due to the difficulties to establish species of *Dinophysis* in culture, sensitive molecular techniques for single-cell DNA amplification were developed (Marín *et al.* 2001a, 2001b; Edvardsen *et al.* 2003; Hart *et al.* 2007). Most phylogenetic studies of dinoflagellates have used the ribosomal DNA (rDNA) (Bhattacharya and Medlin, 1995; Guillou *et al.* 2002). For *Dinophysis* spp., sequence comparisons of the D1–D2 LSU regions failed to establish intra-specific differences between *Dinophysis* isolates from different locations (Edvardsen *et al.* 2003; Hart et al. 2007). Also, internal transcribed spacers, ITS1 and ITS2, who have less conserved regions, were unsuccessful to establish inter-specific differences (Marín *et al.* 2001a; Edvardsen *et al.* 2003). Mitochondrial DNA, still rather unexplored within dinoflagellates, is a useful candidate as a molecular marker in closer phylogenetic reconstructions because its genes are generally conserved, but they are subject to more variations than nuclear coding genes (Saccone *et al.* 2000). Saunders (2005) verified the mitochondrial cytochrome c oxidase subunit I gene (*cox*1) as a suitable marker for red algae, and Robba *et al.* (2006) compared results from numerous samples of this algal group and stated that *cox*1 was a more sensitive marker than the plastid RuBisCO. Recently, Zhang *et al.* (2008) used the *cox*1 gene to determine the phylogenetic attribution of several microalgal species including *D. acuminata* and found that this microalgae was sister to Gonyaulacoids. We compared the *cox*1 sequences of two close

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species of the "*D. acuminata* complex" -*D. acuminata* and *D. ovum*- and found 25 mismatches between them, concluding that *cox*1 could be a more useful candidate marker than the rRNA genes and the ITS to discriminate between close species of *Dinophysis* (Raho *et al.* 2008). Also, in 2010, Papaefthimiou *et al.* followed the same approach to explore the identity of a *Dinophysis* in Greek waters similar to *acuminata* and *ovum*. These authors also concluded that *cox*1 is a more robust phylogenetic marker, allowing increased resolution. Here we used *cox1* sequences of the most common species of *Dinophysis* found in Galician coastal waters to generate a sequence bank that enable us to differentiate local species and with those from others localities.

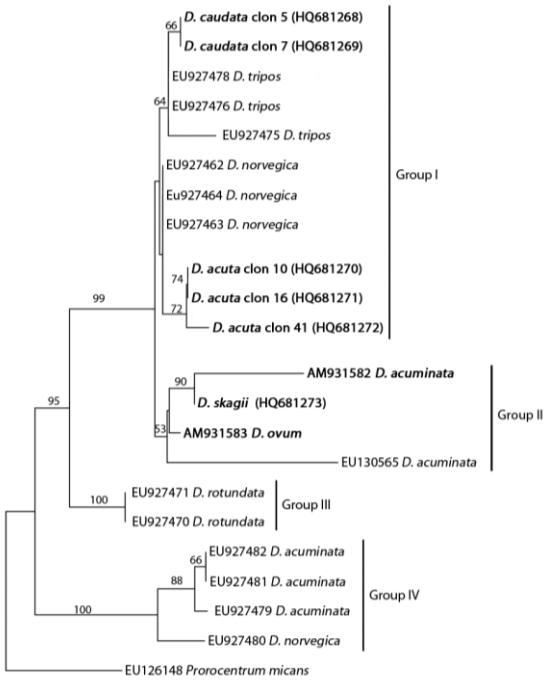

Fig. 1. Phylogenetic tree based on mitochondrial cox1 gen inferred by Neighbour Joining showing the relation of different Dinophysis species. The bootstrap values (1000) are shown at the internal branches. The sequences are in bold.

Material and Methods

Plankton samples. Opportunistic weekly samplings were carried on board RV J.M. Navaz at a fixed station (P2, 42° 21.40' N, 8° 46.42' W) in Ría de Pontevedra (NW Spain) between 2005 and 2007. Seawater from 3 to 5m depth was pumped and passed through a set of superimposed meshes. The 20–77 µm size-fractioned concentrate was selected for *Dinophysis* spp. On arrival to the laboratory, aliquots of these samples were used directly for *Dinophysis* single-cell isolation, or filtered through 20 µm mesh, and the slurry re-suspended in cold methanol and kept at -20ºC.

Single cell isolation and PCR amplification. Cells from *Dinophysis acuta, tripos, acuminata, skagii, ovum* and *caudata* were picked from plankton concentrates, with a microcapillary pipette under inverted microscope (Zeiss Axiovert 200), at 100 and 400X magnification. The isolated cells where treated and PCR amplified with primers Dinocox1F and Dinocox1R as Raho *et al.* (2008). PCR products were cloned into pGEM-T Easy Vector System (Promega, Madison, WI, USA) and transformed to *Escherichia coli* strain DH5α according to manufacturer´s protocol and correct length amplicons were selected for sequencing.

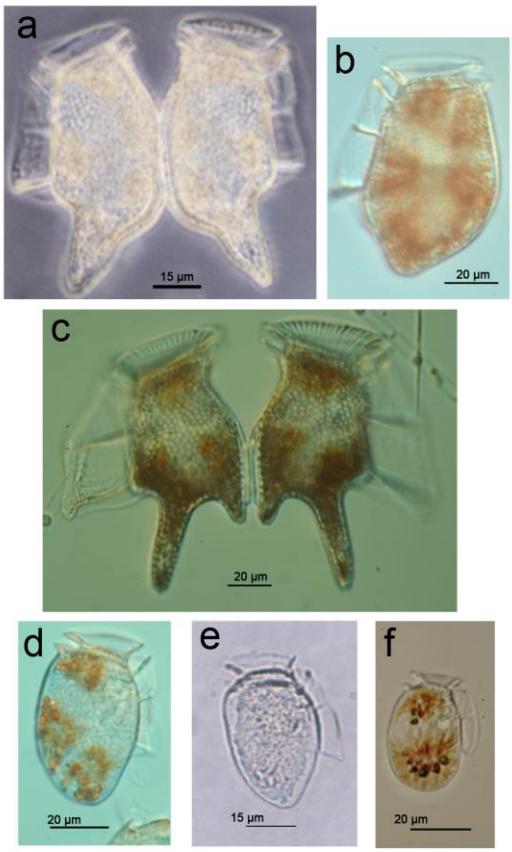

Figure 2. Micrographs (DIC, 400X) of live specimens of *Dinophysis* from galician waters presented in this work: a) *Dinophysis caudata*; b) *D. acuta;* c) *D. tripos*; d) *D. acuminata*; e) *D. skagii* and f) *D. ovum.*

***DNA sequencing and analysis*.**
PCR products were sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) and an Applied Biosystem ABI 310 (PE Applied Biosystems, Foster City, CA, USA) automated sequencer. The selected sequences were aligned with CLUSTAL X (Thompson *et al.* 1997) and Neighbor Joining (NJ). Phylogenetic constructions were performed with PAUP* 4.0 (ver. 4.0b10) (Swofford, 1998) using the Kimura 3 parameter (Kimura, 1981). Finally, support for the NJ branches was tested by Bootstrap (Felsenstein, 1985) analysis of 1000 repetitions

Results

The phylogenetic tree inferred by Neighbour Joining (Fig. 1) was formed by four well-defined groups where Group I showed three clusters. One contained the "*caudata* group", represented here by *D. caudata* and *D. tripos*. The two others were closely related and contained *D. norvegica*, which is related to all *D. acuta* clones branch, respectively. Individuals of the "*acuminata* complex" formed group II, with *D. acuminata* grouped together with its small form, *D.skagii*, (Group III clustered all *D. rotundata* sequences, and finally, there was a divergent Group IV, formed with three *D. acuminata* sequences and one *D. norvegica*. The presence of the same *Dinophysis* species (e.g. *D acuminata* and *norvegica*) in different genetic clusters cannot be explained so far. However, as most of these sequences arise from single cells, not cultures, intraspecific morphological variability could lead to some misidentification at species level. To rule out this possibility, we think it would be important to provide images of the species included in the analyses (Fig.2). We suggest that mtDNA phylogenies of *Dinophysis* should be revised incorporating new sequences, from individuals or cultures at best, unambiguously identified by morphological criteria.

Acknowledgements

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Morphological variability, toxinology and genetics of the potential lipophilic toxin-producer *Dinophysis tripos*

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Abstract

In recent years, Park *et al.* (2006) succeeded in cultivating the toxic dinoflagellate *Dinophysis acuminata* using a three-species approach including the ciliate *Myrionecta rubra*, as *Dinophysis* prey, grown with a cryptophyte (*Teleaulax* sp.). After this achievement, several other *Dinophysis* species have been brought into culture (*D. caudata, D. fortii* and *D.infundibulus*; Nagai et al., 2008; Nishitani *et al.*, 2008a; b). In the present study we describe for the first time the morphological variability, genetics and toxin content in cultures of *D. tripos.*

Introduction

Dinophysis tripos (Gourret, 1883) exhibits a marked morphological variability and small and intermediate forms may get confused with those from *Dinophysis caudata* when blooms co-occur. Sequences of ribosomal DNA (LSU) place it with *D. caudata*, in a clade separated from the other larger clade that includes all mixotrophic species of *Dinophysis*. *D. tripos* has been included in the list of toxin-producing *Dinophysis* spp on the basis of one single HPLC analyses with fluorescence detection. Here, we examined morphological, toxinological and genetic characteristics of *D. tripos* from laboratory cultures fed on *Myrionecta rubra*. During late autumn 2009, *D. tripos* was the dominant *Dinophysis* species in the Galician Rías Baixas (NW Iberia) and there were no harvesting closures associated with its occurrence. OA derivatives and pectenotoxins were under detection levels in LC-MS analyses of single cell isolates, net hauls rich on this species and laboratory cultures. Our results indicate that the Galician strains of *D. tripos* are toxin-producers (PTX-2) and emphasize the need to re-examine, with last generation analytical techniques, the checklist of toxin-producing *Dinophysis* spp in different parts of the world.

Methods

Cultures. Single cells of *Dinophysis tripos* were picked from a coastal station (B1) at Ría de Vigo (NW Spain) in October 2009. Cultures were maintained in L20 medium adding *Myrionecta rubra* as prey.

Genetic analyses. 23SrRNA plastid sequences were amplified by PCR using published primers (Sherwood and Presting 2007) from single cells of *D. tripos* and *D. caudata* and cultures of *M. rubra* and *Teleaulax* sp.

Toxin analyses. A LC-MS system was used following the method of Pizarro *et al* (2008).

Results

Cultures. In optimal conditions of prey and environmental conditions cultures of *D. tripos* were dominated by typical vegetative cells, reaching up to 250 cells mL^{-1} in L20 medium. Cultures of *D. tripos* were able to grow in a temperature range of 15 - 26 °C. When cultures became prey-limited, it was observed a continuum of intermediate and small forms (like varieties of *D. diegensis* and *D. taylorii* in the literature). Typical cells from cultures of a closely related species (*D. caudata*) and their small forms (*D. diegensis*) were also measured ($n \geq 50$ specimens (Fig. 1).

Genetics. NJ tree of partial 23SrDNA (400 bases) showed identical sequences of two *Dinophysis* species (*D. tripos* and *D. caudata*), the ciliate *Myrionecta rubra* and *Teleaulax* sp. used as prey in these *Dinophysis* cultures (Fig. 2).

Toxins. LC-MS analyses of the *D. tripos* culture showed the presence of PTX-2 (Fig. 3).

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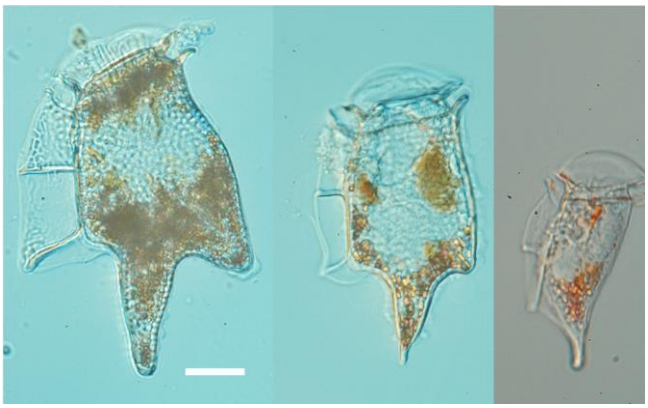

Fig 1. Morphological variability of *D. tripos* (normal, intermediate and small sized cells) in culture.

Conclusions

Small forms of *D. tripos* can be distinguished from *D. diegensis* by the body length and the left sulcal list —which in the case of *D. tripos* extends to the antapical projection. In addition, dorsal and ventral margins are parallel in small cells of *D. tripos* (yellow lines) where the antapical process is always differentiated. In contrast, the antapical process disappears in small cells of *D. caudata* (*D diegensis*).

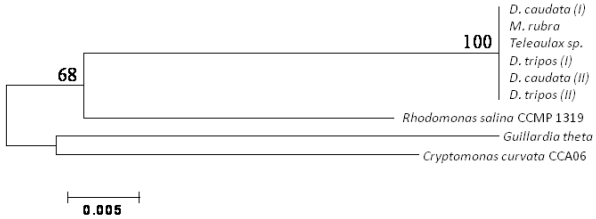

Fig. 2. Neigbor-joining tree based on 23SrRNA plastid partial sequences of *Dinophysis tripos*, *D. caudata* and associated cultures of the ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax* sp.

Partial sequences of plastid 23SrDNA (400 bases) from cultures of *D. tripos* and *D. caudata* were identical to those of *Myrionecta rubra* and *Teleaulax* sp., but different to the three available cryptophyte sequences in GenBank database (0.93-95 similarity).The only previous study detecting any toxins in *D. tripos* (Lee *et al* 1989) suggested the presence of DTX-1. To our knowledge, this is the first report of established cultures of *D. tripos* (fed on *M. rubra*) and on the presence of PTX-2 in this species.

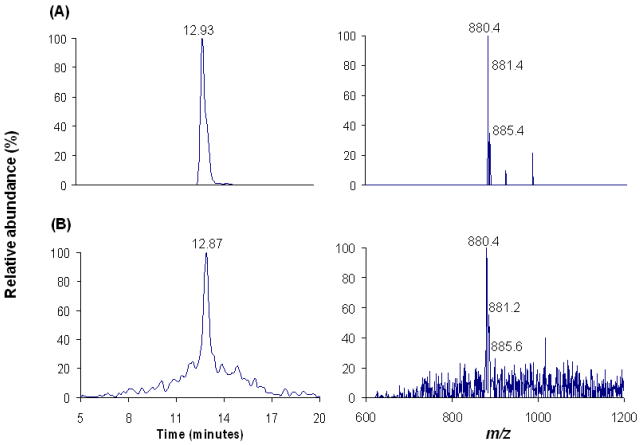

Fig 3. Selected LC/MS chromatograms (left) and mass spectra (right) obtained in positive ionization mode showing m/z 881 [M + Na+]+ ion characteristic of PTX 2. (A) PTX 2 standard. (B) Metanolic extract from a *Dinophysis tripos* culture

Acknowledgements

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Potentially Toxic *Dinophysis* in Southern Brazil (winter 2005; summer 2007)

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Abstract

We present data on oceanographic conditions and the distribution and cell density of six potentially toxic *Dinophysis* species in coastal and oceanic waters off Santa Marta Grande Cape (SMGC; 28°40'S) and Albardão-Chuí (AC; 34°S) in southern Brazil. Water samples (from the surface and from the depth of the chlorophyll maximum were collected by means of Niskin bottles) and plankton vertical hauls (plankton net 20 µm) were collected at 33 stations in the austral winter of 2005 (August/September) and the summer of 2007 (February) from the R. V. *Atlântico Sul*. *D. acuminata*, *D. caudata*, *D. fortii*, *D. mitra*, *D. rotundata* and *D. tripos* were recorded. their occurrence was clearly associated with the presence of particular water masses. *D. acuminata* was the most important toxic species in winter and summer, occurring primarily in the plume of the La Plata River, within a wide range of temperature and salinity, but also in STSW and upwelled SACW. *D. fortii* showed a preference for warmer water, while *D. caudata* was most abundant in winter at both sampling localities under a wide range of temperature and salinity.

Introduction

In southern Brazil, the contamination of bivalve molluscs due to toxic *Dinophysis* blooms represents a severe threat to the shellfish industry. The first closure of shellfish trade following a DSP (Diarrheic Shellfish Poisoning) outbreak occurred in the summer of 2007 (Proença *et al*. 2007). Little is known of the distribution of *Dinophysis* species or of the factors influencing their occurrence on the southern Brazilian continental shelf (Haraguchi & Odebrecht 2010). In this area, complex oceanographic conditions prevail; strongly influence by outflow from the La Plata River, and the presence of subantartic waters in winter and tropical waters in summer (Seeliger *et al*. 1997; Piola *et al*. 2000). We aim to provide information on the occurrence and distribution of potentially toxic *Dinophysis* species in relation to oceanographic conditions off Santa Marta Grande Cape (SMGC; 8°40'S) and Albardão-Chuí (AC; 34°S) in Southern Brazil, influenced mostly by tropical water and La Plata River outflow, respectively.

Material and Methods

Sampling of 33 oceanographic stations (Figure 1) was undertaken in the austral winter of 2005 (August/September) and the summer of 2007 (February) from the R. V. *Atlântico Sul*. Water samples from the surface and the depth of the chlorophyll-maximum (Niskin bottles) were fixed with lugol's neutral solution and formalin (1%) (Throndsen 1978) for cell enumeration , following the method of Utermöhl (Andersen & Throndsen 2004). Plankton samples obtained from vertical hauls (plankton net 20 µm) were fixed with formalin (4%) and used for identification and the establishment of relative contributions. Species identification followed morphological criteria according to the Dinoflagellates Atlas from the Southwestern Atlantic Ocean (Balech, 1988).

For *Dinophysis acuminata* we observed a wide range of morphological variability and therefore refer to these cells as *D.cf.acuminata*. Salinity and temperature data were obtained from *in situ* CTD measurements and were used to classify water masses following Möller *et al*. (2008), as shown in Table 1. Cell count data were included in temperature-salinity diagrams, to establish the association of particular species with particular water masses.

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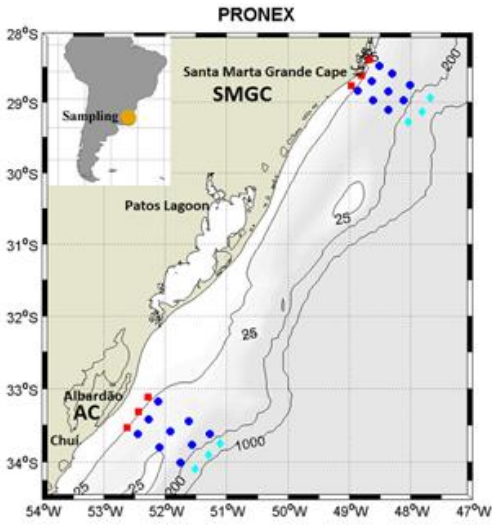

Fig 1. Southern Brazil showing coastal (red), continental (blue) and shelf break (blue-green) stations off Santa Marta Grande Cape (SMGC) and Albardão-Chuí (AC).

Table 1: Water masses classification (Möller *et al*. 2008)

	Winter 2005	
Water mass	**Temperature**	**Salinity**
Sub Tropical Shelf Water (STSW)	T>14°C T>18.5°C	33.5<S<35.3 35.3<S<36
Tropical Water (TW)	T≥18.5°C	S≥36
La Plata Plume Water (PPW)	T>10°C	S≤33.5
Sub Antartic Shelf Water (SASW)	T≤ 14°C	33.5<S<34.2
South Atlantic Central Water (SACW)	T≤18.5°C	S≥35.3
	Summer 2007	
Water mass	**Temperature**	**Salinity**
Sub Tropical Shelf Water (STSW)	T>18.5°C T>21°C	33.5<S≤36 33.5<S≤35.3
Tropical Water (TW)	T≥18.5°C	S≥36
La Plata Plume Water (PPW)	T>10°C	S≤33.5
Sub Antartic Shelf Water (SASW)	T≤21°C	33.5<S≤34.2
South Atlantic Central Water (SACW)	T≤18.5°C	S≥35.3

Results

Six potentially toxic species were observed on the Southern Brazilian shelf: *Dinophysis acuminata*, *D. caudata*, *D. fortii*, *D. mitra*, *D. rotundata* and *D. tripos*. The most abundant species were *D. cf. acuminata* (SMGC in summer) and *D. caudata* (AC & SMGC in winter; AC in summer) (Fig 2). *D. fortii* occurred off SMGC in summer and winter, and off AC in summer, but only at stations influenced by Tropical Water. *D. tripos* was observed during both periods but only off SMGC. *D. mitra* was present off AC in the summer, while *D. rotundata* was rare during both periods of sampling at both localities. Cell densities of *Dinophysis* were relatively low (*D. acuminata* max 300 cells L^{-1}) and the TS diagrams (Fig 3a-c) show that *D. acuminata* was widely distributed in PPW, STSW and SACW; *D. fortii* occurred only at higher temperatures and salinity (STSW); and *D. caudata* occurred in STSW mixted with PPW.

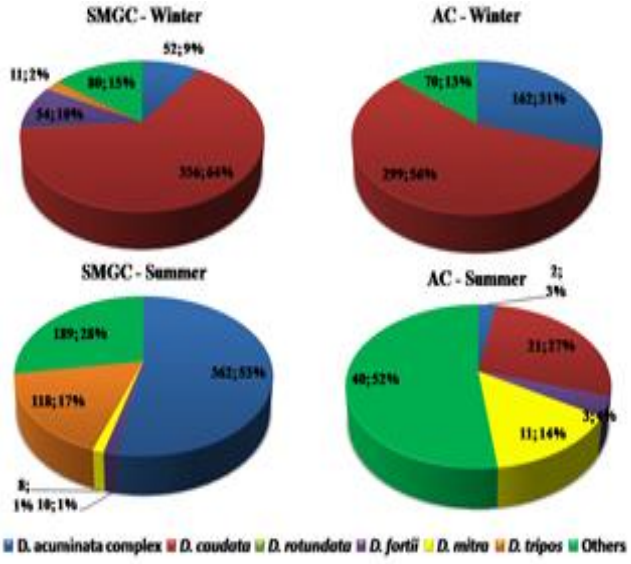

Fig 2. Relative abundance in net plankton of toxic *Dinophysis* species off Santa Marta Grande Cape (SMGC) and Albardão-Chuí (AC) off Southern Brazil, Winter 2005 and Summer 2007.

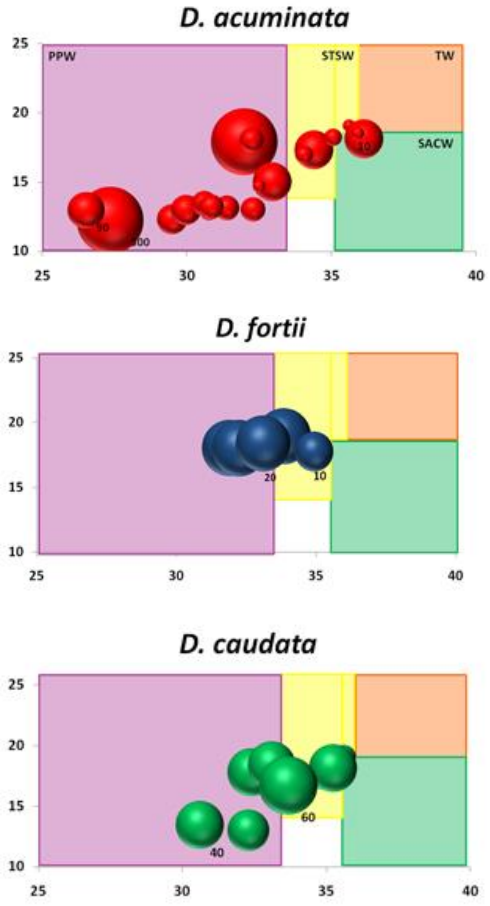

Fig 3. Cell concentrations off the coast of southern Brazil in Winter 2005 and Summer 2007 of *D. acuminata* (a), *D. fortii* (b) and *D. caudata* (c) in T (y axis)- S (x axis) diagrams.

Conclusion

In Southern Brazil, species of *Dinophysis* are clearly associated with the presence of particular water masses. *D. acuminata* was the most important toxic species in winter and summer, occurring primarily in the plume of La Plata River under a wide range of temperature and salinity, but also in STSW and upwelled SACW. *D. fortii* showed a preference for warmer water, while the highest occurrence of *D. caudata* was observed in winter at both sampling localities under a wide range of T and S.

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Spatio-temporal distribution of *Dinophysis* spp. in relation to particulate organic matter and other parameters in Thermaikos Gulf, Greece (Eastern Mediterranean)

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Abstract

Dinoflagellates of the "*Dinophysis acuminata* complex", producing mostly the Diarrheic Shellfish Poisoning (DSP) toxin okadaic acid, are the main for harmful algal blooms (HABs) in Greece. The aim of this study was to investigate *Dinophysis* populations in relation to the availability of organic matter and other parameters in Thermaikos Gulf, north Greece. A network of stations was sampled from November 2002 to March 2004 and the spatio-temporal distribution of *Dinophysis* spp., chlorophyll *α*, inorganic nitrogen and phosphorus, particulate organic carbon and particulate nitrogen were studied. *D.* cf. *acuminata* was dispersed throughout the Gulf and reached high cell numbers. Maximum abundance and toxicity levels (10.7 X 10^3 cells L^{-1} and 63.06 pg OA $cell^{-1}$) were observed during or right after the spring phytoplankton bloom, forming HABs in most of the Gulf area. The spatial and temporal distributions of *D.* cf. *acuminata* were significantly correlated with particulate organic carbon and particulate nitrogen (ANOVA, $p<0.05$, Pearson coefficient 0.56-0.66) but not with chlorophyll *α* or inorganic nitrogen and phosphorus. Blooms of *D.* cf. *acuminata* in Thermaikos Gulf during or after the spring phytoplankton bloom appear to be favored by the high particulate organic matter availability at that time, given the mixotrophic character of this species.

Introduction

Diarrhetic Shellfish Poisoning (DSP) incidents have been reported along the European coast over the last twenty years. The main responsible species have been *Dinophysis acuminata* and *D. acuta* as well as *D. fortii* and *D. sacculus*, which produce okadaic acid derivatives (DSP toxins) and pectenotoxins (Blanco *et al.* 2005). In the Gulf of Thermaikos, north Greece (eastern Mediterranean Sea), cultures of the mussel *Mytilus galloprovincialis* have been increasing since 1990's, reaching 85% of national production and 10 million Euros annual profit (Karageorgis *et al.* 2005). Every year over the last decade, toxic species of the "*Dinophysis acuminata* complex", which morphotypes were shown to be more related to *D. cf. ovum* (Papaefthimiou et al. 2010) have been mostly responsible for shellfish intoxications and long harvest closures in Thermaikos Gulf (Koukaras & Nikolaidis 2004; Pagou 2005). Although increased organic pollution has been documented in the area (Pagou *et al.* 2000), the impact of organic matter availability on *Dinophysis* cf. *acuminata* distribution and variation remains unknown. The aim of this study was to investigate *Dinophysis* populations in relation to organic matter and other biotic and abiotic parameters in Thermaikos Gulf.

Materials and Methods

A network of stations was sampled at the inner part of Thermaikos Gulf from November 2002 to March 2004 (Fig. 1). This is a shallow (max depth <50m) eutrophic coastal area, receiving discharges of five rivers, low water exchange with open sea and high inputs of agricultural, industrial and domestic waste (Pagou *et al.* 2000). The spatio-temporal distribution of *Dinophysis* spp. was studied in the area. Seawater samples (120-150 mL) were fixed with Lugol's solution and examined with light inverted microscopy for the identification and enumeration of *Dinophysis* spp. (Utermöhl 1958). Seawater samples were also collected for the analysis of the following

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parameters: chlorophyll *α* (Holm-Hansen *et al.* 1965), inorganic nitrogen as nitrates, nitrites and ammonium (Strickland & Parsons 1968; Koroleff 1970), inorganic phosphorus as phosphates (Murphy & Riley 1962), particulate organic carbon and particulate nitrogen (Verardo 1990; Cutter & Radford-Knoery 1991). Seawater was filtered on precombusted GF/F filters and only the toxin okadaic acid (OA) was analysed on the cells retained onto the filters by LC/FD, according to the method of Zhou *et al.* (1999), modified by Pyrgaki *et al.* (2010). The software Statgraphics Plus (Statistical Graphics Corp.) was used for the statistical analyses and computations performed.

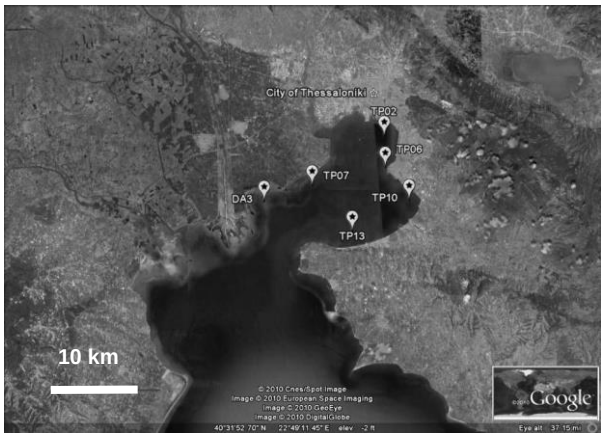

Fig 1. The network of sampling stations in Thermaikos Gulf, north Greece, eastern Mediterranean Sea.

Results and Discussion

The abundance of *Dinophysis* cf. *acuminata* (Fig. 2) ranged from 2.1 to 10.7 10^3 cells L^{-1} and peaked during spring, with maximal values usually in the northern part of the Gulf (station TP02 in Fig. 3). Intracellular toxin content reached 63.06 pg OA $cell^{-1}$ and the mean value was 27.8±6.1 SD pg OA $cell^{-1}$.*D.* cf. *acuminata* increased during and right after the maximum phytoplankton biomass, expressed as chlorophyll *a* (chl *a*, Fig. 4) corresponding to the spring bloom conditions in the area, whereas a lower peak recorded in winter (January), reported also by Koukaras & Nikolaidis (2004). Concentrations of total inorganic nitrogen (N) and phosphorus (P) maximized in winter due to high freshwater inputs in the gulf (Fig. 5). N and P decreased in spring due to consumption by the abundant phytoplankton. N:P ratio was constantly below the Redfield ratio (16:1), indicating an overall N deficiency in the area.

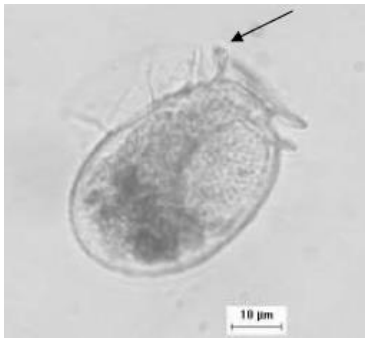

Fig 2: *Dinophysis* cf. *acuminata (*more related to *D. cf. ovum* (Papaefthimiou *et al*. 2010) with a ridge on the anterior cingular list (arrow).

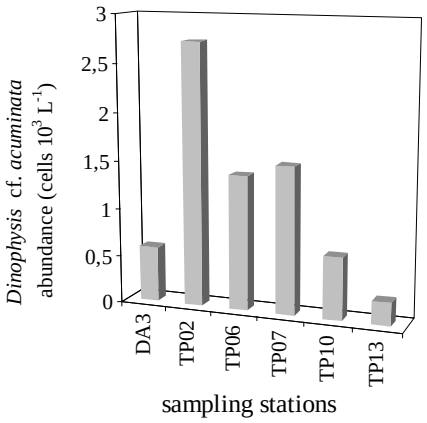

Fig 3. Spatial distribution of *D.* cf. *acuminata* abundance in Thermaikos Gulf (values are means of depth integrated values for each station from all samplings).

The concentrations of particulate organic carbon and particulate nitrogen increased in spring (Fig. 6), followed the increase of phytoplankton biomass (as chl *a*). The spatial and temporal distributions of *D.* cf. *acuminata* were significantly correlated with particulate organic carbon and particulate nitrogen (ANOVA<0.05 Pearson correlation coefficient 0.56 - 0.66) but not with chl *a* or inorganic N, P or N:P ratio. Chl *a* was significantly correlated with particulate organic carbon and particulate nitrogen but not with inorganic N, P or N:P (ANOVA<0.05 Pearson correlation coefficient 0.53 - 0.96) but not with inorganic N, P or N:P.

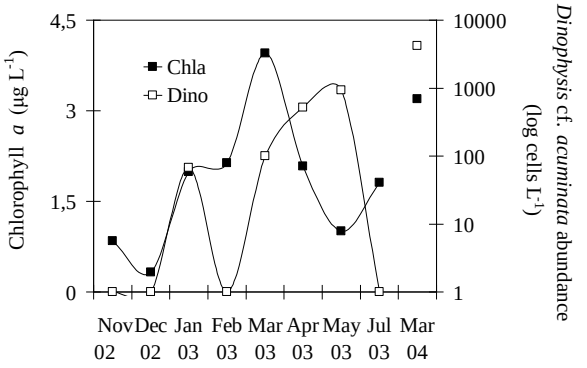

Fig 4. Temporal distribution of phytoplankton biomass (as chl *a*) and *Dinophysis* cf. *acuminata* abundance in Thermaikos Gulf (values are means of depth integrated values from all stations per sampling).

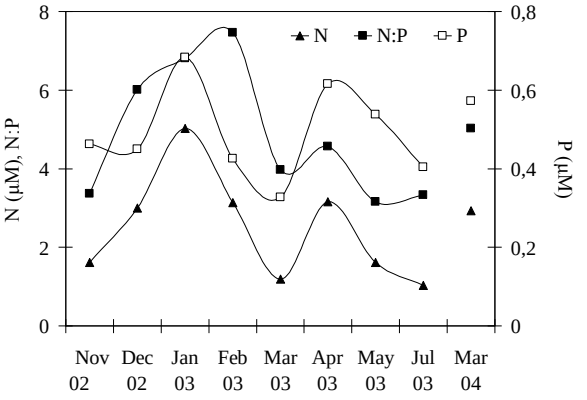

Fig 5. Temporal distribution of total inorganic nitrogen (N), inorganic phosphorus (P) and N:P ratio (values are means of integrated depth values from all stations per sampling).

In conclusion, these results suggest that the increase of *D.* cf. *acuminata* abundance coincide or follow the spring phytoplankton biomass peaks, but despite the mixotrophic character of this dinoflagellate (Granéli *et al.* 1999) the relationship with high organic matter cannot resolve whether this is due to particulate matter or to the high production of dissolved organic matter (DOM) accompanying phytoplankton blooms. However, organic matter may be a crucial component to be considered in ecosystem scale models and prediction tools for *Dinophysis* HABs in Thermaikos Gulf.

Acknowledgements

Financial support was provided by the European Commission through the FATE project ‘‘Transfer and Fate of Harmful Algal Bloom (HAB) Toxins in European Marine Waters’’ (contract EVK3-CT01-00055), as part of the EC-EUROHAB cluster, contract holder E.G.

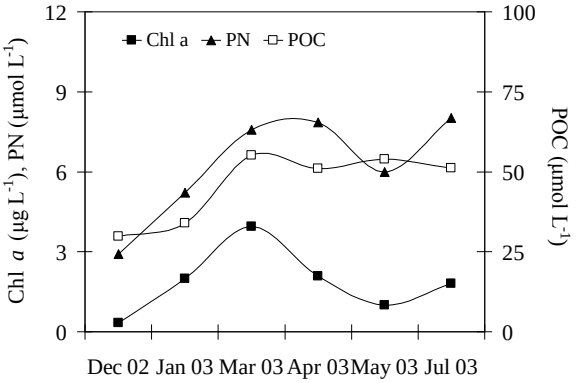

Fig 6. Temporal distribution of phytoplankton biomass (as chl *a*), particulate organic carbon (POC) and particulate nitrogen (PN) concentrations in Thermaikos Gulf (means of integrated depth values from all stations).

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First Report of *Dinophysis tripos* bloom in Norwegian Coastal Waters

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Abstract

The Norwegian Food Safety Authority has since 1992 monitored the Norwegian coast for potentially toxic HAB species. About 50 localities, covering the whole Norwegian coast from the eastern part of the Skagerrak to the Barents Sea, are now sampled weekly, from mid January to the mid of December. Several institutions are involved in the monitoring. Norwegian Institute for Water Research (NIVA) is monitoring the Norwegian west coast and the northern coastal areas. During 2009 *Dinophysis tripos* was observed for the first time in Norwegian waters. It first appeared in four different localities at the west coast in mid August and a week later in the Norwegian Sea (northern Norway). During the following weeks it was detected weekly in these areas, and at the end of September it was also registered in the Barents Sea region. Paired cells were frequently detected indicating that *Dinophysis tripos* actually was growing in Norwegian waters.

Introduction

Several species of the genus *Dinophysis*, i.e. *D. acuminata, D. acuta, D. norvegica* and *D. rotundata*, commonly occur in Norwegian coastal waters. *D. acuta* seems to be the most toxic causing a lot of problems to the shellfish farming industry and having a threshold value of 200 cells/L, or only 100 cells/L during three following weeks. In autumn, *Dinophysis* spp. of a more southern, origin, such as *Dinophysis odiosa*, reached the Norwegian coast transported by Atlantic waters. *Dinophysis caudata* is a less frequent guest, but is not uncommon during autumn (Throndsen et al. 2007). Even though it is a species of warm temperate to tropic waters and rarely found in cold waters, it occasionally occurs, possibly as an intruder in warm water masses (Steidinger & Tangen 1996).

Material and Methods

Norwegian coastal waters have been routinely monitored since 1992. Currently, there are two governmental monitoring programs: The National Monitoring Program for Mussel Production financed by the Norwegian Food Safety Authority and The Norwegian Coastal Monitoring Program "Long term monitoring of environmental quality in coastal regions of Norway" financed by the Climate and Pollution Control Directorate.

Norwegian Food Safety Authority is monitoring the Norwegian coast for potentially toxic HAB species. 40-50 localities, covering the whole Norwegian coast from the eastern part of the Skagerrak to the Barents Sea (Figure 1), are sampled weekly, most of them having a sampling period from the middle of January to the middle of December. Several large institutions are involved in this monitoring. These are Norwegian Institute for Water Research (NIVA), Institute of Marine Research (IMR), and SINTEF. In addition a small company Marine Phytoplankton Consulting (MPC) participates. In this program NIVA is monitoring the Norwegian West Coast and the most northern areas.

Results and discussion

In 2009 *Dinophysis tripos*, originally considered an inhabitant of warm temperate to tropic waters, had a rather massive and long lasting occurrence in Norwegian waters (Figure 2), re-ocurring in 2010. As far as we know, this is the first time this species is observed to bloom in the region. Earlier it has just occasionally been found in colder regions

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(Taylor et al. 1995). *D. tripos* is a rather large *Dinophysis*-species (L: 80-125 µm; D: 50-60 µm). It has been reported as a toxic species containing dinophysistoxin (DTX1) (Lee et al. 1989), but has not been associated with blooms (Larsen & Moestrup 1992). In 2009 and 2010 *D. tripos* was normally found in net-hauls, and occasionally in low numbers in water samples from Norway (maximum 40 cells L^{-1}). During 2009 it first appeared in mid-August at the west coast where it was detected at four different localities. Just a week later, it

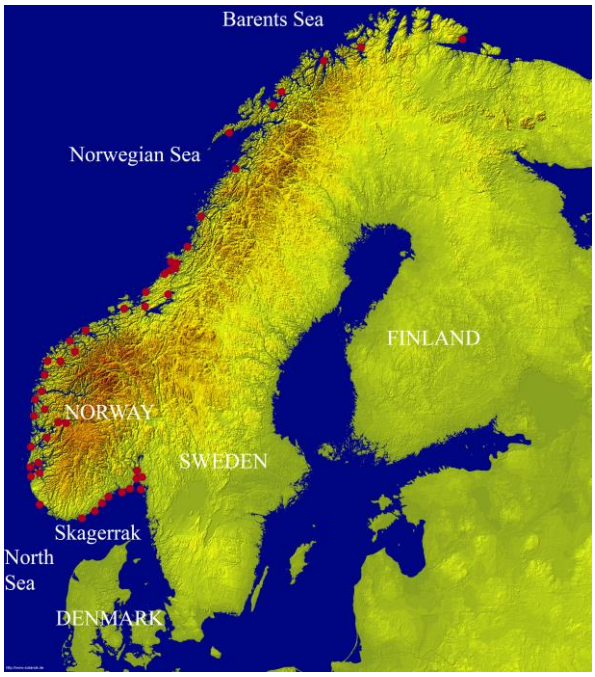

Fig. 1. Map of Norwegian coastal waters. Red circles mark the location of the monitoring stations of The National Monitoring Program for Mussel Production.

also appeared further north in the Norwegian Sea area. During the following weeks it was detected weekly in these areas, and at the end of September it was also registered in the Barents Sea region. Later on it was also detected in the south east part of Norwegian waters, in the Oslofjorden, so *Dinophysis tripos* seemed to be an inhabitant of the whole Norwegian coastal waters during autumn 2009. Paired cells (Figure 2) were frequently detected, suggesting that *D. tripos* was in healthy conditions and even growing in these waters (Reguera et al. 2003). The last detection of *D. tripos* in 2009 was at the beginning of November, after almost four months of occurrence. *D. tripos* reoccurred in 2010. It first appeared at the end of August, and was frequently registered to the end of October. Also in 2010 paired cells were frequently observed.

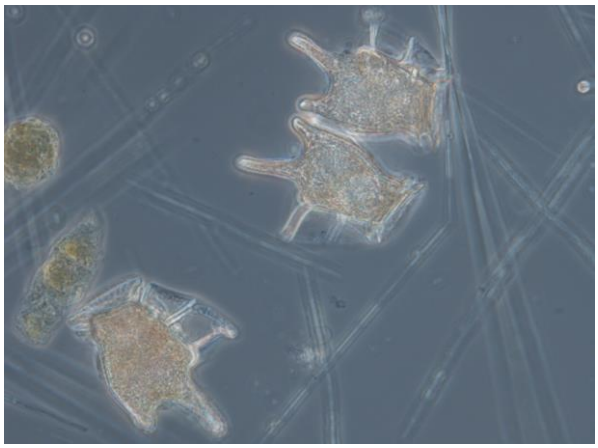

Fig. 2. *Dinophysis tripos*, single and paired cells.

Occurrence of species of a more southern distribution has increased in Norwegian coastal waters the last decades. The diatom *Ditylum brightwelli* occurred 30 years ago occasionally in net samples during autumn. Now in 2010 it is a far more common species in our waters, occurring frequently in water samples, even as a participant of the spring bloom event. Especially during the last decade other species from warmer waters, such as *Neocalyptrella robusta, Pseudosolenia calcar-avis, Peridinium quinquecorne* and *Chattonella globosa*, have been observed possibly indicating that global warming affects the phytoplankton flora of Norwegian coastal waters.

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POPULATION DYNAMICS

Population dynamics of *Alexandrium fundyense* in the Gulf of Maine: outlook for improved management and forecasting

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Abstract

Paralytic shellfish poisoning (PSP) is a recurrent and widespread problem in the Gulf of Maine (GOM) caused by the dinoflagellate *Alexandrium fundyense*. Blooms have been the subject of more than a decade of investigation through the ECOHAB-GOM and GOMTOX research programs. Multiple large-scale field surveys have provided data that were combined with mooring observations, satellite-tracked drifters, and numerical model simulations to document the complex dynamics of *A. fundyense* blooms within this region. A conceptual model of *A. fundyense* bloom dynamics and PSP toxicity in the region is summarized here, highlighting key physiological, behavioral, and environmental or oceanographic factors underlying blooms. A numerical model has also been developed and evaluated against cruise observations and other data. The status of those modeling efforts is discussed, including recent efforts to provide seasonal forecasts of *A. fundyense* bloom magnitude, and near-real time hindcasts and forecasts of use to resource managers.

Introduction

Paralytic shellfish poisoning (PSP) toxicity is a recurrent and widespread problem in the Gulf of Maine (GOM; Fig. 1), affecting vast expanses of the region's nearshore and offshore shellfish (Shumway *et al.* 1988; Anderson 1997). Toxicity is not uniform, but instead reflects *Alexandrium fundyense*[1] growth and toxin accumulation in several separate zones or habitats defined by circulation patterns and the temporal distribution of the dinoflagellate (Anderson 1997). This biogeographic diversity in *A. fundyense* blooms has been the subject of sustained investigation through the ECOHAB-GOM (Anderson *et al.* 2005d) and GOMTOX (www.whoi.edu/gomtox/) research programs. A series of large-scale field surveys provided data that were combined with mooring observations, drifter tracks, and numerical model simulations to document the complex dynamics of blooms within this region.

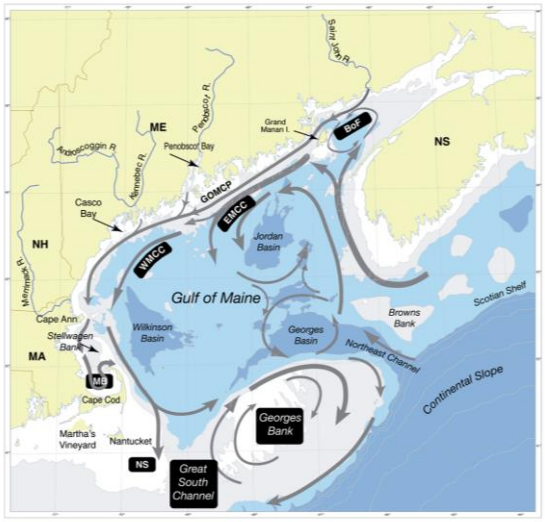

Fig.1. Map of Gulf of Maine showing ocean currents.

[1] Both *A. tamarense* and *A. fundyense* occur in the Gulf of Maine region. We consider these to be varieties of the same species (Anderson et al. 1994; Brosnahan et al. 2010). Neither antibody nor oligonucleotide probes can distinguish between them, and only detailed analysis of the thecal plates on individual cells can provide this resolution. This is not practical for field samples. Accordingly, for this study, the name *A. fundyense* is used to refer to both forms.

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This paper highlights major features and mechanisms underlying the widespread, coastal blooms that can close hundreds of kilometers of coastline for shellfish harvesting. A numerical model that provides realistic simulations of these blooms is also presented, emphasizing progress towards short-term (weekly) and seasonal (annual) forecasts of bloom dynamics and toxicity.

The *Alexandrium fundyense* conceptual model

A dominant feature underlying *A. fundyense* regional bloom dynamics is the Maine Coastal Current or MCC (Fig. 1; Lynch *et al.* 1997) - a composite of multiple segments and branch points. The two major transport features in this system are the eastern and western segments of the MCC, hereafter termed the EMCC and WMCC. Conceptual models of *A. fundyense* bloom dynamics within the MCC have been provided by Anderson *et al.* (2005c) and McGillicuddy *et al.* (2005).

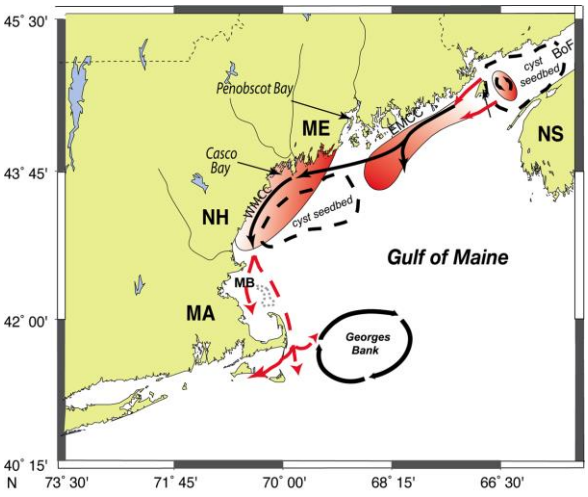

Fig.2. Map of cyst beds.

Key features in the models are two large cyst "seedbeds"- one in the Bay of Fundy and the other offshore of mid-coast Maine (Fig. 2; Anderson *et al.* 2005c). Cysts germinate from the BOF seedbed, causing recurrent coastal blooms that are self-seeding with respect to future outbreaks in that area. In effect, the BOF is an incubator for localized populations in that area, but the incubator is leaky, as cells escape into the EMCC, where they bloom, particularly at the distal end of that coastal current, where waters warm and stratify (Townsend *et al.* 2001). Some cells travel south and west with the EMCC, while others deposit cysts in the mid-coast Maine seedbed. In subsequent years, these latter cysts (combined with vegetative cells from populations within the EMCC) inoculate WMCC blooms that cause toxicity in western portions of the Gulf and in offshore waters as well. Toxicity in southerly and western regions of the GOM such as Massachusetts Bay is regulated by coastal current transport, with northeasterly winds accelerating the alongshore and cross shor movement of the populations (Anderson et al. 2005a).

Hindcasting and forecasting efforts

A coupled physical/biological model of *A. fundyense* population dynamics in the Gulf of Maine has been developed that is consistent with the above conceptual model (e.g., McGillicuddy *et al.* 2005; Anderson *et al.* 2005c; He *et al.* 2008; Li *et al.* 2009). The model is initiated from large-scale maps of cyst distribution, with germination rates parameterized through laboratory experiments. Likewise, the growth of the resulting vegetative cells is regulated by light, temperature, and salinity, again parameterized using laboratory cultures. In a novel application of this model, observations were combined with model hindcast simulations to identify the dominant factor leading to a 2005 *A. fundyense* bloom considered to be the largest in at least three decades (He *et al.* 2008). Anderson *et al.* (2005b) proposed three factors to explain the historic 2005 outbreak: 1) high abundance of resting cysts that provided a large inoculum; 2) storms with strong northeast winds that carried toxic cells towards, and along the coast; and 3) abundant fresh water runoff, providing macro- and micro-nutrients, a stratified water column, and an alongshore (towards the southwest) transport mechanism. These factors were evaluated using a sensitivity analysis that utilized field observations in the *A. fundyense* population dynamics model (He *et al.* 2008). A snapshot from the 2005 hindcast simulation that used the 2004 cyst data (hereafter termed the central hindcast) illustrates the bloom's regional-scale characteristics (Fig. 3A). Recently germinated cells swimming upward from the western GOM and BOF cyst seedbeds are evident in vertical transects. Germinated cells inoculate the coastal current system,

which flows from northeast to southwest and then spreads offshore in the south. Large-scale characteristics of the simulation are generally consistent with field observations.

Initial conditions of the three sensitivity experiments were identical to the central hindcast in all respects except: experiment 1 utilized the 1997 cyst map instead of 2004; experiment 2 was forced by winds from a more typical year (2004) instead of the strong downwelling-favorable winds of 2005; experiment 3 used riverine discharge from a typical year (2004) instead of the anomalously large discharge of 2005.

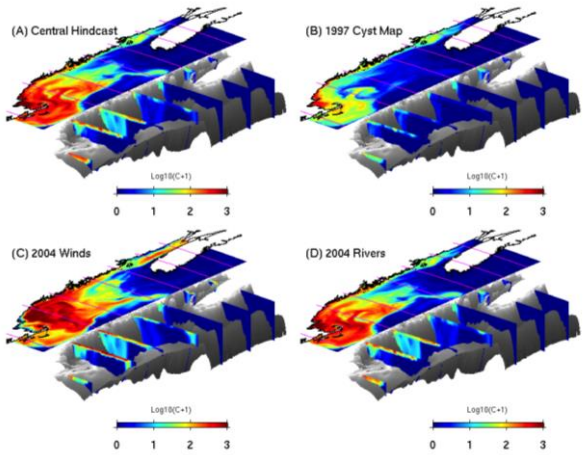

Fig. 3. Bloom predictions based on a. hind casting;b.cyst beds; c. wind forcing; d. riverine discharge.

This sensitivity analysis suggested that high cyst abundance in the WGOM was the main cause of the 2005 bloom. Wind forcing was an important regulator, in the form of both episodic bursts of northeast winds and the downwelling-favorable mean condition, causing onshore advection of offshore populations. Anomalously high river runoff enhanced alongshore transport near the coast. These and other results demonstrate that model simulations initiated from *A. fundyense* cyst distributions capture large-scale seasonal patterns in the distribution and abundance of vegetative cells. Cyst abundance is a first-order predictor of regional bloom magnitude the following year in the WGOM, suggesting that cyst abundance may hold the key to interannual forecasts of PSP severity, recognizing that other factors will determine the extent of population growth and delivery to shore. This is a major finding that is of significant importance in terms of bloom management and forecasting in the region.

Weekly and annual forecasts

The model has been used to produce near-real-time quasi-operational nowcasts and forecasts for 2006 - 2010. Each of these synoptic simulations was initiated using a regional map of *A. fundyense* cyst abundance in the GOM (e.g., Anderson *et al.* 2005c) obtained in the winter before the next bloom season. Each year, weekly model updates were made available to a listserve of more than 150 managers and other officials and scientists involved with PSP outbreaks in the northeastern US. These weekly updates allowed the listserve members to go to a website where they could view the latest model simulations of that year's *Alexandrium* bloom, extended one week forward in time using weather forecasts. An example forecast can be seen at http://omglnx3.meas.ncsu.edu/yli/08forecast/dino_08.htm. Forecasts were also sent to researchers at sea to aid in the planning of sampling activities. Readers are also encouraged to visit the forecasting web site cited above to scrutinize the comparisons between simulated and predicted *A. fundyense* concentrations in 2008 as one example of the skill of the model. Other analyses of model skill are given in Stock *et al.* (2005) and He *et al.* (2008). The reception for this information has been highly positive, as it gave managers a view of the entire bloom in the Gulf through weekly updates during the bloom season. This information was complementary to shellfish toxicity measurements made on a weekly basis at scattered locations along the coast. Seasonal or annual forecasts have also been made. This effort began when a cyst survey in late 2007 revealed that cyst abundance offshore of mid-coast Maine was 30% higher than in fall 2004, just prior to the historic bloom of 2005. Those cyst maps are shown in Fig. 4. The 2008 field season thus offered an exceptional opportunity for testing the hypothesis that the magnitude of the bloom in the western Gulf of Maine is set by the abundance of resting cysts. In advance of the bloom season, the coupled physical-biological model was used to make a seasonal

forecast using an ensemble of scenarios based on archived hydrographic simulations from 2004-2007 model runs.

The ensemble forecast was made available to resource managers on the web at http://omglnx3.meas.ncsu.edu/yli/simulation_new/08forecast/dino_08.htm. The simulations were initialized with zero cell concentration throughout the domain and the cyst map prescribed from fall 2007 observations. Each member of the ensemble was based on the hydrodynamic hindcast for each specific year, which affected the abundance and distribution of *A. fundyense* cells through environmental influences on germination, growth, mortality, and transport. Although the hindcasts for 2004-2007 did not span the range of all possible outcomes, they provided contrasting conditions including one with strong downwelling-favorable winds and anomalously high river discharge in May (2005) and one with near climatological conditions (2004). They also spanned the range from major PSP outbreak (2005) to moderate (2006, 2007) to low (2004) levels of regional toxicity.

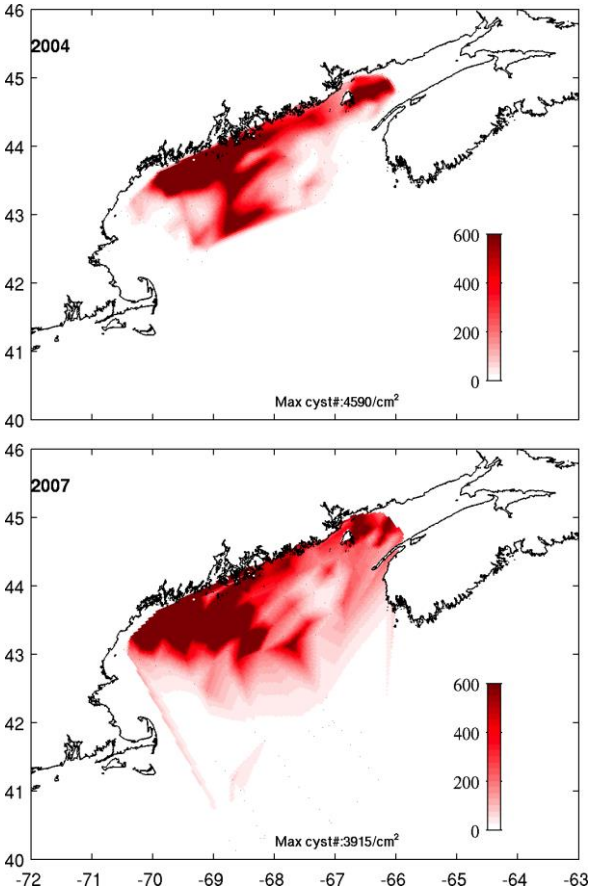

Fig.4. Cyst maps for 2004 and 2007.

All of the simulations indicated a severe bloom in the western GOM, on par with the historic bloom of 2005. A press release was issued (http://www.whoi.edu/page.do?pid=9779&tid=282&cid=41211&ct=162). This information was used by resource managers in staffing decisions in advance of the bloom and was seen by many as a major factor in the controlled and moderate response of the public and press during the outbreak, and thus in the reduced economic impacts compared to the 2005 event. The seasonal forecast was confirmed when a major bloom occurred, extending from Maine through New Hampshire and much of Massachusetts, leading to federal emergency assistance to these three states because of the "failed fishery".

This seasonal forecast of the 2008 outbreak is a major breakthrough, as it represents the first prediction of a red tide or HAB on a regional scale, and speaks to the advanced nature of our understanding of the *A. fundyense* bloom dynamics in the GOM, and to the sophistication and accuracy of our numerical model.

For 2009, a "moderately large" outbreak was forecast, based on the cyst abundance observed in fall, 2008 (http://www.whoi.edu/page.do?pid=24039&tid=282&cid=56567). This forecast was generally accurate, since the toxicity was more limited in scale than in the previous year, extending only to the middle of Massachusetts Bay. However, a resurgence of toxicity in June and July occurred in Maine, leading to very high and prolonged toxicity in that state. This second wave of toxicity could not have been anticipated in the seasonal forecast, and reflected unusual wind patterns in June and July.

For 2010, the forecast that was issued was similar to that for 2008– i.e., a. "significant" *A. fundyense* bloom was anticipated since even more cysts were documented in late 2009 than were present in 2007, immediately before the large-scale 2008 outbreak (http://www.whoi.edu/page.do?pid=24039&tid=282&cid=69586). This forecast was, however, not borne out by the subsequent bloom that year. Relatively small sections of the Maine coast were closed because of toxicity, with no closures in coastal New Hampshire or Massachusetts. GOMTOX

research cruises documented very low *A. fundyense* cell abundances in both nearshore and offshore waters of the GOM, so the issue was not a lack of onshore transport, but rather the overall lack of a bloom. Our working hypothesis is that a mesoscale GOM water mass change occurred that lies outside the envelope of observations from the six years used as the basis of the 2010 ensemble forecast.

This hypothesis is currently being evaluated using GOMTOX survey data for 2010, satellite measurements of ocean color, as well as moored observations from instrumented buoy networks (McGillicuddy *et al.* in prep.) Preliminary analyses suggest that the deep basins of the GOM were fresher and warmer than was observed in prior years. This water mass anomaly would have affected intermediate and surface waters, the latter being where *A. fundyense* resides. For example, surface waters were several degrees warmer than in 2008, when a large *A. fundyense* bloom took place. Stratification, nutrient concentrations, grazers, and other factors critical to *A. fundyense* growth could all have been affected. Should we be able to deduce the mechanisms responsible for the lack of a bloom in the WGOM in 2010, those processes could then be included in the population dynamics model. Furthermore, we note that the water mass changes mentioned above relate to the large-scale circulation of the northwest Atlantic, and therefore are observable months in advance of the *A. fundyense* season using moored instruments in ocean observing systems. Therefore, it is conceivable that forecasts can be made taking into account this type of variability. It is indeed fortunate that GOMTOX cruises were scheduled for 2010, as this will allow us to understand the factors that prevented a bloom and thereby allow us to improve our model.

Overview

The conceptual and numerical models described herein are a result of more than a decade of detailed study of *A. fundyense* dynamics over a large area in the GOM. The models are extraordinarily useful research and management tools that help to guide decisions about closures and re-openings of harvest sites, support forecasts and predictions that are of use to shellfish industry and resource managers, and that in general, provide a context against which blooms and toxicity observations can be viewed. Looking back, one can highlight the information needs and analytical approaches that can help other countries or regions develop similar models for HABs and their waters. First and foremost, one needs a detailed understanding of the hydrography of the area under investigation, including adjacent waters that influence the localized flows. Major current systems need to be identified and characterized, as well as the episodic movements of water associated with storm runoff, upwelling, downwelling, and other factors. Moored instruments and survey cruises are needed to characterize this hydrography to provide data to numerical models that are critical in the development of an understanding of HAB dynamics. Initially, the numerical models should focus entirely on the physics of the region, but ultimately, biological elements can be added (e.g. Stock *et al.* 2005) that can be very useful in understanding HAB dynamics. For a cyst-forming HAB species like *Alexandrium fundyense*, much of the biological model formulation has already been accomplished, and can be adapted to the strains of this or related species in a different area following laboratory studies to derive growth rate and germination rate as a function of temperature, light, and salinity. More sophisticated efforts might include nutrient uptake kinetics, as this can be useful in forecasting the decline of blooms in the locations where cysts will be formed and deposited. This is important as the initial condition for physical/biological models for cyst-forming species. Grazing may need to be considered as well, but this is a difficult issue to parameterize in any detail. In our formulations, we have utilized a mortality rate that varies with temperature according to a Q_{10} formulation (He *et al.* 2008). It is simplistic, but thus far, does an adequate job with bloom termination judging from the match between our simulations and observations. This is one example where a simple approximation can replace a complex submodel and still provide acceptable simulations.

Another key feature in the development and application of conceptual models like that

described here is the documentation of the nutrient environment that the HAB species will occupy. Survey cruises will provide large-scale snapshots of the nutrient fields, but these change constantly, and are quickly out of date. We have found it useful to utilize "climatological" or long term average nutrient fields for the modeling efforts. These have been derived for the GOM region on the basis of numerous shipboard surveys conducted throughout the years, with those data being compiled and related to parameters such as temperature and salinity. The development of climatological nutrient fields is thus an important priority for those wishing to develop models in a particular region. However, as demonstrated in 2010 in the GOM, there may be years in which the nutrient fields differ dramatically from the climatology

Our numerical model for *A. fundyense* in the GOM will undoubtedly be refined and modified through time. In its present form, however, it is already proving very useful as a management tool and as a means to communicate the nature of the HAB phenomenon to the public, the press, and to agency officials. Development of such models for HABs in other regions requires a systemic approach whereby the key hydrographic and biological features of the system are identified, characterized, and ultimately modeled. Conceptual models and numerical models are best formulated in parallel, as each provides information and insights to the other. Effective management and mitigation of HABs are greatly facilitated by these efforts.

Acknowledgements

This work was supported by grants from the ECOHAB (NOAA Grant NA06NOS4780245) program and the Woods Hole Center for Oceans and Human Health (NSF Grants OCE-0430724 and OCE-0911031**;** NIEHS Grant P50ES012742-01. This is contribution number xxx from the ECOHAB program.

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Interannual variability in *Alexandrium* spp. cyst densities in Cork Harbour, Ireland and it's relation to bloom intensity

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Abstract

The distributions of *Alexandrium* cysts in the sediments of Cork Harbour, Ireland, have been investigated since 2003. Cyst densities of *A.minutum* and *A.tamarense* were examined during the winter dormant season in the North Channel of Cork Harbour, where blooms are known to initiate. The means and variances of the horizontal cyst distribution in the top 1 cm of sediment in the North Channel were compared between years, and the data were also analysed in conjunction with the maximum observed vegetative cell densities in subsequent summers. The results show a decreasing trend in cyst densities since 2003. An analogous decreasing trend in the maximum observed bloom cell density was also apparent since 2004 when an exceptional *Alexandrium* bloom ($5*10^5$ cells l^{-1}) occurred. The potential of the winter cyst density in controlling the intensity of summer blooms is discussed.

Introduction

Shellfish aquaculture is a significant component of the economy of the southwest coast of Ireland (Parsons 2006). The occurrence of Harmful Algal Blooms has hindered the development of the industry, in particular through contamination of shellfish with algal biotoxins (McMahon and Silke 1998). Cork Harbour, Ireland's most industrialised harbour located on the southern Irish coast, has a history of episodic contamination of shellfish with Paralytic Shellfish Toxins, and blooms of *Alexandrium* spp. can occasionally reach high cell density (ca. 10^3 ml^{-1}; Ni Rathaille 2007). The first documented outbreak occurred in 1987, and subsequently there have been frequent bans on shellfish harvesting in the area. Notable exceptions have been the years 1999 and 2001. Cork Harbour is the only site in the Republic of Ireland where PSP contamination has occurred, and toxin contamination usually occurs between mid-June to mid-July. The *Alexandrium* genus is large (~28 species) and to date *A. minutum*, *A. tamarense* and *A. ostenfeldii* have been identified in Cork Harbour (Touzet *et al*. 2008). It was originally thought that *A. tamarense* was the causative organism of these toxic events. However, toxin profiles of cultures isolated from the estuary have indicated that the causative organism is *A. minutum* (Touzet *et al*. 2007a). Isolates had a toxin profile of the two potent gonyautoxins GTX2 and GTX3 which have been characteristic of contaminated stock (Furey *et al*. 1998; Touzet *et al*. 2007).

The life cycle of *Alexandrium* has a resting cyst stage, in which state it can overwinter or stay dormant for several years in adverse conditions (Anderson *et al.* 1997). Excystment into the planktonic vegetative form initiates *Alexandrium* blooms. Knowledge of the distribution and cyst density is therefore potentially vital information if the effects of toxic *Alexandrium* blooms are to be managed and mitigated. This study examined the variation in the distribution and density of *Alexandrium* cysts in Cork Harbour since 2003.

Materials and Methods

Cyst surveys were carried out in the north-eastern section of Cork Harbour known as the North Channel (Figure 1). It is in this part of the estuary, which has dimensions 8 km by 0.5 km, where *Alexandrium* blooms initiate and where the highest dinoflagellate cyst densities can be found (Ni Rathaille 2007). Sub-tidal sediment samples were obtained using a manually operated Ekman Birge bottom sampler (HydroBios, Kiel). Triplicate sub-

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samples of surface sediment were taken from this using sawn off 50 ml syringes that were filled to the depth of the sediment in the sampler. Intertidal samples were collected from the shoreline manually, also using 50 ml core syringes. The sediment was kept in the dark at 4º C until further analysis in the laboratory. In order to quantify the number of cysts in the sediment, the surface 1 cm of each sediment core was removed, mixed to ensure homogeneity, and from this a measured, weighed volume (usually 0.4 ml) was put into a beaker of water, ultrasonicated, and the cysts were extracted using density gradient centrifugation with sodium polytungstate. Prior to 2007 Ludox was used for the density gradient. The number of *Alexandrium* cysts was counted using a Sedgwick-Rafter cell at x100 magnification. Cyst surveys were carried out between mid-September and mid-April when *Alexandrium* can be expected to be in the dormant phase (Table 1).

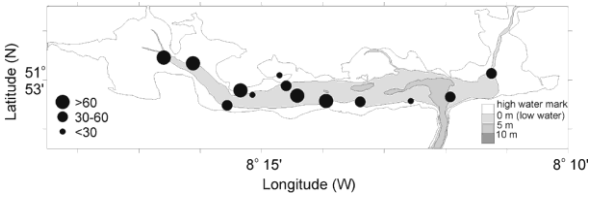

Fig. 1. Distribution of *Alexandrium* cyst densities, North Channel, April 2010. Units are in cysts g dry sediment^{-1}.

Results

The most recent survey carried out on 6-7 April 2010 involved thirteen sampling sites, three of which were intertidal (Figure 1). Cyst densities of *A. tamarense* ranged from 5-20 cysts . g dry wt sediment^{-1}. Cyst densities of *A. minutum* were more variable and generally higher in the range of 5-80 cysts . g dry wt sediment^{-1}. Higher cyst densities were found in the western (inner) end of the North Channel (Figure 1). Mean cyst densities of *A. tamarense* and *A. minutum* were 11 and 38 cysts g^{-1} respectively. These data can be compared with those obtained during the dormant season in previous years (Table 1). A decline in *A. tamarense* and *A. minutum* cyst densities is evident in years proceeding 2003. Results from the annual surveys identified six locations containing notably increased cyst densities (>mean + 2*s.d) during the annual dormant season (Figure 2). These were located in the regions of the North Channel with the most retention near the furthest east and west of the channel and in a lagoon situation along the north shore. The other three locations were where gyres exist on ebb and flood tides near the mouth of the channel. Cysts can be regarded as passive, positively buoyant particles within the water column, and might be expected to form 'seed shadows', analogous to terrestrial systems (Wyatt 2003).

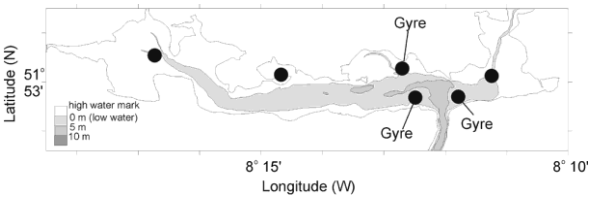

Fig. 2. Locations where increased *Alexandrium* cyst densities have been recorded, North Channel, Cork Harbour.

Thus characteristic water movement patterns could influence the distribution of the cysts within the sediment. Figure 3 illustrates the variation with time in the mean observed *Alexandrium* resting cyst densities in the North Channel. It is evident that densities never achieved as great a value as was found in the winter of 2003/2004. *A. minutum* cyst densities of up to 1680 cysts . g dry wt sediment^{-1} were found during the winter of 2003/2004, with a mean value of 437 cysts . g dry wt sediment^{-1}. In the following September, after the summer bloom, the highest count recorded for this species was 301 (mean 88) cysts . g dry wt sediment^{-1}. A similar pattern was observed for *A. tamarense* cyst densities, although counts were relatively lower than *A. minutum* (Table 1). Maximum and mean recorded densities of 956 and 302 cysts . g dry wt sediment^{-1} in the winter of 2003/2004 fell to 239 and 54 cysts . g dry wt sediment.$^{-1}$ respectively in September of 2004.

When these data were plotted against observed *Alexandrium* vegetative cell densities, the decrease in *Alexandrium* cyst densities corresponded with a decrease in the vegetative cell densities after the winter period of 2003-2004. A bloom of 620,000 cells l^{-1} was observed in the western half of the N. Channel in the summer of 2004. Yet during the same period in 2005, the maximum count recorded was only 31,000 cell l^{-1}. With the exception of the year 2009 cell densities were recorded annually, but never peaked above 55,000 cells^{-1}.

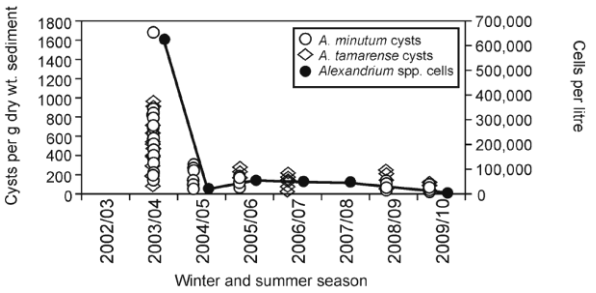

Fig 3. *A. minutum* and *A. tamarense* cyst densities and *Alexandrium* spp. bloom cell densities, North Channel, Cork Harbour 2003-2010.

The data derived from four subsequent winter cyst surveys and summer bloom studies all showed low densities of both species (Table 1; Figure 3).

Table 1. *A. tamarense* and *A. minutum* cyst density data (cysts/g dry sediment), North Channel (2003-2010).

Dormant Season	Date	*A. tamarense*			*A. minutum*		
		max	mean	sd	max	mean	sd
2003/2004	22-25 Oct 2003 9-12 Mar 2004	959	302	232	1680	437	325
2004/2005	21 Oct 2004	239	54	77	301	88	91
2005/2006	16 Sept 2005	264	88	85	175	96	64
2006/2007	3-5 Oct 2006	213	75	51	-	-	-
2008/2009	Oct 2008	247	108	69	140	39	32
2009/2010	6-7 Apr 2010	20	11	5	76	38	23

Discussion

The monitoring of HABs is crucial in order to provide enough information to predict bloom events and, if possible, their intensity. There are, however, relatively few time series of cyst surveys. In the Gulf of Maine, studies have been carried out on *Alexandrium* blooms for over two decades. In 2005, a massive *A. fundyense* bloom occurred there. It has been suggested, through modelling, that the bloom intensity was linked to an unusually high cyst density, which had been measured the previous autumn (He *et al*. 2008). Furthermore, these observed high cyst levels reflected deposition from an autumn bloom of *A. fundyense* that occurred in late 2004 (Anderson *et al*. 2005). A parallel case can be made for the situation in Cork Harbour. In September 2003, cell densities of *Alexandrium* of the order 20,000 cells l^{-1} were observed in samples in the North Channel (Ni Rathaille 2007). Such levels would be unusually for this time of year. The late summer bloom could have deposited fresh cysts causing increased densities in the sediment, as measured in October of that year, which then promoted a large bloom the following spring. No September *Alexandrium* bloom has however been observed since, and cyst densities have remained low. On the other hand, the 2004 *Alexandrium* bloom is known to have provided a substantial number (ca. 5000 cm^{-2}) of cysts to the western section of the North Channel through direct measurement with sediment traps (Ni Rathaille 2007). Such high levels were not evident in the autumn cyst survey of 2004. This could have been due to their burial, export or some may even have been lost through germination after a mandatory dormancy period. Increasing the cyst density by a factor of two in a model of *Alexandrium* blooms in Hiroshima Bay did not alter the bloom intensity but did bring the timing forward by about a week (Yamamoto and Seike 2003). It might be expected that if environmental conditions were favourable then planktonic cell numbers would be more influenced by the speed of vegetative reproduction than by excystment rate. If the size of the inoculum of cells (provided by excystment) is not important, then substantial blooms could occur in any year. There has not however been a substantial bloom of *Alexandrium* in Cork Harbour since 2004.

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Influence of environmental variables on *Alexandrium catenella* motile stage and other harmful taxa in southern Chile (43° - 55° S) (January – December 2009)

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Abstract

Phytoplankton records during the last decade in Southern Chile (41º-55º S) show that *Alexandrium catenella* blooms are recurrent, have an annual frequency in the area between 43º50’ and 55º00’, but sporadic (2002, 2006 y 2009) between latitudes 42º20’and 43º20’. These blooms are always associated with the presence of Paralytic Shellfish Poison (PSP) in molluscs. The temporal and geographical distribution by *A. catenella* during each event seems to be associated with the characteristics of different sectors of the study area. Remarkable hydrographic and meteorological differences occur in this vast geographical area. Based on biological, toxicological, hydrographic and meteorological information generated during 2009, the structure and interrelationships of recorded variables are analyzed to identify key variables linked to *A. catenella* blooms, which will serve to generate explanatory models for these phenomena.

Introduction

Phytoplankton records during the last decade in the fjords of Southern Chile (41º-55º S.L.) show that *Alexandrium catenella* blooms are recurrent, have an annual frequency between 43º50’ and 55º00’ S, but occur sporadically (2002, 2006 and 2009) along latitudes of 42º20’and 43º20’. These blooms are always associated with Paralytic Shellfish Poisons (PSP) in sentinel molluscs (Guzmán et al., 1975, 2007, 2009). The temporal and geographical distribution of *A. catenella* during each event seems to be associated to the features of different sectors of the study area. Remarkable hydrographic and meteorological differences can be found in this vast geographical area. At least three important physical phenomena that affect *A. catenella* abundance and distribution have been identified. The first refers to abundance gradients of this dinoflagellate, frequently found in fjords and channels connected to the continental shelf, showing an abundance increase from the head to the mouth of the fjord (Guzmán et al. 1975), the second is related to dispersion phenomena forced by winds, tides and currents that spread the motile stage of this organism, and the third is associated with advection phenomena and shear probably

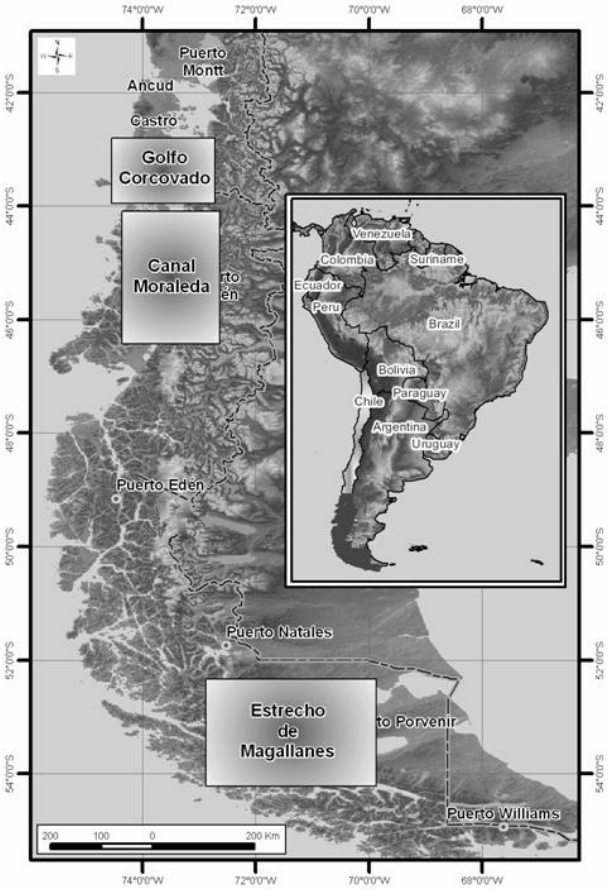

Fig. 1. Sampling area. forced by winds, tides and currents that lead to a local higher dinoflagellate

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concentration generating toxicity hotspots, such as the Magellan region (Guzmán et al., 2002, 2009).

Material and Methods

During 2009 27 biological, toxicological, hydrological and meteorological variables were monitored under a quasi-monthly sampling scheme along 151 stations located in southern Chile between 41° and 55° S (Fig. 1). Quantitative phytoplankton was obtained by hose sampling from two layers (surface to 10m and 10m to 20m depths) and qualitative phytoplankton was sampled by vertical hauls between surface and 20m using a 23µm net. Toxicological information was obtained from bivalves by mouse bioassay (PSP and DSP) and HPLC (ASP). Hydrographic information and oxygen were obtained by CTD and meteorological data were measured with a thermal anemometer and weather stations. The data were sorted, transformed to base 10 logarithms and analyzed using principal components analysis (PCA) without rotation. The study area was divided into 3 sectors: Corcovado Gulf (37 variables), Moraleda Channel (35 variables) and Strait of Magellan (16 variables). Variables were: Relative abundances of *A. catenella*, *Dinophysis acuta*, *D. acuminata*, *Pseudo-nitzschia cf. australis*, *P. cf. pseudodelicatissima*, *A. ostenfeldii* and *Protoceratium reticulatum*, besides G Index, phytoplankton species richness, transparency, air temperature, air pressure, wind speed, cloudiness, wind direction, PSP, diarrhetic shellfish poison (DSP), surface, 5m, 10m and 20m water temperatures, salinities and oxygen concentrations, and phytoplankton abundance.

Results and Discussion

PCA results show that the proportion of the explained variance for the first two components in the three analyzed situations is between 39.5 and 49.8%. However the eigen values of the significant variables are above 0.75 reflecting a strong association between them. The principal PCA results are summarized in Table 1 and Figs. 2-4.

Table 1. Principal Component Analysis statistics.

% explained variance first component	% explained variance second component	Determinant correlation	Kaiser-Meyer-Olkin
Corcovado Gulf			
24,99	18,67	$7{,}84 * 10^{-5}$	0,73
Moraleda Channel			
26,93	12,61	$6{,}56 * 10^{-10}$	0,77
Strait of Magellan			
31,69	18,15	$2{,}11*10^{-19}$	0,81

Results for the analyzed sectors show that air and surface water temperatures are correlated with phytoplankton and consequently with harmful phytoplankton abundance. This correlation is primarily due to higher irradiance during Spring and Summer. In contrast, surface salinity shows a close association with species richness and phytoplankton abundance.

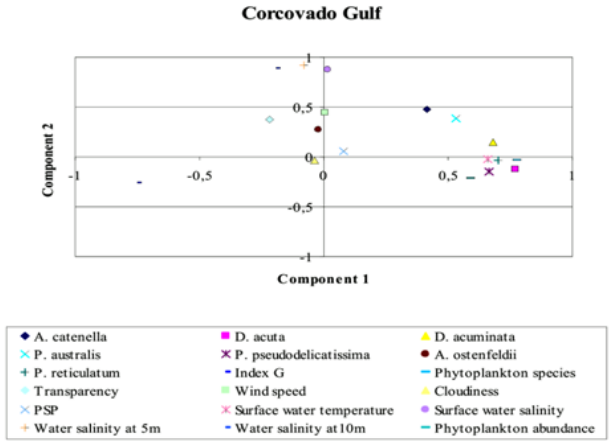

Fig. 2. Results of principal component analysis applied to information of Corcovado Gulf.

At a more localized level, the Corcovado Gulf show that surface water temperature is associated with species richness and phytoplankton abundance, but not always with harmful taxa abundances, since *Pseudo-nitzschia* species show a stronger relationship, in contrast to dinoflagellates speciesfor which this relation is less clear. Salinity is associated to microalgal diversity. During Spring and Summer, along the Moraleda Channel the air temperature is related to water temperature and favours freshwater inputs due to snow and ice melting, affecting surface salinity and water transparency due to particulate matter input, but transparency is also affected by phytoplankton abundance. Apparently, freshwater runoff

favours also the phytoplankton development, as is shown by PCA reflecting an increase of species richness and phytoplankton abundance, including an increment of harmful taxa concentrations. As was expected *A. catenella* relative abundance segregates together with PSP concentrations.

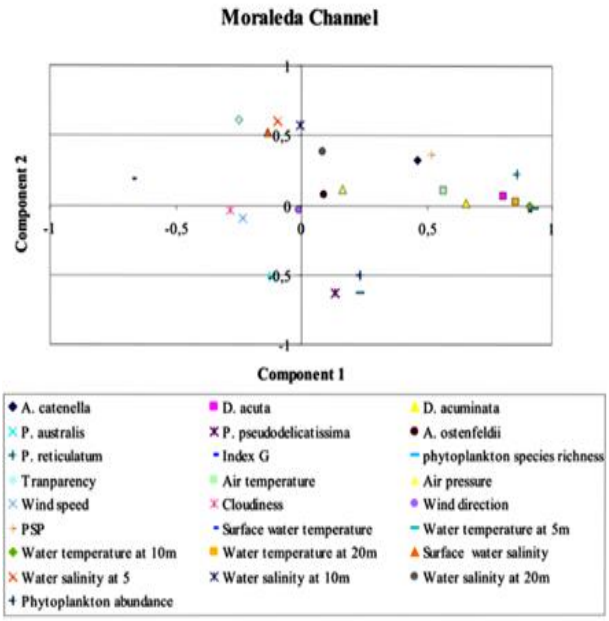

Fig. 3. Results of principal component analysis applied to information of Moraleda Channel.

In the Strait of Magellan, although temperature shows the narrowest variability, the air and water temperatures are positively correlated to species richness and phytoplankton abundance, as well as, to the relative abundances of *A. catenella*, *D. acuminata*, *P. cf. australis* and *P. cf. pseudodelicatissima*.

Conclusions

The meteorological and hydrographic forcing affects the geographical distribution of *A. catenella* and also other harmful species, with air temperature and water temperatures the most important. The greatest abundance of *A. catenella* occurs when the rest of the phytoplankton is also more abundant.

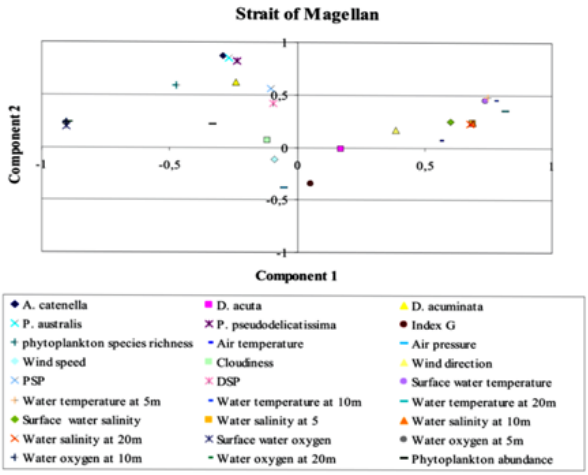

Fig. 4. Results of principal component analysis applied to information of the Strait of Magellan.

Acknowledgements

Study financially supported by the Fisheries Undersecretary of Chile.

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Spatial and temporal variability of *Alexandrium catenella* and PSP in southern Chile (43° - 55° S) (May 2006 – July 2010)

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Abstract

Since 1972 *Alexandrium catenella* and Paralytic Shellfish Poison (PSP) are known from Magellan Strait, being sporadic and restricted to the Magellan region until 1990. Afterwards, recurrent annual blooms and PSP outbreaks and a northern expansion occurred. *A. catenella* was detected in the Aysén region in 1992 and in 1998 in the southeast of Chiloé Island. In October 2009 it reached its most northern distribution at Calbuco (41°48'S; 73°10'W) and was cited at the Pacific coast of Chiloé Island. The blooms - PSP outbreaks - show different annual patterns encompassing vast geographical areas. In its northern area of distribution, they are sporadic, reaching densities of 1,000 cells l^{-1} and a toxicity of 1,419 µg STX eq. 100 g^{-1} shellfish meat. In the southern area, densities have been up to 53,800 cells l^{-1}, toxicity has reached 27,159 µg, nevertheless the highest densities, 1,132,200 cells l^{-1}, the highest relative abundances and the greatest mean PSP levels have been observed in the region of Aysén. The interannual variability and its wide geographic coverage, including sectors and periods with higher probabilities to detect the microalga and PSP, suggest that the bloom initiations and PSP outbreaks are of climatic - oceanographic origin.

Introduction

Since October 1972 *Alexandrium catenella* blooms and PSP outbreaks are known for the Magellan region (Guzmán et al., 1975a). Since the nineties, blooms increased in frequency, annual toxicity outbreaks occurred and an expanded distribution to the North was observed. The microalga was detected in the region of Aysén in 1992 (Muñoz et al., 1992) and PSP in 1994 (Guzmán et al., 2002) and in the region of Los Lagos, it was detected in 1998 in the southeast area of Chiloé Island (Lembeye et al., 1998) and PSP detected in 2002 (Mardones et al., 2010). Today this microalga is present along the fjords between 41° and 55° S, but PSP encompasses from 43° to 55° (Guzmán et al., 2010). These facts triggered the initiation in May 2006 of a phytoplankton and marine toxin monitoring program to protect public health and minimise impacts to productive activities. This contribution presents an analysis of *A. catenella* and PSP temporal and geographic patterns along the Chilean fjords and channels from May 2006 to July 2010.

Material and Methods

A total of 151 sampling sites have been established between 41° and 55° S (Fig. 1). Sampling sites are visited monthly in Spring, Summer and Autumn, and during Winter about every 40 days. Sampling was conducted since May 2006, with gaps between March-October 2007, October-November 2009 and January 2010. The variables considered here are relative abundance and density of *A. catenella* and PSP concentrations in bivalve molluscs. Relative abundance is estimated from net samples (23 µm), collected by vertical tows from 20 m depth to the surface. At each site three replicates at two points are collected, integrating a single sample from the six hauls. Relative abundance

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estimations are made on sedimented samples counting the microalgal vegetative phase. Details on relative abundance scale and its estimations are presented in Guzmán et al. (this proceeding). Density was estimated from samples taken with a 2.5 diameter hose, in two strata: surface - 10 m and 10 - 20 m depths. Density was estimated by the Utermöhl (1958) method. Paralytic Shellfish Poison (PSP) was estimated by mouse bioassay (A.O.A.C., 1990) in *Aulacomya ater*, *Mytilus chilensis* and *Venus antiqua* and analyses conducted at the Ministerial Regionals Secretariat for Health.

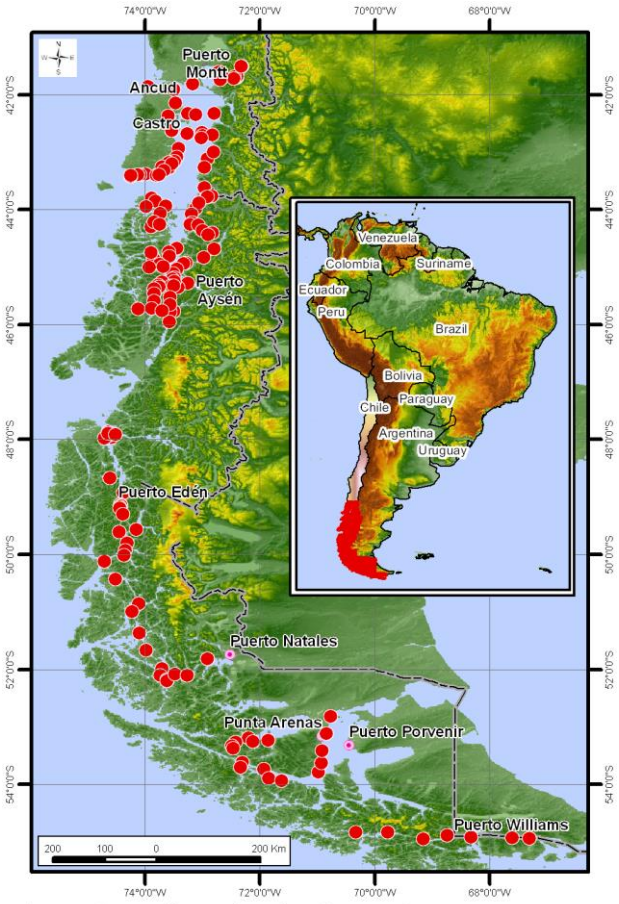

Fig. 1. Sampling sites in the study area.

Results and Discussion

The relative abundance in comparison to density estimates for *A. catenella* reflects more appropriately variations in shellfish toxicities but the three variables show similar tendencies, particularly during periods of increase. Toxicity peaks are preceded or coincident with relative abundance peaks and in some occasions with density peaks (Figs.2-4). The region of Aysén and the Magellan region are the geographic areas with greater persistence and spatial coverage of blooms of *A. catenella* and PSP outbreaks, the first presents on average highest toxicities, but the highest records have occurred in the Magellan region (Figs. 2-3). This region has the longest historical record, with toxicities as high as 52.920 µg at the Magellan Strait (Uribe et al. 1995).

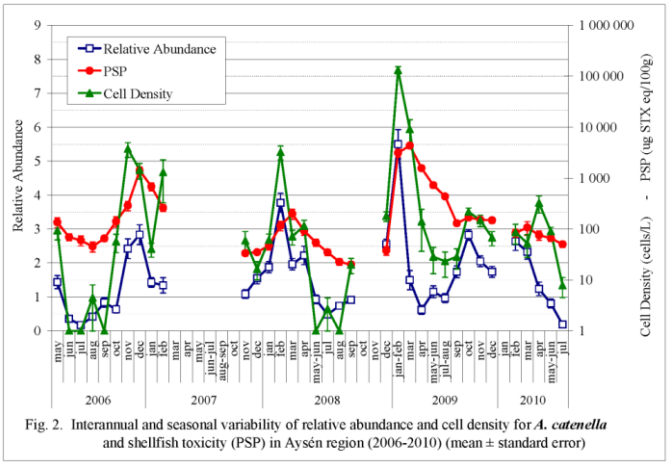

Fig. 2. Interannual and seasonal variability of relative abundance and cell density for ***A. catenella*** and shellfish toxicity (PSP) in Aysén region (2006-2010) (mean ± standard error)

In the region of Los Lagos, blooms and outbreaks are sporadic, and preferably cover the far southern and eastern coast of Chiloé Island, not reaching densities, relative abundances and toxicities as high as in the other regions (Fig. 4). Also an increase with latitude of toxicity highest records along the study area exists. Previous to 2009, *A. catenella* blooms and PSP outbreaks were characterized by its initiation during spring in the Magellan region, and one or two months later the bloom and PSP outbreak appeared in the region of Aysén, and in some years reached the southern tip of Chiloé island, 2002, 2006, 2009. However, during the summer of 2009 (March) occurred a very intense bloom of *A. catenella* and PSP toxic outbreak covering almost all the Aysén region, not affecting the Magellan region, which subsequently, during spring presented a toxic bloom reaching the highest toxicities of that period and independently on what was happening in the region of Aysén, excluding its southernmost area. During 2010 the conditions in microalga abundance and toxicity in bivalve

molluscs have been milder (Figs. 2-4). There are interannual differences, with periods of toxicity levels and *A. catenella* abundance relatively low (e.g. 2007), as well as periods of intense blooms and PSP outbreaks with very high toxicity and abundances of the microalga (e.g. 2009) (Figs. 2-4).

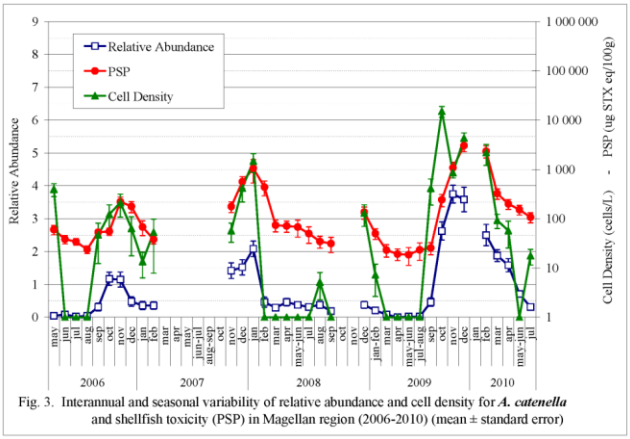

Fig. 3. Interannual and seasonal variability of relative abundance and cell density for ***A. catenella*** and shellfish toxicity (PSP) in Magellan region (2006-2010) (mean ± standard error)

In the Aysén region *A. catenella* blooms usually occur during summer (January to March) but can occur also in Spring (October and December), whereas in the Magellan region, may occur in Spring, Summer and Fall, depending on the sector in this region. In the region of Los Lagos blooms have occurred only in late Spring (December) and Summer (January to March) (Figs. 2-4). The microalga is not present or appears at very low concentrations during the winter months (July-August), but toxic shellfish can be detected during this period as a result of previous blooms and toxin levels reached in previous months (Fig. 2-4).

Conclusions

A. catenella blooms and toxicity outbreaks not always coupled between regions and may even show differences within each region in the distribution and abundance of *A. catenella* and consequently in PSP distribution. The blooms of the microalga and the PSP outbreaks show interannual differences, characterized by mild and intense periods. Within the Chilean fjords, the extensive geographic coverage of *A. catenella* blooms associated to PSP outbreaks suggests that climatic oceanographic factors are responsible for the initiation of these phenomena.

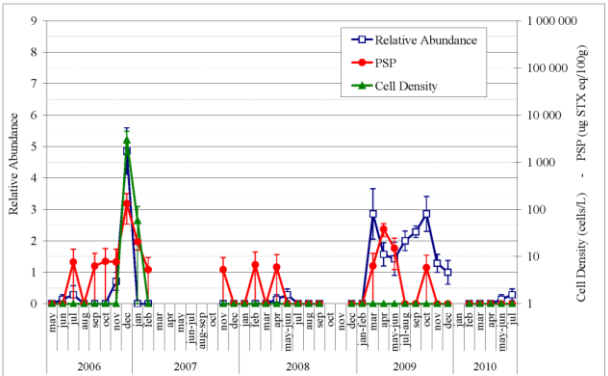

Fig. 4. Interannual and seasonal variability of relative abundance and cell density for ***A. catenella*** and shellfish toxicity (PSP) in Chiloé Island Southern area (2006-2010) (mean ± standard error)

Acknowledgements

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The effect of Light on Growth Rate and Primary Productivity in *Pseudo-nitzschia australis* and *Pseudo-nitzschia turgidula*

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Abstract

A neritic isolate of *Pseudo-nitzschia australis* and an oceanic isolate of *P. turgidula* were assessed for rates of growth and primary production as a function of photosynthetic photon flux density (PPFD). Maximal exponential growth rates were 1.44 d^{-1} for *P. australis* and 1.00 d^{-1} for *P. turgidula*, and were attained at the highest PPFD examined (334 µmol quanta m^{-2} s^{-1}). Maximum carbon (^{14}C) uptake rates (P^B_m), determined via photosynthesis versus irradiance (PE) curves, were 0.707 µg C (µg Chl *a*)$^{-1}$ h^{-1} for *P. australis* and 0.563 µg C (µg Chl *a*)$^{-1}$ h^{-1} for *P. turgidula*. Reduced carbon uptake rates for the oceanic *P. turgidula* isolate were not unexpected, as previous work has demonstrated a reduced photosynthetic efficiency for this oceanic species. The higher growth rates for the neritic species *P. australis* may demonstrate an ability to adapt to rapidly changing coastal environments, in contrast to the more invariant oceanic environment.

Introduction

Access to light is an absolute requirement for phytoplankton metabolism and the synthesis of organic molecules needed for their growth and reproduction. Photosynthesis, as the essential process needed for primary metabolism, requires visible light (photosynthetically active radiation [PAR]; 400-700 nm wavelengths), which in the natural environment is highly variable on both temporal and spatial scales. Here, we investigate the effects of light on the exponential growth rate and primary productivity in two species of the toxigenic diatom genus *Pseudo-nitzschia* – a neritic isolate of *P. australis* and an oceanic isolate of *P. turgidula*. Each species' photosynthetic response is quantified using exponential growth rate (µ) and short-term ^{14}C uptake measurements (photosynthesis versus irradiance [PE] curves) to derive parameters which characterize the light- and dark-dependent reactions of photosynthesis, thereby gaining an assessment of these species' response to photosynthetic photon flux densities (PPFDs). The present study provides a comparative analysis of the effects of light on two *Pseudo-nitzschia* species isolated from widely contrasting environmental habitats.

Materials and Methods

P. turgidula (unialgal strain NWFSC 254) was collected at Ocean Station PAPA (OSP: 50°N, 145°W) in the subarctic, northeast Pacific Ocean in June 2006. *P. australis* (unialgal strain 0771B) was collected at Point Reyes, California in March 2007. Maintenance of stock cultures, growth medium, sample fixation and enumeration, chlorophyll *a* and *in vivo* fluorescence determination are described in detail by Bill (2011). Briefly, all experiments were conducted at 13°C (± 0.2°C), illuminated on a 12:12 hr, light:dark cycle and cell growth was monitored twice daily for cell abundance and *in vivo* fluorescence. Specific growth rates for the eight PPFDs tested were determined for the exponential portion of the growth curves using least-squares linear regression of the natural logarithm of *in vivo* fluorescence as a function of time. Prior to the PE experiments, cultures of each species were subdivided and grown for a minimum of two weeks under sub-saturating (50 µmol quanta $m^{-2}s^{-1}$) and saturating (225 µmol quanta m^{-2} s^{-1}) PPFDs. PE curves were obtained using short-term (30 min) incubations of $NaH^{14}CO_3$ using a temperature-controlled photosynthetron equipped with white (halogen) light at 16 PPFDs between 0-800 µmol quanta m^{-2} s^{-1}. The carbon uptake rates obtained

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from liquid scintillation counting of incorporated ^{14}C were normalized to Chl *a* concentration, and fitted to the three-parameter PE model of Platt and Gallegos (1980) using a non-linear, least-squares regression technique to obtain maximum carbon fixation (P^B_m) and initial slope (α).

Results

The maximum exponential growth rates (μ_{max}) obtained for *P. australis* (1.44 d^{-1}) and *P. turgidula* (1.00 d^{-1}) were observed at the highest PPFD examined (334 µmol quanta m^{-2} s^{-1}; Fig. 1). Both cultures exhibited a linear, positive relationship between growth rate and PPFD at the lower PPFDs - *P. australis* from 21 to 71 µmol quanta m^{-2} s^{-1} and *P. turgidula* from 21 to 52 µmol quanta m^{-2} s^{-1}. At PPFDs above these ranges, the relationship between growth rate and PPFD gradually plateaued, approaching their respective saturation values. PPFD saturation for *P. australis* was approximately 200 µmol quanta m^{-2} s^{-1} while *P. turgidula* PPFD saturation was approximately 100 µmol quanta m^{-2} s^{-1}, indicating that PPFDs at or above these irradiances should be used to achieve maximal growth rates in culture.

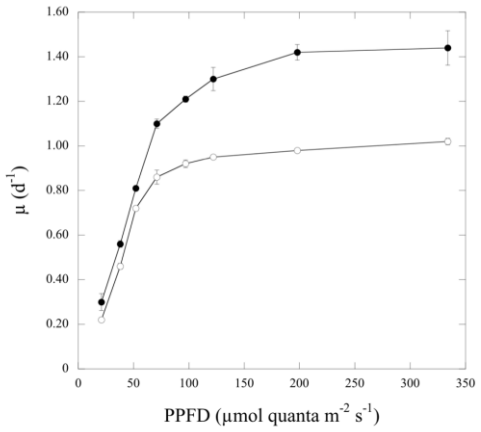

Fig. 1. The mean exponential specific growth rate (µ: d^{-1}) of replicate cultures (n=2) plotted as a function of PPFD for *P. australis* (●) and *P. turgidula* (○). Error bars are the range of replicates.

The PE curves of *P. australis* and *P. turgidula* cultures grown under saturating (225 µmol quanta m^{-2} s^{-1}) and sub-saturating (50 µmol quanta m^{-2} s^{-1}) PPFD are shown in Figure 2. Cultures of *P. australis* previously acclimated to saturating PPFD reached a maximum carbon (^{14}C) uptake rate (P^B_m) more than double than that achieved for *P. turgidula* (0.707 and 0.316µg C (µg Chl *a*)$^{-1}$ h^{-1}, respectively). Sub-saturating PPFD-acclimated cultures of *P. australis* attained a lower P^B_m of 0.572 µg C (µg Chl *a*)$^{-1}$ h^{-1} compared to the saturating PPFD-acclimated culture while the reverse was observed for *P. turgidula* where the sub-saturating PPFD-acclimated culture attained a higher P^B_m of 0.563 µg C (µg Chl *a*)$^{-1}$ h^{-1} than the saturating PPFD-acclimated culture.

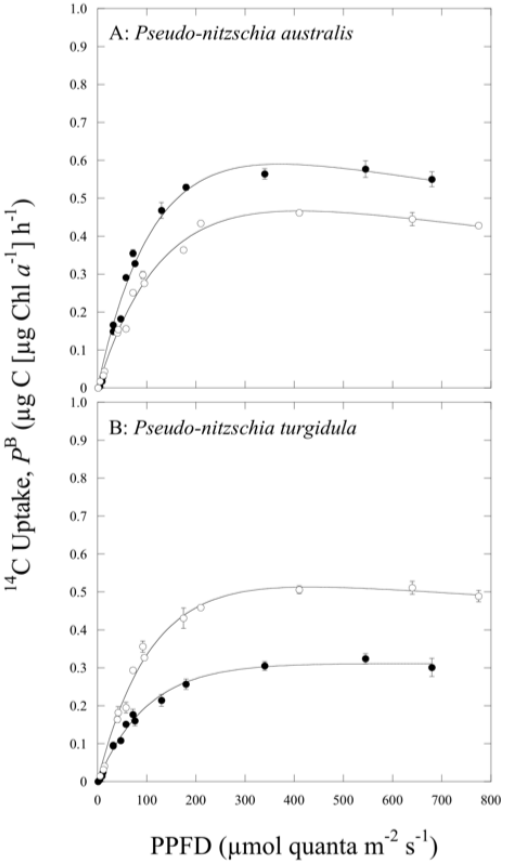

Fig. 2. Photosynthesis versus irradiance (PE) curves: mean carbon (^{14}C) uptake rate normalized to Chl *a* as a function of PPFD for replicate (n=2) cultures of A: *P. australis* and B: *P. turgidula* acclimated to saturating PPFD (●) and sub-saturating PPFD (○). Error bars are the range of replicates.

Discussion

Maximum growth rates (μ_{max}) attained at saturating PPFD ranged from 1.29 - 1.44 d^{-1} for *P. australis* and from 0.92 - 1.00 d^{-1} for *P. turgidula*, and are similar to those reported previously for strains of these two species

(Lundholm *et al*. 2004; Marchetti *et al*. 2006; Howard *et al.* 2007). Maximum growth rates for *P. australis* were consistently greater (*ca.* 35 - 45%) than those achieved for *P. turgidula* at the same saturating PPFD employed. The difference in the *in situ* light environment typically experienced by these two species may explain the consistently higher growth rates achieved by *P. australis* over *P. turgidula* at saturating PPFDs. *P. australis* is considered primarily a neritic species and the isolate used here was collected from a coastal California upwelling system. Phytoplankton in such systems typically experience larger and more frequent fluctuations in environmental parameters such as light and nutrient availability than oceanic areas (Kudela *et al.* 2010). In addition, physical parameters such as vertical and horizontal water movement, via fast moving currents, can have large impacts on the availability of PPFD to free-floating organisms such as phytoplankton. *P. australis* may have adapted to these abrupt and inconsistent changes in its light environment by developing the capacity for higher growth rates over a wider range of PPFDs.In contrast to the coastal environment described above, the oceanic waters of OSP where *P. turgidula* was isolated, is situated in the high-nutrient, low-chlorophyll (HNLC) region of the subarctic northeast Pacific Ocean. The surface waters of this region are characterized by less intense variability in water movement, PPFD, and nutrient form and concentration, and likely provide this isolate of *P. turgidula* with a relatively stable growth environment. The PE curves generated here were obtained from both a sub-saturating PPFD-acclimated (low light-adapted) culture and a saturating PPFD-acclimated (high light-adapted) culture. The uptake curves generated for *P. australis* are typical of those generally observed when comparing low and high light-adapted cells; high light-adapted cultures yield greater values for the initial slope (α) of the PE curve, and higher maximum C uptake rates (P^B_m) than low light-adapted cultures. However, the opposite was observed for *P. turgidula*. Here the low light-adapted culture yielded greater values of both α and P^B_m than the high light-adapted culture. This may be a result of the overall reduced photosynthetic efficiency observed for the oceanic species, *P.* cf. *turgidula* (Marchetti *et al.* 2006). The results of the present laboratory study indicate that maximum growth rates for *P. turgidula* do not increase substantially above 100 µmol quanta m^{-2} s^{-1}, suggesting that maintenance of these cells for multiple generations near 225 µmol quanta m^{-2} s^{-1} (an *in situ* PPFD not normally expected at OSP) may have compromised their photosynthetic capabilities, resulting in depressed photosynthetic rates upon exposure to elevated rates of PPFD in culture. This study provides evidence for differential maximum growth and carbon uptake rates for two species of *Pseudo-nitzschia* from contrasting environments. These differences are likely attributed to the variable oceanographic abiotic parameters experienced by these isolates and highlights the need for comparative studies of genera with cosmopolitan distributions.

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Annual cycle of *Pseudo-nitzschia* species in Outer Oslofjorden, Norway

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Abstract

The annual cycle of the diatom genus *Pseudo-nitzschia* was examined in Outer Oslofjorden, Norway. Vertical net tows (2 5- 0 m depth) and seawater samples (1m depth) were collected monthly for one year (June 2009 -June 2010). The diversity of *Pseudo-nitzschia* species were recorded from live and preserved material with light microscopy and from acid cleaned samples viewed in scanning (SEM) and transmission electron microscopy (TEM). *Pseudo-nitzschia* species were present in every sample collected during the year. Nine species of the genus were identified, of which eight are potentially toxic: *Pseudo-nitzschia delicatissima, P. pseudodelicatissima, P. calliantha, P.* cf. *cuspidata, P. pungens, P. multiseries, P. frau-dulenta* and *P. seriata.* Two species, *Pseudo-nitzschia calliantha* and *P. delicatissima,* were the most frequently observed and present in 9 out of 11 samples. The highest concentration of *Pseudo-nitzschia* spp. was recorded in January 2010 (1, 61 x 10^6 cells L^{-1}).

Introduction

Species of the genus *Pseudo-nitzschia* H. Peragallo (H. & M. Peragallo, 1900) are widely distributed and present in all biogeographic zones (Hasle 2002; Casteleyn *et al.* 2008). Earlier investigations from Norwegian waters have shown a considerable variation in the species composition of the genus *Pseudo-nitzschia,* both geographically and seasonally (Hasle *et al.* 1996). The genus contains more than 30 species and several of them are found in Norwegian waters (e.g. *P. delicatissima, P. fraudulenta, P. granii, P. heimii, P. pungens, P. mul-tiseries, P. calliantha, P. seriata, P. obtusa, P. americana).* Twelve species of *Pseudo-nitzschia* have been documented to produce domoic acid (DA) a neurotoxin that causes amnesic shellfish poisoning (Moestrup 2005; Moschandreou *et al* 2010). The ability to produce DA varies among species, thus an exact identification and knowledge about their geographical and seasonal occurrence at the species level is important. Monitoring of microalgae (including toxic species like *Pseudo-nitzschia*) is based on light microscopy; nevertheless a precise identification of *Pseudo-nitzschia* at the species level requires verification by electron microscopy and/or molecular biological tools. The genus *Pseudo-nitzschia* is a common component of the phytoplankton in Norwegian waters. The aim of the present study is to examine the composition and abundance through the annual cycle of *Pseudo-nitzschia* in Outer Oslofjorden in the Northern Skagerrak.

Materials and methods

Samples were collected monthly during one year period from June 2009 to June 2010, at station OF2 (59.186668N, 10.691667E) in Northern Skagerrak, Norway. Seawater samples collected at 1 m depth were preserved in Lugol's solution (1% of final concentration) and cell counts were made according to the protocol of Uthermöhl (1958). Vertical net-tows (20 µm mesh size) from 25 - 0 m depth were preserved with formaldehyde (2% final concentration) and with Lugol's solution (1% final concentration). In order to remove organic material the formaldehyde preserved net samples were acid cleaned (Throndsen *et al.* 2007). The cleaned frustules were mounted on stubs and grids and viewed in a Hitachi FEG S-4800 SEM and Philips CM-1000 TEM.

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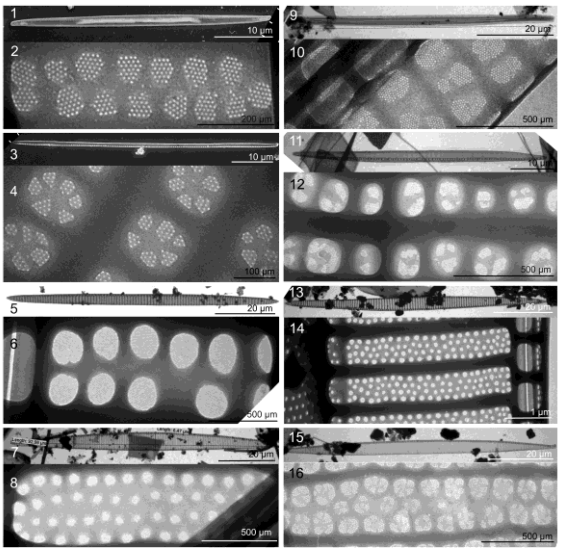

Fig. 1. SEM and TEM micrographs of eight potentially toxic *Pseudo-nitzschia* species; *delicatissima* – group: (1, 2) *P. delicatissima*; (3, 4) *P. calliantha*; (9, 10) *P. pseudodelicatissima*; (11, 12) *P.* cf. *cuspidata.; seriata* – group: (5, 6) *P. pungens*; (7, 8) *P. seriata;* (13, 14) *P. multiseries*; (15, 16) *P. fraudulenta.*

Results

Occurrence and cell counts

Pseudo-nitzschia species were present in all samples with densities ranging from 500 cells L^{-1} in June 2010 to 1.61 million cells L^{-1} in January 2010 (Table 1). In January 2010, the high cell density was mainly related to a bloom of *P. delicatissima* and *P. calliantha*. The *delicatissima*-group (species with valve width less than ca. 3 µm) dominated all samples except the April and May samples of 2010.

Species diversity and identification

Pseudo-nitzscia species identification was based on morphometric characteristics (Hasle *et al.*1996, Lundholm *et al.* 2003).

A total of ten species of the genus *Pseudo-nitzschia* were found, nine of them were identified: *P. calliantha, P. delicatissima, P. pseudodelicatissima, P.* cf. *cuspidata*, *P. fradulenta*, *P. seriata*, *P. pungens*, *P. multiseries* and *P. americana*. An unidentified *Pseudo-nitzschia* species resembles *P. pseudodelicatissima* in all morphometric characteristics but differs in poroid structure. *Pseudo-nitzschia calliantha* and *P. delicatissima* were the most frequently observed and present in 9 out of the 11 examined samples. The species composition changed through the year and the number of species present in one sample varied from one in June 2010 to seven in June and October 2009 (Table 1).

Table 1. Abundance and monthly occurrence of *Pseudo-nitzschia* spp. during June 2009- June 2010 in Outer Oslofjord, Norway.

***Pseudo-nitzschia* species**	**22.06 2009**	**05.08 2009**	**22.09 2009**	**20.10 2009**	**17.11 2009**	**09.12 2009**	**21.01 2010**	**11.03 2010**	**13.04 2010**	**11.05 2010**	**22.06 2010**
***Pseudo-nitzschia* spp. cells L^{-1}**	**99100**	**15000**	**217500**	**91500**	**150100**	**11400**	**1615900**	**141200**	**1400**	**800**	**500**
***delicatissima*- group**											
P. calliantha	x	x	x	x	x	x	x	x		x	
P. delicatissima	x			x	x	x	x	x	x	x	x
P. pseudodelicatissima	x			x							
*P.*cf. *cuspidata*					x	x					
Pseudo-nitzschia sp.	x			x							
***seriata*- group**											
P. fraudulenta	x	x		x						x	
P. seriata	x	x						x	x	x	
P. pungens	x		x	x	x	x				x	
P. multiseries				x	x						
P. americana						x	x				

Discussion

Occurrence and cell density of *Pseudo-nitzschia* spp. varied through the seasons, with a more or less gradual increase from June 2009 until January 2010 (bloom) followed by a subsequent decrease. The *delicatissima*- group dominated the samples and the most common *Pseudo-nitzschia* species were *P. calliantha* and *P. delicatissima* (the only representative of the genus in June 2010). Eight potentially toxic *Pseudo-nitzschia* species were detected in the present study: *Pseudo-nitzschia pungens, P. multiseries, P. frau-dulenta, P. seriata, P. delicatissima, P. pseudodelicatissima, P. calliantha* and *P.* cf. *cuspidata* (Fig. 1). Seven of the nine identified *Pseudo-nitzschia* species have earlier been encountered regularly in Norway; the exceptions are P. *cuspidata* and *P. pseudodelicatissima* that are recent additions to the Skagerrak phytoplankton flora.

Acknowledgments

This study was supported by the EU - project MIDTAL and NCR- funded project Toxic algae. Special thanks to Nina Lundholm for useful comments on the manuscript.

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An integrated approach for the assessment of HAB dynamics in two NW Mediterranean bays from a GEOHAB perspective

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Abstract

Alfacs and Fangar Bay in the Ebro Delta, NW Mediterranean are the major sites in Catalonia for shellfish cultivation. These bays are subject to occasional closures in shellfish harvesting due to the presence of phycotoxins. Fish kills have also been associated with harmful algal blooms. The comparison of phytoplankton dynamics in both bays offers the opportunity to reveal differences in bloom patterns of species known to be harmful for the ecosystem and aquaculture activities. Field research is underway under the GEOHAB framework within the Core Research Project on HABs in Fjords and Coastal Embayments. The overall objective of this study is to improve our understanding of HAB biogeographical patterns, and key elements driving bloom dynamics in time and space within these semi-constrained embayments. Via the comparative approach we aim to improve the prediction for monitoring purposes, with a focus on *Karlodinium* spp. associated with massive kills of aquaculture species. This objective is addressed by incorporating long-term time series of phytoplankton identification and enumeration with the first results of recent field work in both bays. The latter includes the application of optical sensors, to yield a complementary view with enhanced spatial and temporal resolution of bloom phenomena.

Introduction

The two semi-enclosed embayments Alfacs and Fangar Bay in the Ebro Delta system, NW Mediterranean, are the major aquaculture sites in Catalonia. Due to the presence of phycotoxins, both bays are subject to occasional harvesting closures. The ichthyotoxic species *Karlodinium veneficum* and *K. armiger* (in this area previously referred to as *Gyrodinium corsicum* (Garcés *et al*. 2006)) have been found in Alfacs Bay since 1994. In 2010 the species were also detected in Fangar Bay. In spite of their proximity and similar climatic conditions, Alfacs and Fangar Bay profoundly differ in HAB dynamics. Circulation patterns and retention time of water in both bays are differently affected by winds, coastal currents, and freshwater inflow from agriculture. The comparison of environmental forcing functions and bloom characteristics in both bays therefore provides the opportunity to improve our understanding of the key element

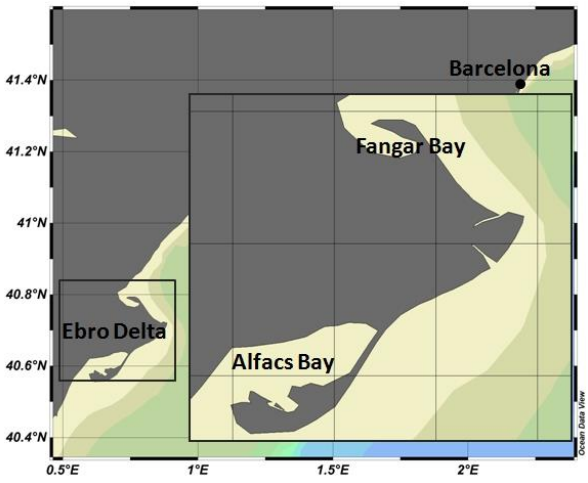

Fig.1 Study area in the Ebro Delta, Spain, NW Mediterranean.

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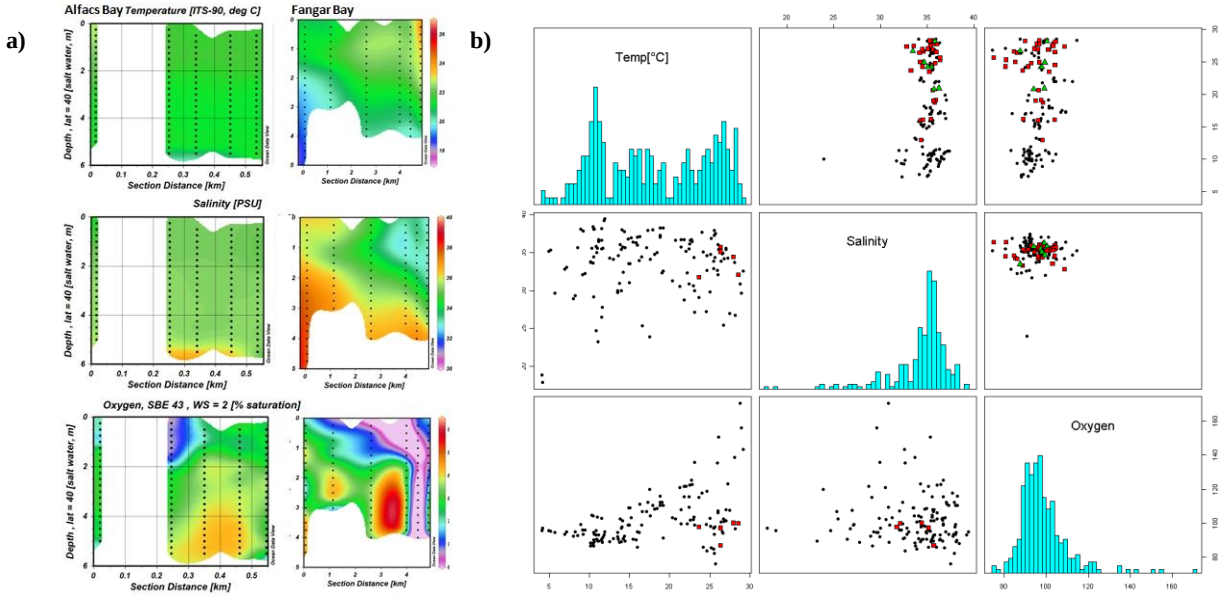

Fig.2 a) CTD casts on a transect of sampling stations (vertical dotted lines) in Alfacs (left) and Fangar (right) Bay. The first station is at section distance 0km). b) Pair plot of *Karlodinium* spp. detection in three abundance classes (<1000cells L-1=black dots; <10,000 cells L-1 =red squares; >10,000 cells L-1=green triangles) from 4 Jan – 30 Aug 2010 in Alfacs (upper three scatter plots) and Fangar (lower three scatter plots) with respect to temperature, salinity and oxygen saturation. The distribution of total *Karlodinium* spp. abundances in environmental ranges of both bays is given in the bar charts.

that drive bloom dynamics in time and space.Via the comparative approach we aim to improve the prediction for monitoring purposes, with a focus on *Karlodinium* spp. associated with massive kills of aquaculture species (Delgado *et al.* 1995). This objective is addressed by incorporating long-term time series of phytoplankton identification and enumeration with the first results of recent field work in both bays. The latter includes the application of oceanographic and optical sensors, to yield a complementary view with enhanced spatial and temporal resolution of bloom phenomena. The objective of the presented work is the comparison of high *Karlodinium* spp. abundances in both bays.

Material and Methods

Cell numbers for abundance class generation of *Karlodinium* spp., temperature, salinity, and oxygen values were taken within the regular monitoring project in five stations in each of both bays for the time between January and August 2010. Complementary, an intensive depth resolved comparative field study was conducted between May and July, here only CTD casts along a transect of regular monitoring stations in both bays are taken into account.

Results and Discussion

Variation in time and space of the environmental conditions in both bays (temperature, salinity, and oxygen saturation) as well as maximum abundances of *Karlodinium* spp. are reported. The highest abundances and maximum cell concentrations were reached in Alfacs Bay. At the time of maximum abundance of *Karlodinium* spp. in Alfacs Bay and first detection in Fangar Bay, we identify differences in environmental characteristics between bays (Fig. 2a). At the stations of high algal abundance in each bay (first station in the transects, at 0km section distance), we report a difference of >1 °C in temperature and >1 in salinity from surface to bottom, indicating a certain degree of stratification (Fig. 2a). A trend of *Karlodinium* spp. blooms in stratified waters was recognized in a 20 years´ time series of monitoring in the Ebro Delta Bays

(Fernandéz-Tejedor 2010). During the summer months, dams are open for rice field irrigation in the Delta area. Consequently flow of freshwater increases from the rice fields to the bays and stratification predominates in both bays. This is due to a lateral fresh water inflow through a series of channels of the main land, and sea water inflow from the Mediterranean as a salt wedge along the bottom (Camp and Delgado 1987).

As Fangar Bay is smaller than Alfacs Bay, the influences of freshwater inflow, as well as from the southwesterly coastal currents, have a stronger effect on this bay. In 2010, *Karlodinium* spp. have been detected throughout a wide range of environmental conditions (Fig. 2b). Higher abundances, however, occur in the range of salinity of 32-35, and temperature of 20-27°C in both bays. The combination of these small ranges in salinity and temperature may not be the key elements that trigger high *Karlodinium* spp. abundances, but provide an environmental setting of this year´s bloom patterns. This can be an indication of the presence of proxies for algal proliferations that can be used for the early detection of blooms, e.g. by means of optical sensors.

Outlook on coupling of environmental- and HAB patterns:

- Inclusion of additional environmental parameters such as nutrients and turbidity
- Analysis of high depth resolution of biological and physical parameters from May to July
- Application of an optical sensor system and derivation of physical-optical bloom proxies
- Incorporation of bio-optical datasets into oceanographic models
- Setup of a long-term environmental observatory

Acknowledgements

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Role of cyst germination in the bloom initiation of harmful algal species in Korean waters

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Abstract

To clarify the role of cyst germination in the bloom initiation of harmful algal species, isolation and germination experiments on single cysts were carried out in four harmful algal bloom (HAB) species biweekly or monthly. Germination maxima of *Alexandrium tamarense* were observed in winter, but little or no germination occurred in summer. In the case of *Scrippsiella trochoidea*, mass encystments were detected in the water column in July and August when the vegetative population flourished. The vegetative population of Nostocales flourished from August to November; however, the most active akinete germination occurred from the end of March to early April. In *Peridinium bipes*, the cysts collected at higher temperatures germinated more quickly than those seeded at lower temperatures, while cysts collected in the fall and early winter had a higher cumulative excystment rate than those from the spring or summer. From these results, the types of germination patterns showed that there is a temporal discrepancy between the peak of germination success and the bloom of the vegetative population. These results indicate that active germination is not likely to be a direct trigger that forms the blooms of HABs.

Introduction

A number of phytoplankton species present a dormant resting stage that allows survival under adverse environmental conditions in their life histories. Dinoflagellates resting cysts were found to sink to the bottom of sediments or the near-bottom nepheloid layers, when environmental conditions are unsuitable for growth (Prakash 1967; Wall 1971). The cyst population can provide an inoculum for a new bloom after a specific dormancy period (Kremp & Heiskanen 1999). Certain cyanobacteria of Nostocales, including the noxious bloom-forming species *Anabaena* and *Aphanizomenon*, form a thick-walled reproductive structure termed akinete which may serve as a dormant cell under adverse conditions (Baker & Bellifemine 2000). When favorable conditions return, akinetes germinate to produce trichomes (Hori et al. 2002; van Dok & Hart 1996), which act as inocula for the new blooming (Hori et al. 2002; Kravchuk et al. 2002; van Dok & Hart 1996). There are no doubts that cysts including akinetes play an important role as the seed of the vegetative population of phytoplankton (Anderson & Wall 1978; Hansson 1996). Sometimes, however, bloom initiation does not correspond to a direct consequence of *in situ* germination patterns of cysts. (Ishkawa & Taniguchi 1996, 1997). Therefore, the relationship between algal bloom formation and cyst germination rates remains unclear.

However, it is important to identify the effect of encystment and excystment on algal blooming initiations. In this study, I focused on the seasonality relationship between the development of vegetative populations and the cyst germination modes.

Material and Methods

Sampling sites were located in a coastal area (Masan and Youngil Bay) and an inland lake (Juam Reservoir and Seokchon Pond) in South Korea. Vegetative cell and cyst abundance were analyzed by using an optical microscope (Axioplan, Zeiss, Germany). The excystment rates of *Alexandrium*, *Scrippsiella*, *Peridinium* and Nostocales such as *Anabaena* and *Aphanizomenon* were measured by a cyst germination experiment. The isolated cysts were inoculated one by one (using a microcapillary) into individual wells of a 96-well tissue culture plate (Falcon, USA) containing filtered water. The inoculated cysts were incubated at water temperatures similar to those of the sampling station bottom layer, under 12–30 μmol photons m^{-2} s^{-1} with a 12 h light: 12 h dark cycle.

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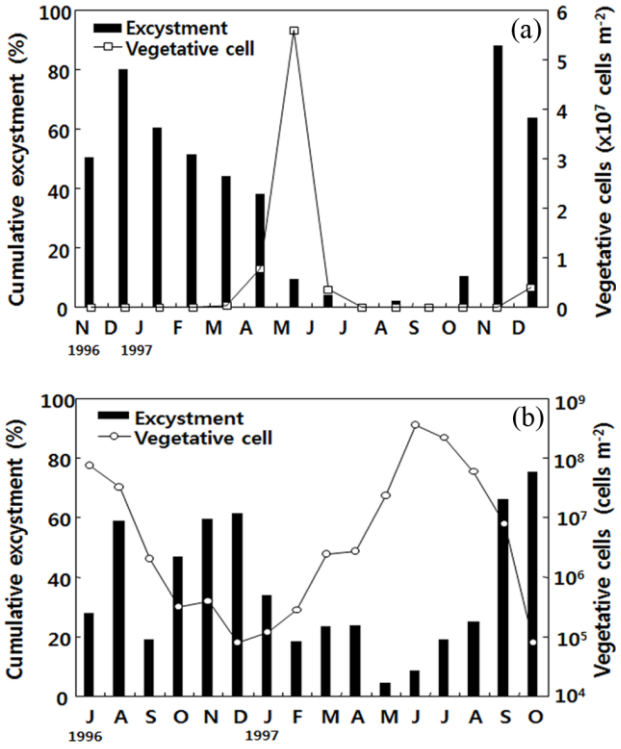

Fig. 1. Seasonal changes of excystment (%) and vegetative cell numbers of HAB species (a) *Alexandrium tamarense* (Kim *et al*. 2002), (b) *Scrippsiella trochoidea* (Kim & Han 2000)

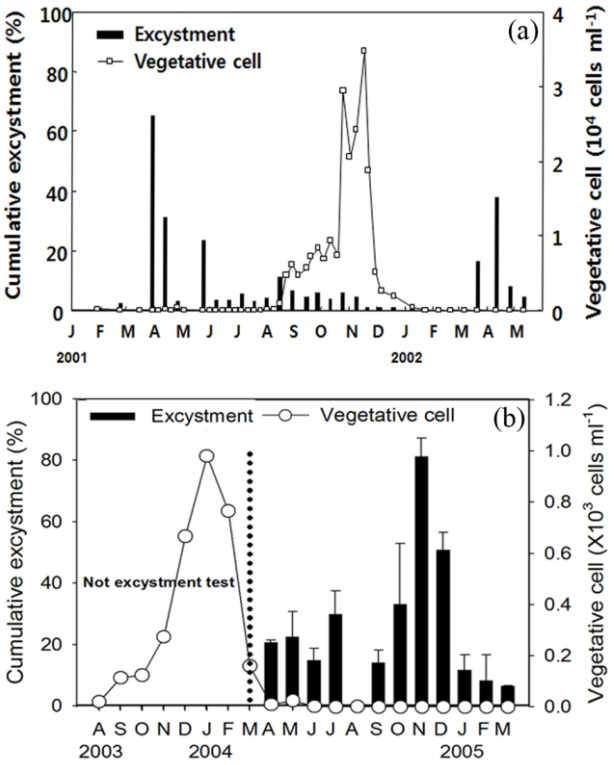

Fig. 2. Seasonal changes of excystment (%) and vegetative cell numbers of HAB species (a) species of Nostocales (Kim *et al*. 2005), (b) *Peridinium bipes* (Kim *et al*. 2007)

Results and Discussion

The vegetative population of *Alexandrium tamarense* peaked in spring, but germination success was not higher. Germination maxima (80–90%) were observed during the winter season when the *A. tamarense* vegetative cells were almost not detectable in the water column (Fig. 1a). Similar germination results for *A. tamarense* were observed in Hiroshima Bay (Itakura & Yamaguchi 2001).

In *Scrippsiella trochoidea*, the vegetative population peaked in summer; however, the germination ratio was much lower in the bloom season. Active germination was observed in autumn after 3–4 months of blooming and the vegetative population decreased dramatically (Fig. 1b).

The highest vegetative population of Nostocales was observed in November after 7 months of active germination. Of this group, the most active germination was observed from the end of March to early April; however, the vegetative cells were extremely difficult to detect in the water column (Fig. 2a). In the excystment test of *Peridinium bipes*, cysts obtained from samples collected at higher temperatures (over 15 °C) germinated more quickly than those seeded at lower temperatures, while cysts collected in the fall and early winter had a higher cumulative excystment rate than those collected in the spring and summer, suggesting that cysts deposited at higher temperatures may act as a seed population for the winter blooms (Fig. 2b).

In Korean waters, some HAB species have shown a temporal discrepancy between the peak of the germination success and the bloom of the vegetative population, meaning germination success occurs very early and not immediately prior to the bloom of the vegetative population. These results suggest that active germination does not trigger directly an increase to the vegetative population of HABs. In conclusion, cyst-forming phytoplankton species possess their own seasonality in diverse germination modes as a survival strategy.

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Growth and toxin production of *Azadinium spinosum* in batch and continuous culture

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Abstract

Azaspiracids are lipophilic marine biotoxins causing gastrointestinal symptoms similar to DSP toxins. Since 1995, azaspiracids have been encountered in Europe, Africa and more recently in North and South America and Japan. The biological primary producer remained undiscovered during many years and has now been identified as *Azadinium spinosum*. The organism was grown using K modified medium, at 18°C with a PFD of 200 µmol.m^{-2}.s^{-1} and a photoperiod of 16L/8D. Batch cultures were carried out using 75mL and 10L flasks, while continuous cultures were produced in 100L chemostats. Cells were recovered using centrifugation or filtration. Different extraction solvents and procedures as well as evaporation modes were evaluated for yield. Quantitation was carried out using LC-MS-MS. *A. spinosum* had a maximum growth rate of 0.6 d^{-1} with K modified medium, and reached maximum cell concentration of 300000 cells.mL^{-1}. Toxins were mostly intracellular, with 5 to 10% toxin in the culture medium. Analogues detected included AZA1, -2 and the methyl esters of AZA1 and -2, AZA1 being the predominant toxin.

Introduction

Harmful algal blooms might cause severe human illness due to the consumption of bivalves, as microalgae are the principle food for bivalve mollusks. In 1995, contaminated mussels (*Mytilus edulis*) from Killary harbor (Ireland) were consumed in the Netherlands (McMahon and Silke, 1996). The toxic agent caused diarrhea, nausea, vomiting and stomach cramps in consumers, symptoms typical for diarrheic shellfish poisoning (DSP). A new toxin was discovered and was named azaspiracid after structural identification by (Satake *et al.*, 1998). Since then azaspiracids have been encountered in Europe, Africa and more lately in South and North America and Japan (Twiner *et al*, 2010). Recently, the biological source of azaspiracids (AZAs), *Azadinium spinosum* (strain 3D9) a small dinoflagellate, was discovered. This organism produces Azaspiracid-1 and 2 (Krock *et al.*, 2009; Tillmann *et al.*, 2009).

Growth and toxicity of dinoflagellates are dependent of various environmental and nutritional factors. Among various environmental factors, salinity and aeration might affect dinoflagellate growth and toxicity.

This early work on *A. spinosum* aims to assess the effect of salinity and aeration on growth and toxicity; to evaluate the cell growth and toxin production in pilot scale chemostats and to assess analytical scale extraction procedures for best yield and suppression of AZA1 and AZA2 methyl esters, two possible artefacts of extraction.

Materials and methods

Evaluation of protocols for extraction of azaspiracid from *A. spinosum*

Cells were recovered using centrifugation or filtration, different extraction solvents (methanol, acetone, ethanol, acetonitrile, dichloromethane, H_2O) and mixtures thereof as well as evaporation modes were evaluated for yield. Methyl ester formation on AZA1 and -2 was studied using methanol-d_4 for extraction and or reconstitution after evaporation.

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Culture condition

A. spinosum (3D9) was grown using K modified medium (Keller *et al.*, 1987), without NH_4Cl and with Na_2SeO_3 (1.72 $mg.L^{-1}$), at 18°C with a PFD of 200 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 16L/8D, in batch and continuous culture. For experimental purpose, the strain was grown in triplicate at six different salinities (10, 20, 30, 32, 35, 40 psu) adjusted by dilution with Milli-Q® water or evaporation, in sterile 70mL polystyrene flasks. Salinity was checked with a refractometer. The initial culture cell concentration was 5000 $cell.mL^{-1}$ and the culture was grown until stationary phase for each condition.To evaluate the effect of aeration for a future growth in chemostat, *A. spinosum* was cultured in triplicate in 10 L flat bottomed flasks with and without aeration at 35 psu at the same initial cell concentration.After adaptation to aeration and agitation the microalgae were inoculated in a 100 L medium scale chemostat to evaluate cell growth and toxin production. The flow rate was 0.15 d^{-1}. When the first bioreactor was at steady state it was connected to a second bioreactor to increase cell concentration and toxicity.

Cell growth

Every day or two 1 mL sample was collected to asses cell concentration with a particle counter. A Gompertz model was applied to follow growth kinetics in batch culture, to determine the maximum growth rate (μ_{max} in d^{-1}), the maximum cell concentration (A expressed as $\ln(X/X_0)$ with X in $cells.mL^{-1}$) and the latency time (λ in days). The model follows the equation:

$$f(x) = A*\exp(-\exp(\mu_{max}*\exp(1)/A*(\lambda-x)+1))$$

Where x is the time (days).

Extraction procedure used and LC-MS-MS analysis

At the end of the experiment on salinity, every two days for the experiment on aeration and twice a week for the continuous culture, triplicate samples (10 mL) were collected in all flasks and bioreactors for toxin analysis. Samples were centrifuged (2500 g, 20 min, 4°C). The supernatant was discarded and the pellet suspended with 500 µL of acetone 90% and sonicated. After sonication the aliquot were centrifuged (15000 g, 10 min, 4°C). Each supernatant were transferred into a 5 mL glass tube and gently evaporated under nitrogen at 35°C. The pellets were resuspended in 500 µL of acetone 90%, homogenised and centrifuged again. These steps were repeated three times in total. After evaporation of the supernatants, they were reconstituted in 500 µL methanol 90%. Subsequently, the samples were filtered (Whatman nanopore 0.2 µm) at 15000 g, 15 min, 4°C, and transferred into HPLC vials with inserts. The samples were then analysed by LC/MS-MS following method C described in Rehmann *et al.*, (2008).

Results and discussion

Extraction procedure

Less formation of AZA1 and AZA2 methyl esters were measured with centrifugation compared to filtration and with extraction with acetone compared to methanol. Highest azaspiracid yield was obtained with methanol/H_2O and acetone/ H_2O ratio of 100, 90 and 80%, compared to lower ratios and other solvents.

Better recovery was obtained when using 5 mL glass tubes during the evaporation procedure compared to 1.5 mL HPLC vials for the evaporation.AZA1 and 2 methyl esters are believed to be artefacts of extraction due to methanol (figure 1) as they are formed mainly during the extraction and in a lower amount during the reconstitution.

Physiology

Growth of *A. spinosum* was observed between 30 and 40 psu, with a μ_{max} assessed between 0.37 and 0.73 d^{-1}. From 10 to 20 psu all cells died between the beginning and the second day of the experiment. The best cell concentration was obtained at 35 psu and the highest toxicity with 30 and 40 psu (table 1).

Table 1. Summary of results from the experiment on salinities

Salinity (psu)	30	32	35	40
μ_{max} (day^{-1})	0.37	0.52	0.52	0.73
Latency time (days)	1.53	1.65	1.77	4.26
A ($cells.mL^{-1}$)	48 813	55 701	71 379	28 934
AZA1 ($fg.cell^{-1}$)	17.77	16.63	12.47	20.56
AZA2 ($fg.cell^{-1}$)	13.21	12.10	9.22	16.12

A. spinosum had a μ_{max} situated around 0.6 d^{-1} with K modified medium, and reached maximum cell concentration of 200000 to 300000 cells.mL^{-1} with aeration and of 80000±5000 cells.mL^{-1} without aeration, azaspiracid final concentration per cell was also higher with aeration than without aeration, i.e 39±4 and 19±4 fg.$cell^{-1}$, respectively.
The two pilot scale bioreactors (2*100 L, = R1 and R2) were run in series at 0.15 d^{-1} (10 mL.min^{-1}). At steady state, bioreactor 1 had 194 000 ± 6000 cells.mL^{-1} with a toxicity of 62±1 fg.$cell^{-1}$ and bioreactor 2 had 215000±3000 cells.mL^{-1} with a toxicity of 98±5 fg.$cell^{-1}$. Chemostats in series increased algal concentration and toxicity in the second bioreactor compared to the first one and gave a stable concentration of toxins along time at steady state.

Conclusion

Medium scale bioreactors in series allowed for the continuous production of toxic *Azadinium spinosum* in large volumes. It can be used as source of AZA1 and -2 for contamination of bivalves, for toxicological studies, and for purification of azaspiracid-1 and 2.
Extraction procedure of azaspiracid from *A. spinosum* with methanol, the solvent currently used for the extraction of lipophilic toxins from bivalves produced two artefacts of extraction. Although previously reported by Rehmann *et al.* (2008), in mussels, these artefacts were not observed in such high concentrations after extraction of mussels. Thus, with *A. spinosum* acetone must be used in place of methanol.

Acknowledgements

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Effect of Light Intensity on Five Species of *Gambierdiscus*

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Abstract

The effect of light intensity on growth of 5 species of *Gambierdiscus*, the dinoflagellate genus that causes ciguatera fish poisoning, was tested in a series of laboratory experiments. Growth data showed *G. carolinianus* required the lowest irradiance for positive growth (I_{min} = 5 $\mu E \cdot m^{-2} \cdot s^{-1}$), but grew poorly at irradiances >400 $\mu E \cdot m^{-2} \cdot s^{-1}$. In contrast, *G. ruetzleri* and *G. caribaeus* had maximum growth irradiances (μ_{max}) near 200 $\mu E\ m^{-2} \cdot s^{-1}$ and were able to tolerate light levels >700 $\mu E \cdot m^{-2} \cdot s^{-1}$. Projected growth data indicated all 5 species should be able to maintain growth to depths >100 m in optically clear tropical waters.

Introduction

Light penetration through the water column is a critical regulator of primary production in the ocean and limits the distribution of benthic microalgae, including dinoflagellates. One genus of benthic dinoflagellates, *Gambierdiscus*, is of considerable interest because of its causative association with ciguatera fish poisoning (CFP). CFP causes more human illness than all other seafood consumption maladies combined (Lewis and Holmes 1993). CFP events are spatially and temporally unpredictable, probably due to inherently different toxicities among the *Gambierdiscus* species or strains that dominate blooms (Litaker *et al.* 2010). Species dominance is in turn influenced by environmental factors such as temperature, salinity, irradiance, dissolved nutrients, and substrate availability. The extent to which these environmental factors affect the growth of individual *Gambierdiscus* species is unclear. Previous physiological studies examining the effects of environmental factors (e.g. Bomber *et al.* 1988; Chinain *et al.* 1999) were completed before it was possible to accurately identify *Gambierdiscus* species (Litaker *et al.* 2009; 2010). Additional studies using taxonomically defined species will therefore be required to fully characterize the physiology of *Gambierdiscus* species. This study examines how light availability may define the depth distributions and overall habitat space occupied by five different *Gambierdiscus* species.

Methods

Cultures of Gambierdiscus Ribotype 2 (CCMP 1655) and G. pacificus (CCMP1650) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). G. carolinianus, G. caribaeus and G. ruetzleri were established from the Caribbean region or the SE US Atlantic coast (Table 1; Litaker et al. 2009).

Table 1. *Gambierdiscus* species used in this study.

Species	Location
G. carolinianus	Outer shelf, North Carolina, USA
G. caribaeus	Carrie Bow Cay, Belize, Caribbean
G. Ribotype 2	Martinique, Caribbean
G. pacificus	Moorea, Society Islands, Pacific Ocean
G. ruetzleri	Carrie Bow Cay, Belize, Caribbean
G. belizeanus	St. Barthelemy Island, Caribbean
G. carpenteri	Guam, Northern Mariana Islands, Pacific Ocean

Five replicate cultures of each *Gambierdiscus* species were inoculated into flasks containing modified K medium as outlined in Litaker *et al.* (2009). The flasks were incubated at 27°C under full spectrum fluorescent lights at irradiances of 4-664 $\mu E \cdot m^{-2} \cdot s^{-1}$ (12:12 light:dark cycle). At 2-3 day intervals, each replicate flask was shaken and the culture was transferred to a glass screw-capped centrifuge tube. *In vivo* fluorescence was quantified

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immediately using a Turner fluorometer (Turner Designs Inc., Sunnyvale, California, USA) and each culture was poured back into its flask and returned to the incubator. Growth rates, μ (d^{-1}), were determined from the slope of the *ln* fluorescence vs. time curve. Preliminary studies showed that growth rates calculated with fluorescence data are equivalent to those based on cells counts. Growth rate vs. irradiance (*I*) data were fitted to log normal curves using SigmaPlot software (Systat Software Inc., San Jose California, USA). Maximum growth rate (μ_{max}), the corresponding irradiance (I_{max}),

$$z_{max} = \frac{-ln\left(\frac{I_{min}}{I_{surf}}\right)}{K_d} \quad (1)$$

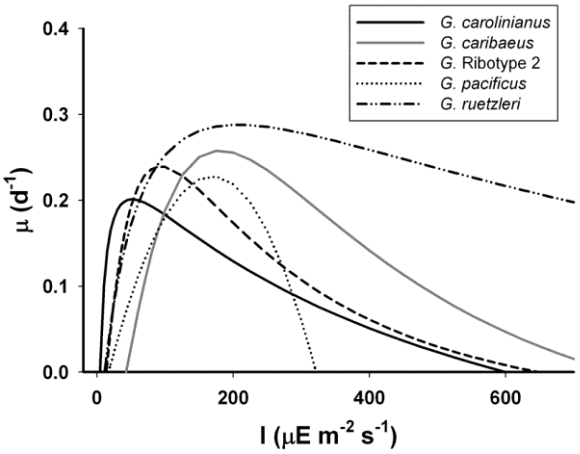

Fig. 1. Growth rates of *Gambierdiscus* species vs irradiance levels

as well as the minimum and maximum irradiances that yielded positive growth (I_{min}, I_{high}), were then determined using the fitted curves. Eq. 1 was used to estimate the maximum projected depth range (z_{max}) for each species after Kemp *et al.* (2004), where K_d (m^{-1}) is the diffuse attenuation coefficient and I_{surf} = irradiance at the ocean surface. K_d values were selected to represent a range of water types from optically clear ocean water (K_d = 0.01 m^{-1}) to highly turbid estuarine water (K_d = 1.5 m^{-1}).

Results

The growth experiments revealed *Gambierdiscus* species exhibited remarkably little variation in μ_{max} (0.2-0.3 d^{-1}, Table 2). The irradiances levels required to achieve μ_{max}, however, varied considerably. For example, under nutrient replete growth conditions, I_{max} ranged from 51 μE·m^{-2}·s^{-1} in *G. carolinianus* to 206 μE·m^{-2}·s^{-1} in *G. ruetzleri*. The I_{min} level was uniformly low for all species tested, varying from 5 μE·m^{-2}·s^{-1} in *G. carolinianus* to 45 μE·m^{-2}·s^{-1} in *G. caribaeus* (Fig. 1, Table 2). These I_{min} values are equivalent to 0.25% and 2.3% of typical irradiance levels at the ocean's surface. *Gambierdiscus* species also exhibited a wide variation in their ability to tolerate high irradiance levels. For example, the species with the lowest tolerance, among those tested was *G. pacificus*. Growth in this species saturated at 170 μE·m^{-2}·s^{-1} and cells began dying when irradiances exceeded 320 μE·m^{-2}·s^{-1} (Fig. 1, Table 2). *G. carolinianus* and *G.* Ribotype 2 achieved maximal growth at lower light levels (<100 μE·m^{-2}·s^{-1}) than *G. pacificus*, but could maintain positive growth to ~600 μE·m^{-2}·s^{-1}, at which point the cells began to die. In contrast, *G. caribaeus* reached maximum growth at ~200 μE m^{-2}·s^{-1} and maintained positive growth at irradiances >700 μE·m^{-2}·s^{-1}. The greatest tolerance to light was shown by *G. ruetzleri*, which reached maximum growth at 206 μE·m^{-2}·s^{-1} and was projected to maintained positive growth at irradiances of >900 μE·m^{-2}·s^{-1} (Fig. 1, Table 2).

Table 2. Maximum growth rates (μ_{max}, d^{-1}), corresponding irradiances (I_{max}), minimum (I_{min}) and maximum (I_{high}) required light levels (μE·m^{-2}·s^{-1}) for *Gambierdiscus* species

Species	μ_{max}	I_{max}	I_{min}	I_{high}
G. carolinianus	0.20	51	5	554
G. caribaeus	0.26	196	45	>700
G. Ribotype *2*	0.24	93	15	589
G. pacificus	0.23	169	21	318
G. ruetzleri	0.29	206	13	>900

When I_{min} was utilized to calculate the maximum projected depth, z_{max} (Eq. 1), the results showed all *Gambierdiscus* species were projected to have a relatively similar depth range. In optically transparent oligotrophic ocean water (K_d<0.05 m^{-1}), all species exhibited a z_{max} >70 m (Fig. 2). Maximum projected depth increased to 90-150 m for water with a K_d of <0.04 m^{-1}. In highly turbid estuarine/coastal water (K_d>1 m^{-1}), z_{max} for each species was <6 m (Fig. 2).

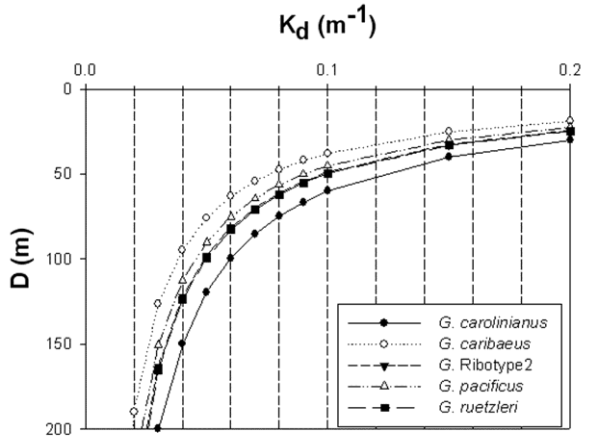

Fig. 2. The diffuse attenuation coefficient K_d (m^{-1}) vs maximum projected depth for each *Gambierdiscus* species

Discussion

The results of the μ vs. *I* experiments indicated *Gambierdiscus* species may be distributed across tropical and subtropical shelf environments to depths of >100 m in oligotrophic waters. There were substantial differences in the tolerance of each species to high irradiances and in their minimum light requirements. With an I_{max} of 51 $\mu E \cdot m^{-2} \cdot s^{-1}$, an I_{min} of only 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (0.25% surface light) and poor growth above 400 $\mu E \cdot m^{-2} \cdot s^{-1}$, *G. carolinianus* is best adapted to low light/deep environments. In contrast, *G. ruetzleri* was better adapted to high light, but could also grow in very low light (I_{min}=13 $\mu E \cdot m^{-2} \cdot s^{-1}$, I_{max}=206 $\mu E \cdot m^{-2} \cdot s^{-1}$, I_{high} >900 $\mu E \cdot m^{-2} \cdot s^{-1}$). For waters with K_d values ≤0.04 m^{-1}, all *Gambierdiscus* species were projected to maintain positive growth to depths of 90-150 m (Fig. 2). Such low attenuation coefficients are not uncommon in the Caribbean region and have been measured in the Gulf of Mexico, Florida Strait s, Lesser Antilles, and across the Caribbean Sea, with slightly higher attenuation coefficients in near shore waters (0.05-0.07 m^{-1}) (NASA 2010).

If *Gambierdiscus* species do commonly occur at the projected depths (Fig. 2), then prevailing ideas about habitat space should be expanded. The current literature suggests that *Gambierdiscus* tends to be most abundant in shallow habitats (<30 m) (Taylor 1985 and references therein), though there are reports of significant population densities to depths of 45 m (Carlson 1985). We have routinely isolated *G. carolinianus* from macrophytes collected at >40 m on the outer shelf off North Carolina, USA, an area influenced by the Gulf Stream. These latter observations are consistent with the fact that *Gambierdiscus* species are likely distributed to greater depths than previously recognized. A future CFP research challenge will be to quantify *Gambierdiscus* cell densities in deeper water habitats and to determine if those populations are contributing significantly to the overall flux of ciguatoxins into the food chain.

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TIME SERIES

Is There a Link Between N:P Ratios and Red Tides in Tolo Harbour?

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Abstract

It has previously been reported for Tolo Harbour, Hong Kong, that the N:P ratio decreased from 20 to 11 while the number of red tide events increased from 10 to 20 from 1982 to 89. When this time series was extended for another 18 years (1990 to 2007), no significant relationship was found between ambient N:P ratio and the occurrence of red tides. When this period was divided into pre- (before 1998) and post (after 1998) sewage treatment, the pre-treatment period was potentially Si limited since the sewage discharge is P-rich (~10N:1P) and has little SiO4. This may be responsible for the increase in dinoflagellates compared to diatoms observed in the 1980s. Post-treatment, there was a 75% reduction in P and a 40% reduction in DIN which led to a significant increase in the N:P ratio (~10 to 30). The inner harbor is now potentially P-limited, but there was no significant change in the number of red tides during this pronounced increase in the N:P ratio from 1998-2007. In fact, the number of red tides started to decrease in 1991 for unknown reasons and has remained relatively constant to 2005.

Introduction

The term "red tide" refers to blooms of a wide variety of phytoplankton species and groups. In Hong Kong, these outbreaks are often dominated phototrophic (e.g. *Prorocentrum minimum*), mixotrophic (e.g. *Ceratium furca*) and heterotrophic (e.g. *Noctiluca scintillans*) dinoflagellates and some diatoms are also included. The ciliate *Myrionecta (Mesodinium) rubra* with its symbiotic cryptophyte also forms frequent red tides. The six most common red tide species in Hong Kong in descending order are: *N. scintillans, G. polygramma, Skeletonema costatum, M. rubra, P. minimum,* and *C. furca.* Red tides caused by heterotrophic *N. scintillans* account for 40% of the red tide events in Hong Kong waters (Yin 2003). Red tides are more common in spring in eastern waters that are far away from the Pearl River and dominated by dinoflagellates, while the summer red tides are dominated by diatoms, especially in southern waters (Yin 2003). Hodgkiss and Ho (1997) presented a provocative data set showing that during a 7 year period from 1982 to 1989, the N:P ratio in Tolo Harbour decreased from 20.3 to 11 and this was accompanied by a statistically significant increase in the annual occurrence of red tides from about 10 to 20. They suggested that one possible reason for this correlation is the fact that the yield of 7 red tide organisms in laboratory experiments was optimized at low N:P molar ratios of between 4 and 16, indicating that they have a high P requirement relative to N. There were rapid environmental changes during the 1978 to 84 period in Tolo Harbour, resulting in a 10-fold increase in PO_4, a 5-fold increase in NO_3, an 8-fold increase in phytoplankton, an 8-fold (2 to 17) increase in red tide events, a 3-fold increase (22-66% inner harbor) in dinoflagellates, a 6-fold increase in catchment population and a 2.5 fold increase in nutrient loading (Hodgkiss and Ho 1997). In this paper, we have analyzed ambient surface nutrient, Chl and red tide data for stations in inner Tolo Harbour using a much longer data set from 1986 to 2007 in an attempt to test if the Hodgkiss and Ho (1997) correlation between N:P ratios and red tide occurrences held up. When sewage was diverted from Tolo Harbour to Victoria Harbour, there was a dramatic decrease in effluent loading from about 2×10^5 m^3 d^{-1} to near zero between 1996 and 1998. We were thus able to compare a pre-treatment interval (1986-1998) with the post-treatment period (1998-2007). We also compared Tolo

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Harbour (always stratified) to Victoria Harbour (hydrodynamically active) to evaluate the role of hydrodynamics in red tide occurrences.

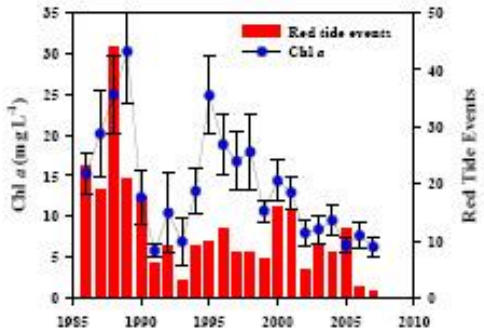

Fig. 1. Red tide events in Tolo Harbour (inner harbor) and the mean annual surface Chl-*a* concentrations (+/- 1 SD, n= 12) during pre-treatment (1986-98) and post-treatment (1998-2007) periods. Data from EPD and AFCD.

Materials and Methods

We used the dataset on nutrients (dissolved inorganic nitrogen (DIN) = NO_3+ NO_2+ NH_4), PO_4, SiO_4, and Chl-*a* from the Hong Kong Environmental Protection Department (EPD) from 1986 to 2007 (EPD 2007). Stations TM2, TM4, TM6 and TM8 represent a pronounced decreasing gradient in nutrient loading from the inner harbor to Tolo Channel, which had the lowest nutrient inputs (location of stations shown in Xu et al. 2010). The number of red tide occurrences was obtained from the Hong Kong Agricultural, Fisheries, and Conservation Department (AFCD 2007).

Results and Discussion

There was an increase in the number of red tide events in Tolo Harbour (Fig. 1) during 1986-90 with a peak in 1988, similar to the increase that Hodkgkiss and Ho (1997) observed. However, there has been a sharp decline starting in 1991, even before the 1998 reduction in sewage discharge. Red tides have remained fairly constant up to 2005 (Fig. 1). In contrast, Chl-*a* showed a significant ~3-fold decrease after sewage diversion (1998) and since there was no accompanying decrease in red tide events, Chl-*a* and red tide events are not necessarily coupled. Since sewage has a low N:P ratio of ~10 (i.e. P-rich), the ambient N:P ratio in Tolo decreased from ~16 to 10 from 1986-1997 and then dramatically increased to ~35:1 from 1998-2007 (Fig. 2) in response to changes in sewage loading. Xu *et al.* (2010) reported that DIN decreased by 40% and PO_4 showed an even larger decrease by 75% after the sewage diversion. Since sewage contains little SiO_4, that nutrient actually increased slightly, probably due to the decrease in uptake as a result of the 40% reduction in Chl-*a* after the sewage diversion. Hence, the Si:P and Si:N ratios increased ~10-fold and 5-fold, respectively (Xu *et al.* 2010) and thereby reduced the potential for Si limitation that was observed in the 1970s and 80s, but apparently switched the system over to potential P limitation because of the large reduction in PO_4 (Fig. 3). Therefore this large scale sewage diversion provided a natural experiment to demonstrate that red tide species did not respond to a significant increase in N:P ratios after the diversion and surprisingly the number of red tides began to decline in 1991, 7 years before the sewage diversion. In fact, Wong *et al.* (2009) suggested that the high number of red tides in 1988 may be due to a low wind year with 6 weak typhoons, while the lowest number of red tides in 1993 may be due to a high wind year with 9 large typhoons (Fig. 1). Since Tolo Harbour is stratified most of the year (Yin 2003, Xu *et al.* 2010), a condition that favors the occurrence of red tides, wind events should play an important role in the extent and duration of the stratification. In addition, Yin (2003) suggested that the NE monsoons produce downwelling which may help to concentrate the red tides in the inner harbor in winter. Generally, N:P ratios are considered to be a weak factor in influencing the competition between diatoms and dinoflagellates compared to Si:N ratios. Yin (unpubl results) also found a significant correlation between Si:N ratios and the occurrence of red tides in several other areas in Hong Kong waters. Yin (unpubl results) also found a significant correlation between Si:N ratios and the occurrence of red tides in several other areas in Hong Kong waters.

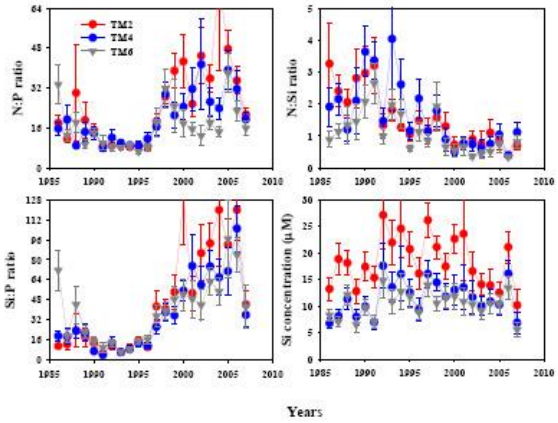

Fig. 2. Time series of N:P, N:Si and Si:P molar ratios (+/- 1 SD, n=12) for surface waters at 3 stations in Tolo Harbour during pre-treatment (1986-98) and post-treatment (1998-2007) periods.

Silicate could be a factor that could explain the shift from diatoms to dinoflagellates that was observed by Hodgkiss and Ho (1997). In 1974-75, Wear *et al.* (1984) observed SiO_4 concentrations of < 1 μM in Feb and March and it periodically decreased to <2 μM especially during April and May, but with high interannual variability (Xu *et al.* 2010). When we plotted the Si:P ratio against the N:P ratio, silicate was shown to be the potential limiting nutrient for the pre-treatment period (Fig. 3). This is not surprising since the sewage input up to 1998 had little SiO_4 compared to N and P and there is little river input into Tolo Harbour from the small Shin Mun River.In contrast, in Victoria Harbour, the highest algal biomass occurred in summer when N:P ratio was the highest and Victoria Harbour is only briefly and weakly stratified in summer due to the inflow from the Pearl River. After sewage treatment in 2001, there was a small but not significant increase in N:P ratio, except in summer when high N:P water from the Pearl River entered the harbor (Xu *et al.* 2010). Red tides seldom occur in Victoria Harbour because it is so hydrodynamically active with a resident time of only a few days and current speeds of up to 1.2 m s^{-1} (Yin 2003, Lee *et al.* 2006).

In summary, *Noctiluca* is the main red tide organism in Hong Kong waters, and since it acts as a grazer mainly on diatoms, it is only indirectly connected to nutrients.

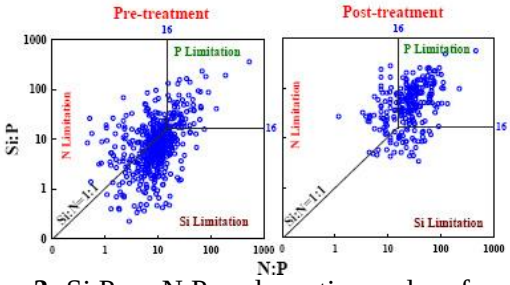

Fig. 3. Si:P vs N:P molar ratios and surface SiO4 concentrations in Tolo Harbour for Stns TM2 and TM4 during pre-treatment (1986-98) and post-treatment (1998-2007) periods indicating the potential limiting nutrient assuming Redfield ratios of 16N:16Si:1P.

In 1988, the maximum number of red tides occurred in both Tolo and Victoria Harbours, suggesting that some larger scale physical forcing was important. Therefore, winds, hydrodynamics, stratification, silicate and grazing likely play a more important role in determining red tides than N:P ratios. However, since there is very large interannual variations in this shallow land-locked inlet, different physical, chemical and biological factors probably interact to produce this variability.

Acknowledgements

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Prymnesium parvum blooms in south-central USA: Concerns of climate change and population growth

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Abstract

Prymnesium parvum blooms have occurred in south-central USA for at least ~30 years, with the last decade experiencing more frequent blooms. In the three lakes examined, bloom thresholds of 10^7 cells L^{-1} were observed as a function of inflow and salinity. In Lake Possum Kingdom, blooms occurred when 7-day accumulated inflows were $<10 \times 10^6$ m^3 and salinities were >1.5 psu. For Lakes Granbury and Whitney, blooms occurred when 7-day accumulated inflows were $<20 \times 10^6$ m^3 and $<40 \times 10^6$ m^3, respectively, and salinities were >0.5 psu. Inflow to these lakes exceeded thresholds during most spring months. Salinities typically exceeded these thresholds during the period of study prior to the spring of 2007, which was a period of high precipitation after which salinities were typically below thresholds. The linkage between incidence of *P. parvum* blooms, inflows and salinity is of concern because combined effects from human population increase and climate change could lead to periods of decreased inflow and increased salinity, which may then increase the frequency and magnitude of *P. parvum* blooms.

Introduction

The influence of inflows on phytoplankton dynamics and species diversity has long been an interest of ecologists and resource managers. The magnitude and timing of inflows produce variations in salinity, nutrients and flushing losses, which in turn, influence plankton community composition and productivity (Buyukates and Roelke 2005, Miller *et al.* 2008). Nutrient pulses and flushing losses associated with inflows have also been linked to the incidence of harmful algal blooms (Spatharis *et al.* 2007, Mitrovic *et al.* 2008), including toxic blooms of *Prymnesium parvum* where blooms terminate with the onset of large inflows (Roelke *et al.* 2010, 2011). In south-central USA, *P. parvum* blooms are recurrent and appear during times when salinity and temperature conditions are far removed from their growth optimum. In fact, maximum specific growth rates during periods of bloom are estimated to be ~0.1 d^{-1}, only ~12% of the optimum. In other words, blooms occur when reproductive growth is stressed. In the absence of significant loss factors, however, these low reproductive growth rates can still lead to blooms of high population density. Production of chemicals that are allelopathic and grazing-inhibiting can facilitate bloom development by minimizing loss factors that would usually arise in the presence of other phytoplankton competitors and healthy zooplankton. This appears to be the case for *P. parvum*, which produces greater amounts of grazing-inhibiting toxin when stressed (Uronen *et al.*, 2005; Granéli and Salomon, 2010), enabling blooms to form despite low rates of growth. Because *P. parvum* blooms occur during a time of year when maximum reproductive growth rates are low, they should be vulnerable to large inflow events, a sensitivity we show here.

Methods

P. parvum cell counts, inflows and salinity were compiled from monitoring activities of Texas A&M University, Brazos River Authority and Texas Parks and Wildlife Department in three lakes along the Brazos River: Lakes Possum Kingdom, Granbury and Whitney (1556 samplings

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total). Locations of sampling stations were system-wide and encompassed shore-based and open-lake locations for each lake. Estimations of *P. parvum* population density in surface waters were achieved using either settling technique or hemacytometer. Daily discharges from the Brazos River were measured at upstream locations for each of the lakes (U.S. Geological Survey monitoring stations). Salinities were measured using a refractometer.

Results and Discussion

Blooms formed during winter months when inflows were below critical levels and ceased when inflows exceeded critical levels during spring through early-summer months. This is shown for Lake Granbury (Fig.1a), a lake representative of others in the region. Critical inflow levels varied between lake systems, which were 10 x 10^6 m^3 for Lake Possum Kingdom, 20 x 10^6 m^3 for Lake Granbury (Fig.1b) and 40 x 10^6 m^3, for Lake Whitney (flow as presented here is cumulative over a 7-day period). These levels of inflow resulted in system-flushing rates comparable to the *P. parvum* maximum specific growth rate during the period of bloom. Inflows above the critical level were not a requirement for bloom decline, however, as blooms ended with only modest inflows during some years. Reasons for this might involve conditions under which toxins are produced. For example, toxin production by the Texas strain of *P. parvum* is sensitive to nutrient pulses (Grover *et al.* 2007). Inflows below the critical level might still load enough nutrients to halt toxin production, thereby removing *P. parvum*'s competitive edge. On at least one occasion, termination of a localized bloom was observed coincident with an inflow event where the lake water level rose, but no out-flow occurred (i.e., no removal of cells through system flushing, Schwierzke-Wade et al. 2011).

The *P. parvum* population declined 52%, where direct dilution only accounted for ~30% of this decrease. Toxicity was completely removed, however, and the bloom did not re-establish. The incidence of blooms was sensitive to small variations in salinity. For example, only when salinity exceeded 1.5 psu

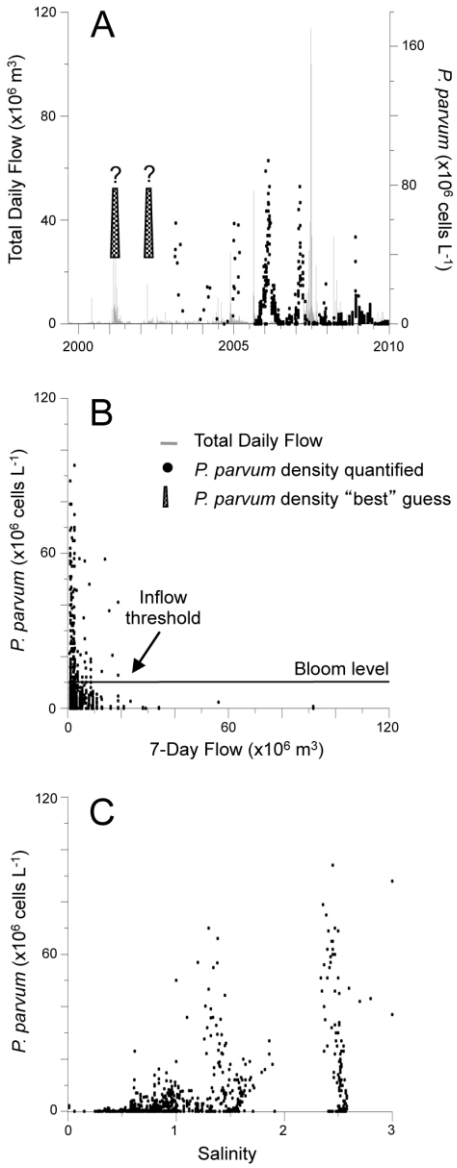

Fig. 1. A decade of lake observations in south-central USA (shown here for Lake Granbury, a representative lake of the region) revealed recurrent *P. parvum* blooms that were terminated with high inflows (A), and bloom threshold relationships with inflow (B) and salinity (C).

in Lake Possum Kingdom did system-wide fish-killing blooms occur. For Lakes Granbury (Fig. 1c) and Whitney, system-wide, fish-killing blooms occurred only when salinity exceeded 0.5 psu. During years after 2007, when salinities were frequently below these thresholds, *P. parvum* population

densities were reduced to ~30% of previous levels, and fish kills were small and localized.

Conclusions

P. parvum blooms are sensitive to inflows because conditions for growth during periods of bloom are sub-optimal. The linkage between incidence of *P. parvum* blooms, inflows and salinity raises concern because sequestration of water with rising human population continues to increase in many areas of the world where *P. parvum* is present. In some areas, this increased water sequestration may be exacerbated by climate change. For example, in the south-central USA variations in precipitation and evaporation predicted from climate change may result in flow decreases of 60%. In addition, it is likely that increased evaporation rates associated with regional warming will result in higher salinity. Consequently, both human population increase and climate change may lead to an increased incidence of *P. parvum* blooms.

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Evaluating the river de-eutrophication gain on the magnitude of *Phaeocystis* blooms in the Southern North Sea between 1985 and 2005: a model study

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Abstract

Despite success obtained in reducing anthropogenic nutrient (mainly phosphorus) loads since 1985, high-biomass blooms of undesirable *Phaeocystis* colonies yet occur each spring in the eutrophied Eastern English Channel and Southern North Sea area. An updated version of the existing MIRO model describing diatom/*Phaeocystis* blooms and related nutrient cycles in *Phaeocystis*-dominated ecosystems is here implemented in the Eastern English Channel and Southern North Sea over the 1985-2005 period to investigate the link between the magnitude of *Phaeocystis* colony blooms and the decrease in nutrient loads. The analysis of model simulations focuses on the Belgian coastal zone (BCZ), for which survey data are available for the period 1988-2000. The maximum cell abundance reached by *Phaeocystis* in the BCZ shows no clear trend, being modulated by the up and down fluctuations of available N stocks. On the contrary a 35% decrease of the bloom duration is simulated for the period and is correlated with the decrease in P loads.

Introduction

Anthropogenic eutrophication in the BCZ results from the input of transboundary (SW - Atlantic waters enriched by the Seine) and local (Scheldt) sources of land-based nutrients (nitrogen N, phosphorus P, silicon Si) (Fig. 1). The eutrophication problem in the BCZ is most visible as massive undesirable algal blooms in spring. These blooms are composed mainly of ungrazable colonial forms of the haptophyte *Phaeocystis globosa* (here referred to as *Phaeocystis*) that impact the ecosystem function and services (Rousseau *et al*. 2004). Accordingly, *Phaeocystis* has been identified as an indicator species of water disturbance (Tett *et al*. 2007). Statistical analysis of the 1988-2000 time series has shown that *Phaeocystis* blooms in the BCZ respond both to climate-driven hydrodynamic conditions and to anthropogenic nutrient loads through local rivers (Breton *et al*. 2006). In the BCZ, N and P delivery to the coastal sea has shown up and down variations over the last 60 years. After 1950, an accelerated increase of nutrient loads was reported due to a combination of increased human population, socio-economic development and intensive agriculture that cumulated in mid-1980s (eutrophication period; Billen *et al*. 2005). After this period, nutrient reduction measures were slowly implemented (de-eutrophication period), leading in particular to an important decrease in P loads (>75% decrease from 1985 to 2005). Here we discuss the potential effect of de-eutrophication on the *Phaeocystis* blooms in terms of bloom magnitude and duration in the BCZ, making use of an upgraded version of the biogeochemical model MIRO (Lancelot *et al*. 2005) over the period 1985-2005.

Material and methods

The biogeochemical model MIRO (Lancelot *et al*. 2005) describes carbon, N, P and Si cycles through aggregated components of the planktonic and benthic realms of the *Phaeocystis*-dominated ecosystem. For this application, the current version of MIRO has been upgraded on the basis of new process studies and a review of recent literature. The temperature adaptation of the diatoms has been reviewed, which has lead to the update of their maximum photosynthetic capacity rate at optimal temperature. The diatom Si:C stoichiometry is now related to the ambient Si (adapted from Rousseau *et al*. 2002). Half-saturation constants for the diatom uptake of nutrients have been updated on the basis of a literature review (Sarthou *et al*. 2005). The light

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adaptation parameter has been individualized for each phytoplankton group (diatoms, *Phaeocystis* and autotrophic nanoflagellates). Finally, in order to reflect the higher abundance of the copepods predators during summer (Daro *et al*. 2006), a temperature-dependant description of their mortality has been introduced.

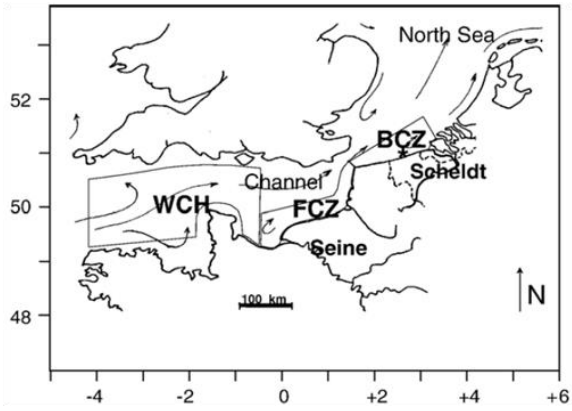

Fig. 1. Map of the studied area and the MIRO multi-box frame.

The MIRO model was implemented in three successive homogeneous boxes (WCH: Western Chanel; FCZ: French Coastal Zone; BCZ; Fig. 1) in order to take into account the cumulated nutrients enrichment of the Atlantic waters by the Seine (FCZ) and Scheldt (BCZ) rivers. The new version has been evaluated using climatologic functions (1989-1999) as in Lancelot *et al*. (2005) and cost function (C; Radach and Moll, 2006). Following the validation assessment of Radach and Moll (2006), model results are 'very good' (diatoms, *Phaeocystis*, dissolved inorganic N: C<1) to 'good' (PO_4, $Si(OH)_4$: 1<C<2).

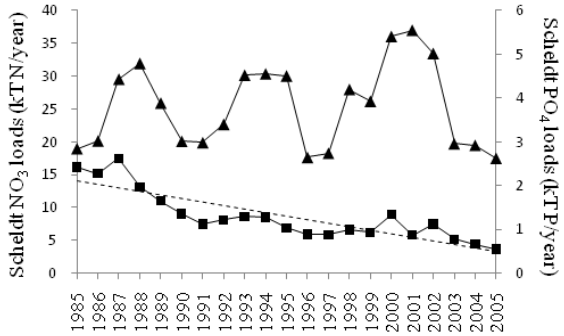

Fig. 2. NO_3 (triangles, left axis) and PO_4 (squares, right axis) Scheldt annual loads to the BCZ. The dotted line is the regression line for PO_4 loads (r^2=0.73).

For the present application, the forcing function included the incident Photosynthetic Active Radiation (PAR), the sea surface temperature (SST) and the nutrients loads as described in Gypens *et al*. (2007) but extended to the 1985-2005 period. The impact of de-eutrophication on the *Phaeocystis* blooms is analyzed in terms of maximum *Phaeocystis* cells reached and duration of the bloom. The latter is computed as the number of days when the *Phaeocystis* abundance is higher than the Lancelot *et al*. (2009)'s disturbance threshold of $4x10^6$ cells l^{-1} + 50%. The analysis of 1985-2005 fluctuations was realized through correlations between year-to-year variations of simulated *Phaeocystis* maximum cell abundance and bloom duration and the winter nutrient enrichment (mean of January, February and March simulated concentrations in the BCZ) computed using a cumulative sum (Cusum) function (*i.e.* a cumulative sum of the anomalies; Beamish *et al*. 1999).

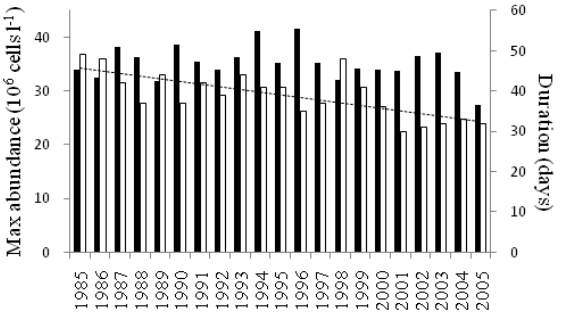

Fig. 3. Maximum abundance reached (black, left axis) and duration (white, right axis) of *Phaeocystis* blooms simulated by MIRO over the 1985-2005 period. The dotted line is the regression line for bloom duration (r^2=0.52).

Results and discussion

Interannual variation of NO_3 and PO_4 Seine and Scheldt loads showed similar trends over the period and is illustrated in Fig.2 for the Scheldt. Clearly PO_4 loads show a general decreasing trend over the period explained by the progressive implementation of waste water treatment and the substitution of PO_4 in washing powders (Billen *et al*. 2001, 2005). On the contrary, no trend is visible for NO_3 loads and their fluctuations correlate significantly with the river discharge (r^2=0.96 and 0.90 for the Seine and Scheldt river respectively). This is explained by the diffuse origin of NO_3 and by the delay in the implementation of agro-environmental measures for reducing NO_3 emission. As a consequence the NO_3: PO_4 ratio of river loads is dramatically increased from <20 in 1985 to

nearly >70 molN:molP in 2005, *i.e.* >4 times the Redfield ratio. The simulated *Phaeocystis* maximum abundance and bloom duration are shown in Fig. 3. No trend is observable for the maximum cell abundance which varies between ~$27x10^6$ cells l^{-1} in 2005 and ~$42x10^6$ cells l^{-1} in 1996, while the bloom duration decreased over the period from 49 to 30 days, although not regularly. The role of changing nutrient loads in modulating *Phaeocystis* bloom over the period was evaluated by comparing Cusum curves of *Phaeocystis* maximum abundance and duration with those of the winter nutrients stocks, all simulated by the model in the BCZ. These winter stocks reflect direct (Scheldt river) and indirect (inflowing Atlantic waters enriched by the Seine river, *i.e.* FCZ waters entering the BCZ) nutrient enrichment. A significant positive correlation (r^2 = 0.57) was found between *Phaeocystis* maximum cell abundance and the winter stock of dissolved inorganic nitrogen (DIN: NO_3 + NH_4) Cusum. This model result agrees with previous observation that NO_3 (the main DIN source) is mainly responsible for the higher occurrence of this HAB species in the area (Breton *et al*. 2006). The concentrations of N available in the water column result from a complex combination of NAO-driven hydro-climatic changes determining the spreading of the Scheldt plume and, hence, modulating the impact of N enrichment from the Scheldt river (Breton *et al*. 2006). These winter DIN stocks show no general trend over the period, and so does the maximum biomass reached by *Phaeocystis* colonies. This analysis over the two decades complements a previous study already pointing N (mostly through NO_3 loads) as the most important factor controlling the inter-annual variability in *Phaeocystis* growth (Gypens *et al*. 2007). The Cusum curves of the bloom duration and the PO_4 winter stock are positively correlated (r^2 = 0.67) suggesting that the *Phaeocystis* bloom duration is linked to the P enrichment and hence decrease over the period. Summarizing, this MIRO model application supports the little visible effect obtained on *Phaeocystis* bloom magnitude by the present-day reduction of N and P river loads. The link highlighted between *Phaeocystis* magnitude and N enrichment implies that a significant decrease in the blooms of this HAB species would not occur without a further significant decrease in N loads. Accordingly, one can expect these significant changes to be delayed by the diffuse nature of the main N source, *i.e.* NO_3 (mainly resulting from agricultural practice) which can be captured in water tables and then progressively restored with a memory effect. A further model study applying different nutrient reduction policies scenario indeed suggests agro-environmental measures to be necessary in order to substantially reduce *Phaeocystis* colony bloom magnitude on the long term (Lancelot *et al*.

Acknowledgments

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A decadal study of harmful algal blooms in three estuarine rivers of North Carolina, USA

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Abstract

A decadal study considering the interactions between phytoplankton populations and photic-zone physicochemical parameters was conducted on three coastal rivers (Neuse, Pamlico, and New Rivers). Over the ten year period, often dynamic inter-annual fluctuations in cell densities were observed in three harmful algal taxa - the cyanobacteria *Cylindrospermopsis raciborskii* and the dinoflagellates *Karlodinium micrum* and *Prorocentrum minimum*. *C. raciborskii*, tended to have a strong temperature response as it frequently bloomed during mid- to late summer (July to September) between 2005 and 2009. In contrast, *P. minimum* favored the cooler temperatures of winter and early spring (December to April), especially in 2006 and 2007. *K. micrum*, however, was prevalent throughout most of the study during all seasons. Nevertheless, peak abundances of *K. micrum* did occur between June 2001 and February 2002, March through October in 2006, and January 2008 through June 2009. To better understand these temporal variations in phytoplankton populations, we employed multivariate analyses to compare cell densities with ambient physiochemical parameters. In the Pamlico and Neuse Rivers salinity was an important factor in shaping algal populations. In the Neuse and New Rivers, nutrients (such as NH_4^+, NO_3^-, total P, and/or N/P ratios) were also important predictors of algal blooms.

Introduction

The Pamlico Sound is largely influenced by the quality of inputs from waters of the Neuse and Pamlico Rivers, and to a lesser extent the New River. The lower reaches of all three rivers are typically shallow oligo- to mesohaline with flushing times varying from weeks to months, depending largely on seasonal river discharge (late winter often being greater than summer and early autumn; Giese *et al*. 1979; Stanley and Nixon 1992). While N is considered the limiting nutrient for algal productivity within the lower reaches of these systems, increased human activities in eastern North Carolina have increased riverine N loadings (Hobbie *et al.* 1972). Thus, accelerated eutrophication and changes in phytoplankton community composition have largely been attributed to industrial, urban, and agricultural expansions within river basins (Piehler *et al*. 2004). As nutrient loadings have increased over the past few decades so has algal boom size and duration within these systems. While N inputs likely shape bloom events in these rivers, other environmental factors may also be involved in regulating these blooms. Therefore the purpose of this study was to characterize how environmental factors (nutrients and physico-chemical parameters) influence populations of two dinoflagelates (*Karlodinium micrum* and *Prorocentrum minimum*) and a cyanobacterium (*Cylindrospermopsis raciborskii*) over the last ten years.

Materials and Methods

This study utilized environmental and biological data collected in three coastal river systems (Neuse, New, and Pamlico Rivers) of North Carolina between January 2000 and December 2009. Samples were collected once monthly, with more frequent collections during periods of high phytoplankton productivity. Within each river, a suite of environmental parameters were monitored including nutrients such as NO_x^-, NH_4^+, total

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nitrogen (TN), and total phosphorus (TP; as described in Fensin 2006). A Hydrolab Surveyer-4 (Hydrolab Corp., Austin, Texas, USA) was used to evaluate subsurface physicochemical parameters such as dissolved oxygen (DO), pH, temperature, and salinity. Phytoplankton samples were collected within the photic zone (twice the Secchi depth) and preserved in the field with acidic Lugol's solution. Phytoplankton were numerated using an Utermöhl settling chamber as viewed under 300x magnification on a Leitz Diavert inverted microscope.

A multivariate canonical correspondence analysis (CAA) was performed for each estuarine river system on data collected over the ten year period using PC-ORD software (version 5; MJM Software Design, Gleneden Beach, Oregon, USA). Algal abundances and chlorophyll *a* values (as an estimate of total algal biomass) were compared against environmental variables, and the resultant factors after 100 randomizations were tested for significance at the level $p < 0.05$ using a Monte Carlo permutation test.

Results & Discussion

Within these rivers, seasonal variations strongly influenced algal density and distribution. There were strong temperature responses for *C. raciborskii*, as it frequently bloomed during mid- to late summer (July to September) between 2005 and 2009 (Fig. 1). *P. mimimum* was more prevalent during cooler periods (December to April), especially in 2006 (Neuse River) and 2007 (Pamlico River; Fig. 1). *K. micrum*, however, was prevalent throughout most of the study during all seasons, with peak abundances between June 2001 and February 2002 (all rivers), March through October in 2006 (all rivers), and January 2008 through June 2009 (New and Pamlico Rivers).

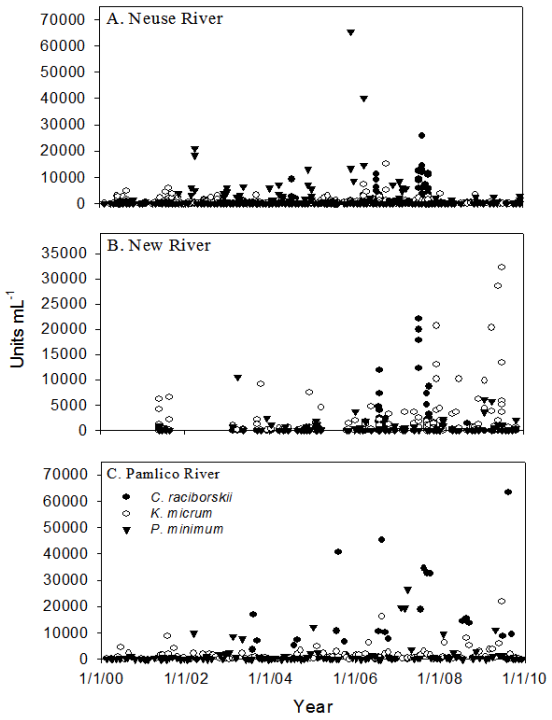

Figure 1. Algal unit abundance for *C. raciborskii* , *K. micrum*, and *P. minimum* in the Neuse River (panel A), New River (panel B), and Pamlico River (panel C). Data were collected over a ten year period for each river system.

CAA revealed significant patterns between harmful algal abundance and environmental physicochemical parameters for the first three components ($p \leq 0.01$ for all three rivers; Monte Carlo permutation tests). The first two factors explained 54% (Neuse River), 42% (New River), and 65% (Pamlico River) of the total variance. In general, eigenvalues were markedly higher for the first axis. Therefore, interpretations of the plots should largely be based on horizontal tendencies (Fig. 2). Overall, the strongest correlations between environmental parameters and the first two components were temperature (Axis-1 for all three rivers), TN (Axis-1 for New and Pamlico Rivers, Axis-2 for Neuse River), TP (Axis-1 for New River, Axis-2 for Neuse and Pamlico Rivers), and salinity (Axis-1 Neuse River, and Axis-2 New and Pamlico Rivers).

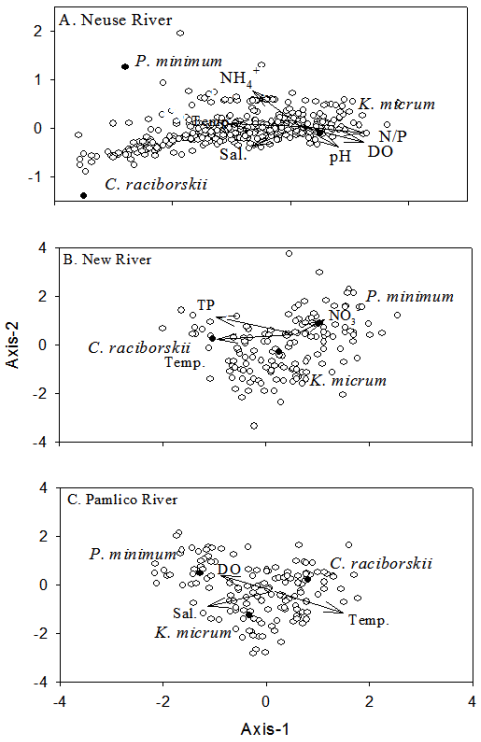

Figure 2. CCA defined ordination of algal abundance within environmental space for each river system. The strongest environmental variables (r^2>0.20 with any axis) are presented as vectors in the biplot overlay.

The only parameter strongly correlated with Axis-1 for all three rivers was temperature (Fig. 2). In temperate waters, seasonal changes can have profound control over the development and maintenance of HABs (Mallin 1994; Fensin and Touchette 2008). In this study, *C. raciborskii* and *P. minimum* had strong seasonal responses, wherein *C. raciborskii* abundances were highest during the warmer temperatures of late summer and early autumn (ca. 28°C), and *P. minimum* was most abundant during cooler winter / early spring temperatures (ca. 11.5°C). Interestingly, in tropical and subtropical systems, *P. minimum* has been observed in considerably warmer waters (between 22.5-33°C; Sierra-Beltrán *et al.* 2005). Nevertheless, our results are consistent with observations in northern Europe wherein the greatest abundance occurred around 14°C (Hajdu *et al.* 2005). While a number of physicochemical parameters likely influence HAB productivity in these river systems, it must be emphasized that these factors often work in concert with ambient nutrient conditions (Christian *et al.* 1986; Mallin 1994). In this study, both nitrogen and phosphorus levels were found to be important explanatory variables for HAB abundance in all three river systems. This finding is consistent with previous studies that observed higher *C. raciborskii* productivity during high P levels and higher dinoflagellate productivity during higher N conditions (Fensin and Touchette 2008).

Acknowledgements

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A Three-Year Time Series Of Toxic *Ostreopsis* Blooming in A NW Mediterranean Coastal Site: Preliminary Results

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Abstract

Epiphytic dinoflagellates of the genus *Ostreopsis* have been related to harmful episodes in many Mediterranean coastal areas since 1998: respiratory difficulties, fever, and skin irritations affected people exposed to marine aerosols by recreational activities on the beach. Although this genus is widespread in most of the Mediterranean coasts, harmful events have been recorded only in some localities and periods. One of those sites has been studied for 3 years in a high frequency monitoring (EBITOX project) in order to elucidate the relationship between the microalgal community and its toxicity.

Introduction

During the last decade, blooms of benthic dinoflagellates belonging to the tropical genus *Ostreopsis* have been recorded in many temperate areas worldwide as the Mediterranean Sea, or regions of New Zealand, Australia and Japan (see references in Shears and Ross 2009). This genus produces palytoxin, one of the most potent non-protein marine toxins known (Penna *et al*. 2005; Riobó *et al.* 2008). In the Mediterranean Sea, blooms of *Ostreopsis* during the summer period have been associated with human health problems such as respiratory irritation, fever, and skin irritations (Sansoni *et al.* 2003; Vila *et al*. 2008). Moreover, blooms of *O.* cf. *ovata* have also been related with mortality of a variety of marine organisms, in particular sea urchins (Graneli *et al*. 2002; Sansoni *et al*. 2003, Aligizaki *et al*. 2008, Shears and Ross 2009). The aim of this paper is to describe the relationship between the *Ostreopsis* population and its toxicity in Sant Andreu de Llavaneres (thereafter Llavaneres) beach (NW Mediterranean), where respiratory symptoms have been detected coinciding with *Ostreopsis* outbreaks. We, therefore, hypothesize that the respiratory symptoms are caused by the inhalation of aerosols containing palytoxin.

Study site

Llavaneres station is located in the central part of the Catalan coast (NW Mediterranean), in a populated area, 40 km north from Barcelona city. The station is a fossil beach in the middle of an extended sandy area. This site is highly colonised by different species of macroalgae (including the genus *Corallina, Stypocaulon, Dictyota and Padina,* among others) which are the substrate of the *Ostreopsis* population.

Background of harmful events in Llavaneres

In August 1998, benthic fauna mortality (1km affected) occurred near Riera d'Argentona after a raining storm, and fish mortality happened in the aquarium of the nearby Restaurant Pins Mar. The maximum *Ostreopsis* concentration detected was $2 \cdot 10^5$ cells$\cdot L^{-1}$. In August 2004, 74 people were identified to be affected by respiratory symptoms (Vila *et al.* 2008). Affected bathers of Llavaneres beach, people living in near shore apartments or working in the restaurant located close to the beach. Since then, many Mediterranean *Ostreopsis* blooms have coincided with respiratory problems in people staying near the beach, suggesting a possible link between them (International Workshop '*Ostreopsis*: is it a problem for the Mediterranean Sea?' Genoa (Italy), November 2005; www.apat.gov.it / site/_files/

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ConvegnoOstreopsis.pdf). Moreover, the usual symptoms (rhinorrea, nose irritation, throat irritation, coughing, expectoration, eye irritation, migraine and even fever) affect some days almost every summer worker of the Restaurant. Occasionally, after sampling in the area during the bloom, some of us suffered those symptoms and also experienced inflammation of skin cuts.

Materials and Methods

Seawater and macroalgae samples were collected monthly or biweekly in winter, and weekly or every 3-4 days in summer since 2007 to 2009 in the framework of the EBITOX project. *Ostreopsis* concentration was determined by light microscopy (LM) as described in Vila *et al.* 2001. *Ostreopsis* were identified by epifluorescence microscopy and molecular PCR-based assay (methods described in Penna *et al*. 2005; Battocchi *et al*. 2010 and Penna *et al.* 2010). Palytoxin was analysed by haemolytic assay (Riobó *et al*. 2008) and Liquid chromatography with fluorescence detection (LC–FLD), and the presence of palytoxin was confirmed by Liquid chromatography coupled to mass spectrometry (LC-MS) (Riobó *et al*. 2006). Seawater was processed following two methods, filtering 8 to 10 L in GF/C glass fibber filters and following the solid phase adsorption toxin tracking procedure (SPATT) described by Mackenzie (2010). This monitoring tool simulates the biotoxin contamination of filter feeding bivalves. Extracts obtained in this case were analyzed by haemolytic activity. Different extraction procedures have been used depending of the sample type. Strains isolated from Llavaneres were cultured in the laboratory, identified by morphology and molecular markers and their toxins analysed by the same chemical and biological methods. The limit of palytoxin detection by the methods tested here is 0.75 and 0.5 x 10^{-3} ng for FLD and haemolytic assay respectively. Aerosol samples were taken once a week during the bloom season in 2009 and during the bloom peak in 2010. Samples were collected with two high volume air samplers fitted with 15 cm diameter quartz fibber filters (Whatman, Maidstone, UK) installed near the beach. In 2010 the air samplers were working continuously for 3 days. The air volume filtered by the samplers was 30 m^3 /h. Filters were replaced every 6 or 7 hours. A total air volume of 1326 m^3 was filtered and then bubbled into a container with 6L of distilled water. Extraction from filters was performed with MeOH in a Soxhlet extractor with 10-12 hours cycles. Distilled water was evaporated to dryness and then dissolved in 30 mL of MeOH for toxin analyses. Aerosol filters were also processed by molecular PCR-based assay. Benthic marine invertebrates (*Paracentrotus lividus*) were collected from this location during the 2009 and 2010 blooms, and were analysed for toxicity. The intestinal content of sea urchins was observed under LM.

Results

Ostreopsis bloom dynamics in Llavaneres

The epiphytic *Ostreopsis* bloom started in late May-early July, and a few weeks later could be detected in the water column, where it reached maximum concentrations of 10^4-10^5 cells·L^{-1} in July-August; maximum epiphytic concentrations (above 10^6 cells·g^{-1} FW) were detected from mid July to mid November. Between December and April, *Ostreopsis* was not detected in the water, but few epiphytic cells were found sporadically.A large bacterial population can be found associated with the mucus and *Ostreopsis* cells. Identification of the bacterial community and their potential role in the toxicity of the bloom are currently being investigated.

Species composition

Two *Ostreopsis* species, *O.* cf. *ovata* and *O.* cf. *siamensis* have been identified to bloom together in Llavaneres by a molecular PCR-based assay using species –specific primers. However, all cells isolated to establish monoclonal cultures from Llavaneres were identified as *O.* cf. *ovata* indicating that *O.* cf. *siamensis* is much less abundant.

Toxicity

The bloom is toxic. Palytoxin, analyzed by haemolysis assay and HPLC-FLD, was detected in epiphytic samples taken during the bloom. Moreover, seawater extracts obtained following SPATT procedure showed typical haemolytic activity due to palytoxin. Instead, filtered seawater samples resulted negative.Toxins were also tested in cultures of *Ostreopsis* established from cells isolated during the bloom period in the study area. A palytoxin analog with a molecular weight of 2647 Da was found. The toxin content in cells from cultures has been estimated to be 0.3 pg/cell. Aerosol samples proved negative for toxins: palytoxin was not detected by LC-FLD in filters neither in distilled water. Filters and distilled water were found not to be toxic by haemolytic assay. However, PCR molecular analysis detected the presence of both *Ostreopsis* species in the aerosol filters.Toxicity was not detected by HPLC-FLD neither by haemolytic assay in sea urchins taken in the study area; although

Ostreopsis cells were observed by LM inside sea urchins.

Resting stages

Resting cysts were not detected in the survey specifically performed during no-bloom season on macroalgae and sediment in order to find the overwintering population. Pellicle cysts were formed in the laboratory from bloom samples which had been incubated in f/2 medium and aeration. These cysts were proven not to have dormancy period and were kept in dark at 15ºC. Thirty cysts were isolated after 2, 4 and 6 months to L/20 medium. Germinations of 100%, 32% and 80% were respectively observed. Due to unknown reasons, cysts from original samples decreased gradually in number and finally, after 8 months, they totally disappeared. Pellicle cysts were seldom observed both during the blooms and during non-bloom season usually associated with stressed vegetative cells.

Conclusions

The *Ostreopsis* bloom in Llavaneres follows a clear seasonality, blooming during summer months. The initiation is quite variable (May-July) and the bloom ends in October-November. Some years, a second peak of cells is detected in autumn, indicating no correlation between maximum cell concentrations and water temperature, as is also reported by Mangialajo *et al.* (2011). The bloom is toxic. Presence of palytoxin has been detected in the epiphytic community and in *Ostreopsis* cultures isolated from Llavaneres. However, palytoxin has not yet been detected in aerosol samples. The positive result of palytoxin in seawater (SPATT procedure), the detection of *Ostreopsis* in aerosol filters (PCR-based assay) and the observation of *Ostreopsis* cells in guts contents of sea urchins suggest us that the toxinological methods used are not sensible enough to detect palytoxin in the lower concentrated samples (filtered seawater, aerosol filters and sea urchins). Thus, the palytoxin concentration that causes human intoxication is below the limit of detection of our methodologies. Otherwise, the symptoms in humans might not be due to palytoxin intoxication but to allergenic processes.Whereas there is a clear coincidence in time between *Ostreopsis* blooms and human intoxications by inhalation, we are not yet able to demonstrate if the causative agent is palytoxin, the entire *Ostreopsis* cells, or another agent that causes an allergic reaction. Further studies, specially focused on toxinology, are needed in order to solve our hypothesis.

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Harmful Algae along Abruzzo coast from 2007 to 2010: correlations with environmental factors and new reports

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Abstract

The presence of new subtropical and tropical harmful species along the Abruzzo coast (Western Adriatic Sea) occurred in 2007 when the first appearance of *Prorocentrum lima* was reported. After that summer, other warm-water toxic species such as *Ostreopsis ovata*, *Coolia monotis* and *Prorocentrum levis* were observed. Besides, the ichthyotoxic species (*Fibrocapsa japonica*) have been detected before 2007. In this study 6 coastal sampling stations, a shellfish farm and four coastal rocky points were monitored from 2007 to 2010. Chemical-physical and nutritional parameters of sea water were correlated with the abundances of Harmful Algal taxa. Temperature has been positively correlated with harmful species ($p = 0.001$) while salinity was negatively correlated ($p = 0.001$). Nutrient factors have been correlated with harmful species such as DIN (Dissolved Inorganic Nitrogen), phosphate and silicate content. Statistical analyses suggest that HA are sensitive to phosphate content ($p = 0.001$). Phosphorus could be considered the limiting factor for HAB species.

Introduction

The presence of new tropical harmful species along the Abruzzo coast (Western Adriatic Sea) was confirmed in 2007 (Ingarao *et al*. 2009). *P. lima* produces DSP toxins (Diarrhetic Shellfish Poisoning) that can contaminate mussels causing gastrointestinal illness in humans (Quilliam & Wright, 1995).*O. ovata* is a common species in tropical areas. Its occurrence in coastal areas of the Adriatic Sea has probably been favoured by the increased water temperatures of the last decade (Russo *et al*. 2005). It clearly poses increasing risks for human health and effects on benthic communities. Blooms of *F. japonica* in the Adriatic Sea, instead, have been regularly observed since 1997, above all in summer periods (July - August). *Prorocentrum levis* was first isolated in 2002 from the Belizean barrier reef system and identified by Faust (2008). Its populations are often associated with floating mangrove detritus and sediments. The first report of this species in the Mediterranean Sea dates back to 2004 (Aligizaki *et al* 2009). Along the Adriatic coast it was observed in 2006 (Honsell *et al* 2008) for the first time, and reached the Abruzzo coasts in summer 2009. *P. levis* is considered toxic and produces the DSP toxins okadaic acid and DTX2. Among the 5000 species of marine phytoplankton, almost 300 species can occur in such high numbers that they obviously discolour the surface of the sea, while only 80 species have the capacity to produce potent toxins dangerous for humans (Hallegraeff, 2003). The main objective of this work was to study the influence of chemical-physical and trophic factors on the dynamics of potentially toxic phytoplankton in an area of the Western Adriatic Sea.

Materials and methods

In this study 6 coastal sampling stations, a shellfish farm and four coastal rocky points have been monitored from 2007 to 2010. The sampling stations were located using the GPS (Global Position System) Garmin GPS45 at the following points: Pescara stations located at 500 m and 3000 m from the mouth of Pescara River and affected by the fluvial inputs; Francavilla stations located at 500 m and 3000 m from the mouth of Alento River; Ortona stations at 500 and 3000 m respectively, shellfish farms located at 3 miles

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from the coast. Besides, rocky stations have been selected to localize epiphytic species like *P. lima*, *C. monotis*, *O. ovata* and *P. levis* due to their behavior to live on macroalgae and inside stagnant environments: Pescara harbor; Ortona harbor; San Vito Rocky point and Fossacesia rocky point (Figure 1). At each station, one seawater sample was taken at 0.5 m from the surface by means of a pump. Cell abundance concentrations were all expressed as cells L^{-1}, reading the entire well using a volume of 50 mL. Algal observations were conducted by the use of the light microscope (ausJENA Telaval 3) at 200, 400 and 1000x magnification. For taxonomic determination and plate tabulation of *O. ovata* and *Coolia monotis* we used an epifluorescent Zeiss Axiophot microscope at 200x, 400x and 1000x magnification following the Calcofluor method of Fritz & Triemer (1985). For SEM analysis of *O.* cf. *ovata*, *P. lima*, *Coolia monotis* and *Prorocentrum levis* cultures, we used a Philips XL–30–CP electron microscope following Zingone *et al.* (1990). Chemical - physical parameters of seawater were measured by a multi-parametric probe. Analyses of chlorophyll *a* and dissolved inorganic nutrients (DIN) silicate and posphate were performed following the method of Strickland & Parsons (1972). Potential nutrient limitation has been calculated as in Justic *et al*. (1995): the criteria of probable limitation are as follows: P limitation (P < 0.1 μM; DIN: PO_4 > 22; Si: PO_4 > 22), N limitation (DIN < 1 μM; DIN: PO_4 < 10; Si: DIN > 1), and Si limitation (Si < 2 μM; Si: PO_4 < 10; Si: DIN < 1).

Results and Discussion

The most representative abundances of HA during the period of study (2007 to 2010) were always measured in summer periods: June 2007 and June 2008 had great abundances of *Prorocentrum lima* species (4.7. 10^5 cells L^{-1} at Ortona harbor station; $6.9.10^3$ cells L^{-1} at San Vito Rocky point respectively).

The highest temperature values, instead, were always measured in August. During these periods, the greatest presence of the harmful species has been registered. Water temperature has been positively correlated with harmful algae abundances (p = 0.001; N = 528). On the contrary, salinity trend has been negatively correlated (p = 0.001; N = 528) meaning that there was a highly positive correlation between

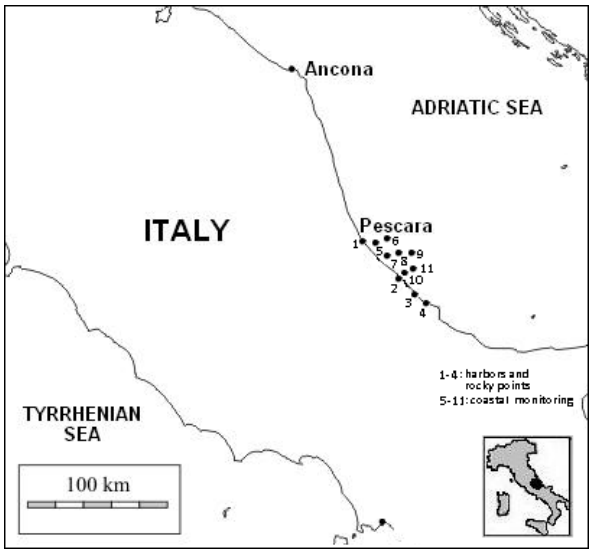

Fig. 1. Sampling sites

August 2009 and August 2010 had a maximum peak of *Fibrocapsa japonica* cell abundances (7.1. 10^4 cells L^{-1} at Pescara harbor; 1.2 $.10^4$ cells L^{-1} at Fossacesia Rocky point respectively).

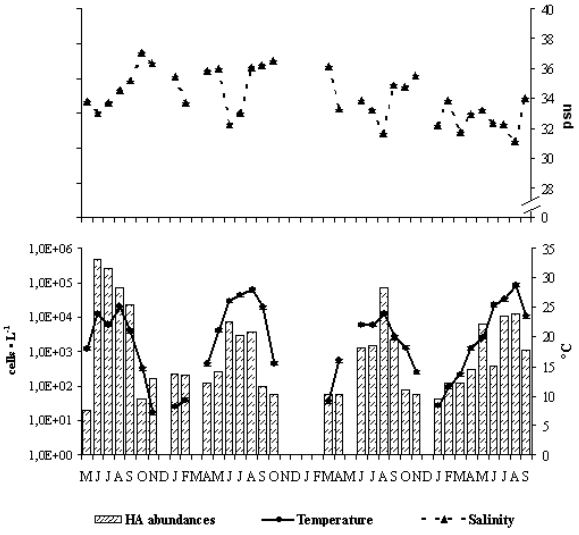

Fig. 2. Harmful algae abundances in relation to temperature and salinity variations

the abundances of HA taxa and low salinity (Figure 2). No significant correlation between DIN trend and silicate trend with the abundance of potentially harmful taxa was found (p = 0,05, N = 528). In contrast, phosphate concentrations were highly negatively correlated with potentially harmful

phytoplankton (p = 0.001, N = 528). Calculations of potential nutrient limitations in the coastal sites monitored over the four-year period were carried out. The DIN:PO4 and the Si:PO4 ratios exceeded the Redfield ratio and Justic adjustment (values of 34.19 and 24.09 respectively). The Si:DIN was 0.31. These data would indicate that P could be the potentially limiting factor in the studied area.Additional studies are needed in order to elaborate the ecology of potentially harmful algae in relation to environmental factors. Monitoring programs represent the most adequate action for the study of new potentially tropical toxic species along the coasts, harbors and rocky banks of Abruzzo Region.

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NEW REGIONAL EVENTS

Karlodinium veneficum as co-occurring bloom species of *Prorocentrum doghaiense* and *Karenia mikimotoi* in the East China Sea

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Abstract

A small unarmored dinoflagellate was recorded as co-occurring bloom species of *Prorocentrum doghaiense* and *Karenia mikimotoi* in the East China Sea (ECS), in late spring. The highest cell concentration of this species was 5.10^6 cells/L during the bloom. The species was confirmed as *Karlodinium veneficum* (D. Ballantine) J. Larsen both morphologically and geneticlly. The mean cell length of 70 cells was 15.3 µm with a standard deviation of ± 1.4µm and the mean width of cells was 11.7 µm with a standard deviation of ±1.3 µm. The ventral pore was located at the left side of the apical groove. Nucleus was large and positioned centrally at hypocone. There are two or four large irregular chloroplasts inside of the cell. The sequence length of ECS strain LAMB090611 is 640bp. The GC content is 49%. The Phylogenetic tree based on rDNA ITS sequence shows that strain LAMB090611 is clustered together with 3 other strains of *K. veneficum*.

Introduction

Special attention has been paid to the unarmoured dinoflagellates, because many species cause fish kill and other harmful events. *Karlodinium veneficum*, a cosmopolitan temperate dinoflagellate, is one of these unarmoured harmful species. Previously, *Karlodinium veneficum* was recorded as *Gyrodinium galatheanum*, *Gymnodinium galatheanum*, *Gymnodinium veneficum* and *Karlodinium micrum* (Bergholtz et al., 2005). It is known that most of the major genera of dinoflagellates were described during the late 1800s or early 1900s and were therefore defined based on morphological criteria visible under light microscope (Daugbjerg et al., 2000). At present, it is assured that *K. veneficum* belongs to the family –*Kareniaceae* that contains at least 20 formally described species (De Salas et al., 2008). Ballantine (1956)
described *K. veneficum* as possessing two to eight chloroplasts, typically with two chloroplasts in epicone and two in the hypocone, also with a sulcal extension and a centrally located nucleus. The species is known to be toxic to a variety of animals (Deeds et al., 2002). Karlotoxin including KmTx1 and KmTx2 has been isolated from strains of this species (Place, 2004; Deeds et al., 2006; Van Wagoner et al., 2008). *K. veneficum* exhibits mixotrophic features which have been also reported from other dinoflagellate species (Legrand et al., 1998; Li et al., 2000a,b; Smalley et al., 2002). Blooms of *K. veneficum* were first described from South Africa (Braarud, 1957; Pieterse et al., 1967), and later Europe (Bjornland et al., 1979; Nielsen, 1996) ,North America (Terlizzi, 2000; Li et al., 2000a) and Australia (Ajani et al., 2001). However, *K. veneficum* still remains poorly recorded globally as cells are fragile and difficult to preserve for LM and SEM. Large scale algal blooms caused by *Prorocentrum donghaiense* often occurred in the East China Sea (ECS) but *K. veneficum* has not been reported. The aim of this paper is to identify and clarify *Karlodinium* originally registered as small *Gymnodinium* isolated from the ECS. The morphological characteristics and molecular phylogeny of the strain were analyzed on the basis of LM, SEM, and ITS sequence data.

Materials and methods

Ballantine(1956)

Isolation and culturing. The natural sample of *K. veneficum* (LAMB090611) from was obtained from coastal water near Nanji island of Zhejiang province. The modified dilution method (Throndsen, 1978) was used for isolating single cells. The strain is maintained at 20°C in F/2 medium at salinity 30 psu and 12:12 L:D cycle at 66 µmol·photons·m^{-2}·s^{-2}.

LM and SEM. Live cells of *K. veneficum* LAMB090611) was observed using an Olympus CX31 and micrographs were taken with a Leica DFC 420 digital camera equipped on a Leica DM 2500 microscope. Cell length and width were

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measured from 50 cells in mid-exponential growth phase. DAPI was used for staining cells, then observed and photographed under fluorescence microscope Leica DM5000B. Mid-exponential growing culture was used for SEM examination. The concentrated culture was fixed by adding 3% OSO_4. The sample was critical point dried in liquid CO_2 in a Hitachi HCP-2 critical point drying apparatus. Cells were examined in a Hitachi S-3000N SEM.

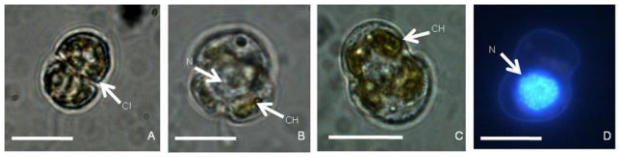

Fig. 1 Light and epifluorescence micrographs of *Karlodinium veneficum* (LAMB090611) (A) Cell in surface focus, showing cell shape and cingulum (arrow); (B) Cell showing chloroplasts and nucleus (arrow); (C) Cell showing four chloroplasts, two in epicone and two in hypocone (arrow); (D) Cell showing nucleus in hypocone (arrow) (Bar=10 μm). CI: cingulum; CH: chloroplasts; N: nucleus

DNA extraction, PCR amplification and sequencing. The genomic DNA was extracted from about 50 mL of early to mid-exponential growth phase culture. The genomic DNA was obtained by using the UNIQ-10 kit (Sangon, China), according to manufacturer's instructions. Subsequently total cellular DNA was used as template to amplify approximately 700 base pairs of the ITS rDNA gene using primers ITS4 and ITS5 (Hou et al., 2005). PCR amplifications were performed in volumes of 50μL: 1μL each primer (10 $\mu mol \cdot L^{-1}$); 5μL, 10XBuffer; 4μL, dNTP; 0.5μL Taq DNA polymerase (5 $unit \cdot \mu L^{-1}$); 2μL DNA extraction, finally adding deionised water to 50μL. PCR conditions were one initial cycle of denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The temperature profile was completed by a final extension cycle at 72°C for 5 min. The PCR products were purified using the Axygen PCR purification Kit (Axygen, USA), and nucleotide sequences were determined using the ABI Big Dye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (ABI, USA). The cycle sequencing reactions were performed using the ABI 3730XL DNA Sequencer (ABI,USA), according to manufacturer's instructions.

Sequence alignment and phylogenetic analyses. ITS sequences of the two strains were aligned with other ITS sequences of *Karlodinium*, *Takayama*, *Karenia* and *Heterocapsa* species in GenBank using the program ClustalW. The data of strains used in the phylogenetic analyses are shown in Table 1. Phylogenetic tree based on the ITS sequences of all strains, were carried out by MEGA 4.1 using the neighbor-joining (NJ), minimum evolution (ME), and maximum parsimony (MP) methods with bootstrap values calculated by 1000 replicates. Jukes-Cantor corrected distances between different strains were calculated by MEGA 4.1 with 1000 boostrap replicates. *Prorocentrum minimum* (FJ823585) was used as an outgroup.

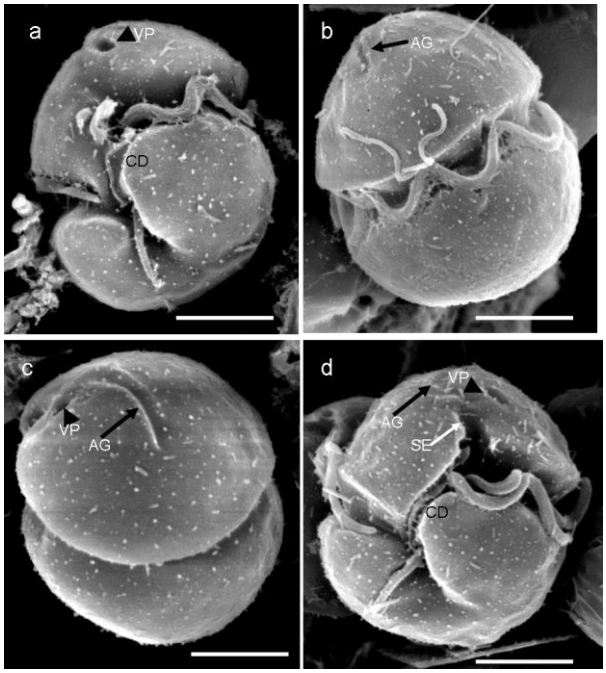

Fig.2 (A) Ventral view showing ventral pore(arrowhead), flagella and cingulum displacement; (B) Dorsal view showing cell shape with flagella in the cingulum and the apical groove (arrow); (C) Apical dorsal view showing apical groove (arrow) and ventral pore (arrowhead); (D) Ventral view of *Karlodinium* planozygote (Bar=5 μm) SE: sulcal extension; AG: apical groove; VP: ventral pore; CD: cingulum displacement

Results and discussions

Morphological observation. Average length of strain LAMB090611 was 14.2±1.8μm (range 11.1–18.7μm) and width 10.8±1.5μm (range 8.2–14.7μm) (*n*=50). Cell shape is oval and epicone and hypocone about equal size. The epicone is conical or rounded, and hypocone is hemispherically rounded (Figs.1, 2). The sulcus extension invading the epicone is clearly present/visible. Cells contain four chloroplasts, two in epicone and hypocone, respectively (Fig. 1c). The nucleus is large, round and located in the hypocone (Fig. 1d) or centrally (Fig. 2b).

Sequence analysis. We obtained corrected ITS sequences of strain LAMB090611 with 640 base pairs. GC content was both 49%. The corrected sequence is compared with *K.*

veneficum (GU263462,AJ557028,AJ534656), and *K. micrum* (AJ557026, DQ459434) Sequences of LAMB090611 and GU263462 were identical.

Phylogenetic analysis. The sequences of different strains of *Kareniaceae* and *Heterocapsa triquetra* were chosen to construct the phylogenetic tree. The NJ, ME and MP methods applied for phylogeny reconstructions. Strain LAMB090611 grouped together with other *K. veneficum* and *K. micrum* strains. Strain GU263462) is closer to *K. veneficum* (LAMB090611) than other strains. All strains of *K. veneficum* and *K. micrum* clustered with *K. armiger*. These groupings are well supported by high bootstrap values (99%). The branching order revealed *Karlodinium* is the sister to *Takayama*, and the two genera formed the sister group to *Karenia*. This result is in agreement with other reports (Garces et al., 2006). *K. veneficum* is the type species of the genus *Karlodinium*. It has been reported from many parts in the world (Bergholtz et al., 2005). *K .veneficum* usually is present at low abundance (10^2–10^3 cells.ml^{-1}) but capable of forming blooms of 10^4–10^5 cells.ml^{-1} associated with fish kills (Deeds et al., 2002; Kempton et al., 2002; Fensin, 2004; Goshorn et al., 2004). The first description of a *Karlodinium*-like species was by Ballantine (1956), who described two cultures isolated from Plymouth as *Gymnodinium vitiligo* and *G. veneficum* (De Salas et al., 2005). The species was described by different scientists under different names. *K. micrum* was widely used in Europe and America, later ultrastructural examination of *K. veneficum* by Bergholtz et al.(2004) from the Plymouth culture suggested that this species was indistinguishable from *K .micrum*, so *K. veneficum* would take precedence. *K. veneficum* has been recorded as small *Gymnodinium* co-occuring with *Prorocentrum donghaiense* and *Karenia mikimotoi* in the ECS. Highest cell concentration was 5.10^6 cells/L (Lu, unpubl.). Therefore fish kill events in the ECS seem not only attributed to *K. mikimotoi* (Li et al 2009) but may also be caused by simultaneous occurrence of *K. veneficum*.

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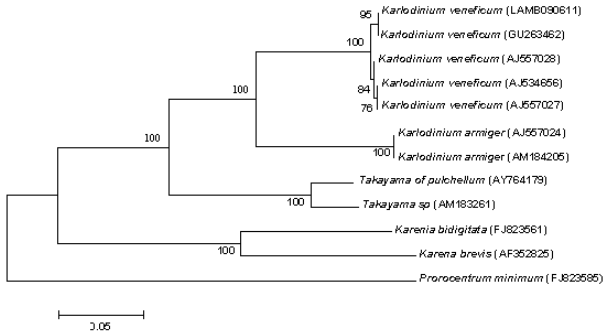

Fig. 3. Phylogenetic trees of the family Kareniaceae based on tITS–5.8S rDNA. (0.05 was the nucleotide substitution).

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Karenia species in the Mexican Pacific

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Abstract

From a review of phytoplankton samples collected in various localities of the Mexican Pacific and especially in Acapulco Bay, nine species of *Karenia* were identified: *K. asteriochroma*, *K. bicuneiformis*, *K.* cf. *breve*, *K.* cf. *brevisulcata*, *K. mikimotoi*, *K papilionacea*, *K. selliformis* and two unidentified taxa. Only *K. mikimotoi* has bloomed in Manzanillo Bay (March-31 to May-22, 2007) with a density of up to $8.7x10^6$ cells L^{-1} in April. The most common species in Acapulco are *K. mikimotoi* and *K. selliformis* with densities up to 1,750 cells L^{-1} in surface water (1-3m). Some organisms like *K. breve*, but smaller, were found (named as *Karenia* sp. 1). They are consistent with *K. papilionacea* life cycle stages, which is rare but widely distributed in the Mexican Pacific. *K. bicuneiformis* is restricted to the Gulf of Tehuantepec and Acapulco, and *K. asteriochroma* to Acapulco. Considering that for *K. brevis* the density threshold for fishing bans is $5x10^3$ cells L^{-1}, and that two of the species reported here (*K. mikimotoi* and *K. selliformis*) are ichthyotoxic , these species could represent a risk in the Mexican Pacific.

Introduction

Karenia species, together with naked dinoflagelates of the *Takayama* and *Karlodinium* genera belong to the Kareniaceae family, established by Berholtz *et al.* (2005). These organisms are photosynthetic and characterized by either straight or S shaped apical grooves and some particular accessory pigments: 19'butanoyloxyfucoxanthin, 19'hexanoyloxy fucoxanthin and 19'hexanoyloxyparacentrone3-acetate (gyroxanthindiester; Bjornland *et al*. 2003; De Salas *et al.* 2003; Pederson *et al.* 2004). Gómez (2006) suggest that *Brachidinium* species are relatives of *Karenia*, based on findings of intermediate forms. Some Kareniaceae are associated with HABs and produce ichthyotoxins (brevetoxins, karlotoxins and gymnodimines) that can cause marine animal illness and mortality (Steidinger *et al.* 2008). *Karenia brevis* is responsible for NSP (Neurotoxic Shellfish Poisoning) mainly in the Gulf of Mexico, affecting fish, dolphins and manatees; *K. mikimotoi* (described from Japanese waters) and *Karlodinium beneficum* cause fish kills affecting the economy of aquaculture industries (Dickman and Tang 1999; Deeds *et al.* 2002). Until 2006, sixteen species of *Karenia* have been recognized, however *K. bidigitata* could be synonymous with *K. bicuneiformis*. Depending on the length-width relationship two groups are recognized, the *brevis* type and the *mikimotoi* type. For the Mexican Pacific there exist no previous reports of *Karenia*.

Study Area

The Mexican Pacific has a winding coast line 7146 km long with a great variety of coastal environments (Moreno-Casasola and Castillo 1992; Lankford 1977). Cross by the Cancer tropic, it presents a subtropical temperate region on the north and a tropical region on the south, interrupted by the Tehuantepec Gulf a high productivity area due to the eolic winter upwellings (Vázquez-Gutiérrez *et. al.* 1998).

In this wide region, a multiyear phytoplankton collection exists, with samples taken from oceanographic ships or from motorboats. Sites where *Karenia* spp. have been found are marked in Figure 1: Cuenca Wagner in the Gulf of California, Manzanillo Bay, Acapulco Bay in the central region and Madero Port in the south.

Methods

Samples were collected with Van Dorn bottles and fixed with acidified Lugol's solution. They were concentrated by Utermhöl´s method to observe

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them in an inverted microscope. Additionally, in Acapulco Bay, living samples were collected and concentrated by inverse filtration in order to observe live organisms. The taxonomic characteristics assessed were: shape, dimensions (including total length (TL)/ width (W) ratio), width transdiameter (Tr), thickness (Th), nucleus position and chloroplasts' shape.

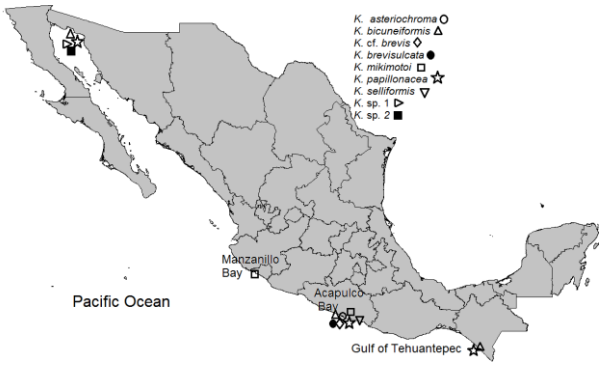

Fig. 1. Map of study area, and distribution of the *Karenia* species in the Mexican Pacific.

Species descriptions

mikimotoi type species

K. asteriochroma de Salas, Bolch *et* Hallegraeff (Fig. 2).

Nucleus in the center of the epicone, chloroplasts elongated, radiating in a star shape. Toxicity non confirmed. TL: 26.6-40.8µm; W: 33.6-50.4µm; Tr: 26.4-42.96µm; TL/W: 0.79-0.82; Th: 17-25µm (De Salas *et al*. 2004).

K. bicuneiformis Botes, Sym *et* Pitcher (Fig. 3).
Synonym: Probably *Karenia bidigitata* Haywood *et* Steidinger.

Hypocone is w-shaped; nucleus in the left side of the hypocone. Chloroplasts circular, dispersed and peripherals. Toxicity unknown. TL: 37µm; W: 35µm; Tr: 27.5µm; TL/W: 1.05; Th: 5-10µm (Botes *et al*. 2003).

K. brevisulcata (Chang) Hansen *et* Moestrup (Fig. 4).
Synonym: *Gymnodinium brevisulcatum* Chang.

Nucleus spherical, in the left side of the hypocone. Chloroplasts two or many, peripherals. Toxic but toxins unknown. TL: 13-18µm; W: 12-20µm; Tr: 8-1µm; TL/W: 1.1-1.16; Th: 5-10µm, 9-11 µm (Chang 1999).

K. mikimotoi (Miyake *et* Kominami *ex* Oda) Hansen *et* Moestrup (Fig. 5).
Synonym: *Gymnodinium mikimotoi* Miyake *et* Kominami *ex* Oda.

Nucleus elongated, located in the left side of the cell. Chloroplasts few or many green-yellowish, the number changes with the edge. Ichthyotoxic (hemolisines). TL: 29-48µm; W: 25.8-42.3µm; Tr: 20.7-35.3µm; TL/W: 1.12-1.13; Th: 21.15 µm; 13-29µm (Oda 1935).
This species produced a HAB in Mexican Pacific in Manzanillo Bay (March-31 to May-22, 2007) with a cell density up to 8.7 x 10^6 cells. l^{-1} in April.

K. selliformis Haywood, Steidinger *et* McKenzie (Fig. 6).

Nucleus ellipsoid, oblongus, horizontaly located in the hypocone, Chloroplasts many and peripherals. Non toxic. TL: 21-22µm; W: 17-21µm; Tr: 12-14µm. TL/W: 1.02-1.25; Th: 5-10µm (Haywood *et al*. 2004).

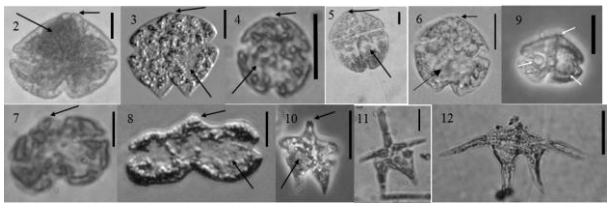

Fig. 2-12. Species of *Karenia* in the Mexican Pacific. 2) *K. asteriochroma*, ventral view; 3) *K. bicuneiformis*, ventral view; 4) *K. brevisulcata*, dorsal view; 5) *K. mikimotoi*, ventral view; 6) *K. selliformis*, ventral view; 7) *K.* cf. *brevis*, ventral view; 8) *K. papilionacea*, ventral view; 9) *K.* sp. 1; 10) *K.* sp. 2; 11) *Asterodinium gracile*; 12) *Brachydinium capitatum*. Arrows show nucleus and carina.

brevis type species

K. cf. brevis (Davis) Hansen *et* Moestrup (Fig. 7).
Nucleus not well observed. Many green chloroplasts. TL: 21-35µm; W: 25-50µm; Tr:

19-32µm; TL/W: 0.76-1.13; Th: 10-15µm (Steidinger *et al*. 2008).

K. brevis is considered an endemic species from the Gulf of Mexico, which is the reason we considered as another species for us.

K. papilionacea Haywood *et* Steidinger (Fig.8).

Nucleus rounded or spherical, located in the left side of the hypotheca. Many green-yelowish chloroplasts and peripherals. Toxicity non confirmed. TL: 17-20µm; W: 28-48µm; TL/W: 0.79-0.82; Th: 10-15µm (Haywood*et al*. 2004).

***Karenia* sp. 1 (**Fig. 9).

TL: 13µm; W: 17µm; Tr: 13 µm; Epi: 6µm; Hipo: 7µm; TL/W = 0.76.

These organisms like *K. breve*, but smaller, that could be consistent with *K. papillonacea* stages of life cycle.

***Karenia* sp. 2** (Fig. 10).
TL: 30 µm; W: 23 µm; T: 16 µm; Epi: 12µm; Hipo: 16µm.

May be a *Brachidinium* species. In the Mexican Pacific we found *Brachidinium capitatum* Fig. 12 and *Asterodinium gracile* Fig. 11.

Distribution

Karenia papilionacea is rare but widely distributed in the Mexican Pacific and in the upper Gulf of California, Acapulco and the Gulf of Tehuantepec. In Acapulco Bay, the most common are *K. mikimotoi* and *K. selliformis* with densities of up to 1,750 cells. l^{-1} in surface water (1-3 m).

The rarest species and restricted to a certain region are: *K. bicuneiformis* in the Gulf of Tehuantepec and *K. asteriochroma* in Acapulco (Fig. 1). In conclusion considering that for *K. breve* the density threshold for fishing bans is 5.10^3 cells. l^{-1}, and that two of the species reported here (*K. mikimotoi* and *K. selliformis*) are ichthyotoxic (hemolysins_and gymnodiminas), we consider that these species could representa risk in the Mexican Pacific, also because they have been found associated with *Gymnodinium catenatum*.

Acknowledgements

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Bloom of *Alexandrium* cf. *tamarense* (Dinophyta) in Oaxaca's Coast, Mexico, during May 2009

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Abstract

A bloom of *Alexandrium*, was detected in 2009 at the coast of Oaxaca, Mexico. The discoloration event (reddish-brown) started on 1st May at Salina Cruz (Gulf of Thehuantepec) and due to the effect of wind, it was moving to the west. Densities were: 1,417-56,644 cells ml^{-1}. The organisms looked like *A. tamarense* (Lebour) Balech due to their morphology and main taxonomic characters. Cells were solitary or in pairs, a ventral pore was in the upper left edge of the plate 1', a connecting pore was in the middle of the pentagonal posterior sulcal plate. Sixth precingular plate was wide. The apical pore has comma shape, and a small plate Po was aside. Mouse bioassay did not showed saxitoxin toxicity, although some times *Alexandrium tamarense* is reported as saxitoxin producer. Because *A. tamarense* is reported for temperate areas, and Oaxaca is in the Tropical Mexican Pacific, it may be a cryptic species.

Introduction

Alexandrium species are dinoflagellates with medium size theca, isodiametric shape or wider than longer, without antapical spines or apical horns: 4 apical plates and absence of intercalar plates. Plate 1' is irregular and rhomboid, somewhat oblique and frequently presents a small grove on the right anterior edge and together with plate 4' forms the ventral pore. Sometimes the 1' plate does not connect directly with the Po plate. Cingulum slightly displaced. Theca with fine pores. Cells frequently in chains (Halim 1960). Several species produce PSP causing saxitoxins (Taylor *et al.* 2003).In the Mexican Pacific there have been reported of 17 *Alexandrium´* species, but only 50% had an illustration (Okolodkov and Gárate-Lizárraga, 2006). Before the present study, there exist no reports of *Alexandrium* HAB in the Mexican Pacific coast.

Study Area

Huatulco Bays on the Gulf of Tehuantepec in the SE Mexican Pacific coast. Located between LN 15°07.8' to 15°51.17', and LW 96°13.47' to 97°07.01' (Fig. 1).

Method

Samples were collected on the water surface with a Van Dorn bottle, fixed with acetate Lugol. For identification, specimens were stained with trypan blue for at least 48 hours while on a dried preparation, then wet again and squashed. Cells were examined with a Leica optical microscope DMLB and Sedgwick-Rafter chambers were used for counting. Toxicity was tested using a mouse bioassay.

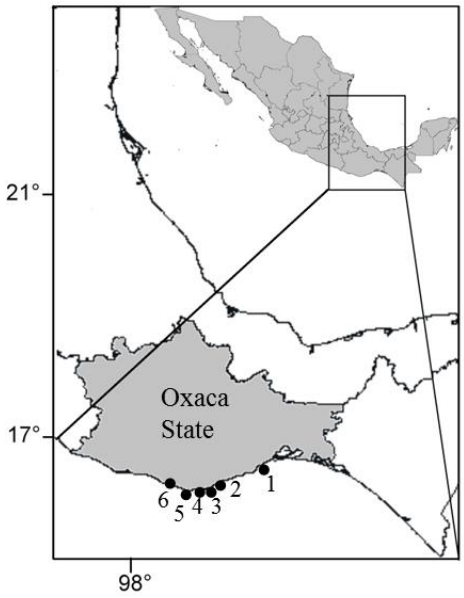

Fig. 1. Study area and sampling locations. 1. Salina Cruz, 2. Sta. Cruz, 3. Riscalillo, 4. Pto. Angel, 5. Zipolite, 6. Sta. Elena

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Results

From May 1st to 7th 2009, a HAB of dinoflagellates was present off the coast of Oaxaca. There was water discoloration (brown-reddish).First cells were found on May 1st in Salina Cruz and possibly due to the effects of wind and currents, the HAB was moving towards the West. Reaching densities up to 56 x 10^3 cells ml^{-1} on May 4th (Table 1; Fig. 1). The test of toxicity was negative: 27 to 60 µg Stxeq /100g oyster tissue. Environmental characteristics during the event were: Temperature 25.6-28.9 °C; Salinity 33.9-49.7; Dissolved oxygen 5.05-9.47 mg L^{-1} (Table 1).

Table 1. Date of sampling, *Alexandrium* cf *tamarense* densities and physico-chemical parameters in different locations.

Location	Santa Elena	Zipolite	Inner Pto. Ángel	Outer Pto. Ángel	Riscal illo	Santa Cruz
Date:05/09	6	6	6	6	4	4
Density cells/ml	49	876	1,146	296	1,417	56,64
Physico-Chemical measurements						
Salinity	33.9	33.6	49.7	28.9	49.38	31.9
Temp (°C)	25.6	26	26	28.9	26.1	25.9
O_2(mg/L)	6.39	6.34	6.89	9.47	5.05	8.77

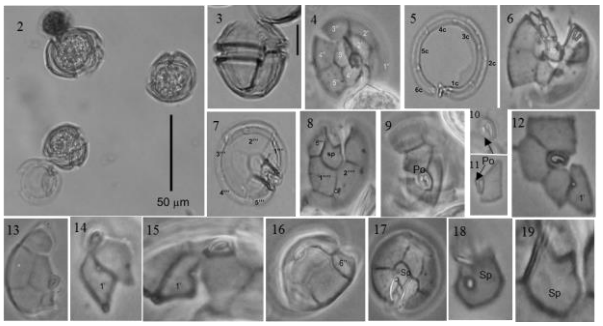

Fig. 2-19. *Alexandrium* cf. *tamarense* from Oaxaca´s coast, Mexico. 2. Solitary cells. 3. Ventral view. 4. Apical view. 5. Cingular plates. 6. Sulcal plates. 7. Antapical view. 8. Antapical and sulcal posterior plates. 9-11. Apical pore with Po plate. 12. Apical pore without Po plate. 13-15. Fist apical plate (internal view). 16. Lateral view.17. Antapical view, Sp with connecting pore. 18. Sp with connecting pore. 19. Sp without connecting pore.

Cell Morphology. The observed organisms (mainly solitary cells, Fig. 2) had a plate formula matching *Alexandrium*: Po, cp, 4', 6'', 6c, 9-10s, 5''', 2'''' (Figs. 3-8). The cell dimensions were: Total Length = 25-31 µm; Transdiameter = 22-24.5 µm; Width = 29.6-32.3 µm; Thickness= 28.7-29 µm; Cingulum displaced 1x. The apical pore has comma shape (length 5.6 µm and width 3.6 µm; Fig. 12), and sometimes a small plate Po (connective anterior pore) was aside. Figures 9-11 show organisms with and without connective anterior pore. A ventral pore was observed in the upper left edge of the plate 1' (Figs. 12, 14-15). Sixth precingular plate was wide (Fig. 16). Frequently a connecting pore was in the middle of the pentagonal posterior sulcal plate (Figs. 17-19). Even though only solitary cells were found, the presence of anterior and posterior connective pores suggests that the presence of organisms in pairs was common and possibly cells separated due to mechanical causes.

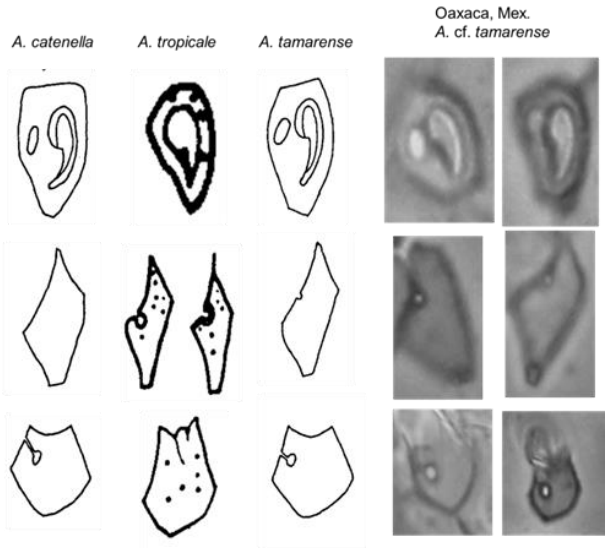

Fig. 20. Comparison of diagnostic characters of *Alexandrium* Mexican material with another species.

The main morphological characteristics were compared (plate pore, first apical and posterior sulcal; Fig. 20) with the following species: *Alexandrium catenella* (Whedon *et* Kofoid)

Balech, *A. tropicale* Balech and *A. tamarense* (Lebour) Balech (Balech 1995**).** The cells looked very much like *A. tamarense*. Lilly *et al. (*2007) in a study of boundaries and biogeography of the *A. tamarense* complex found that the morphologic groups don't agree completely with molecular groups.

We compared the material with other similar species such as *Alexandrium* sp. II Balech (Balech 1995), with a distribution in Mexican Pacific coast: Jalisco; and found differences.

In conclusion: this is the first record of a HAB caused by *Alexandrium* species in the tropical Pacific coast of Mexico. The morphology of the organisms correspond with *Alexandrium tamarense*, however this species has been reported in cold waters and in Mexico it was found in warm waters, which would be a change in its geographical distribution, therefore we think it may be a cryptic species and to prove this hypothesis a molecular study will be necessary.

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Bloom of *Pyrodinium bahamense* var. *compressum* at the Southern region of the Mexican Pacific

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Abstract

A bloom of *Pyrodinium bahamense var. compressum* was registered in Acapulco Bay (Guerrero, México) from July 7th to August 4th 2010. This bloom started in the state of Oaxaca (towards the East). The HAB didn´t cause water discoloration, possibly because the highest densities were from 3 to 5 m below the surface. Heavy rains were present before the event. The longest chains had 16 cells, with a mean of 8 cells; occasionally bigger and less compressed single cells were found. Among the dinoflagellates, *Pyrodinium* was dominant (88%), although few *Prorocentrum (P. gracile, P. triestinum*) and also some naked dinoflagellates (*Karenia* sp., *Gymnodinium catenatum* and *Gyrodinium fusus*) were found. The highest *Pyrodinium* cell densities (1.4. 10^6 cell l^{-1}) were from outside the Bay; inside the Bay the cell densities were 7.7. 10^5 cell l^{-1} at 3 m depth and 5.2. 10^5 cell l^{-1} at 5 m. Small diatoms were present; *Pseudo-nitzchia* spp. were important reaching densities of 14.0 10^3 cell l^{-1}. Saxitoxin concentration reached values of 2092 µg STX eq. 100 g^{-1} in violet oyster (*Chama mexicana*) near Puerto Marques Bay.

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Introduction

Pyrodinium bahamense var. *compressum* is one of the most toxic saxitoxin-producing species that causes PSP. There have been several *Pyrodinium bahamense* HAB events in the South Eastern Mexican Pacific, with an approximate periodicity of five and six years. The late 1995 and early 1996 HAB, on the coast of Guerrero and Michoacan, caused many poisonings and 6 people died. Concentration of saxitoxin in shellfish was 6,337 µg STXeq 100g $^{-1}$ in *Ostrea iridiscens* (Orellana *et al*., 1998). A bloom together with *G. catenatum*, occured from August 2001 to February 2002 and had densities of 3. 10^6 cells l^{-1}, relative abundance 88%, 100 people were poisoned and 9 died, fishing was banned 180 days and 48 tons of dead fish were recovered. Saxitoxin in shellfish was up to 7,309 µg STXeq 100g $^{-1}$ (Ramírez-Camarena *et al*. 2005)

Study Area

Acapulco Bay is located in the tropical Mexican Pacific on the coast of Guerrero state, between 16°35'24'' to 17°28'12'' LN and 99°25'12'' to 110°33' LW (Fig. 1).

Method

Samples were collected from the column water with a Van Dorn bottle in 8 locations at different depths: 1, 3, 5, 10 m and bottom, fixed with acetate Lugol. Sedgwick Rafter and Utermhöl´s chambers were used to count the cells. Weekly samples for cells counting were taken on July 10, 17, 24 and August 4th. Dates for saxitoxin evaluation were July 7, 8, 12, 19, 26 and 29th. Toxicity was tested using a mouse bioassay.

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Fig. 1. Map of study area.

Results

Cell morphology

Solitary or chains up to 16 cells, with a mean of 8 cells (Figs.2-3). Occasionally bigger and less compressed single cells were found or along with cells in chains (Figs. 4-5). Long antapical spine (Fig. 6). Cingulum displaced (Fig.7). Plate 1' is very reduced (Fig. 8) and 2 antapical plates (Fig. 9). Cysts (Figs. 10-11) were found on May 15th, however at that time no vegetative cells were observed. New cysts were observed in the water column again on August 4th.

Cell morphometry

The measurements include small and big cells. Total Length: 32.5-69.5 µm, Average: 55; Body Length: 24.78-49.6 µm, Average: 39.5; Transdiameter: 24.8-54.2 µm, Average: 39.9.

Pyrodinium cell density

The highest *Pyrodinium* cell densities were $1.4.10^6$ celsl l^{-1} outside the bay at 3 m; inside the bay the cell densities were 7.7. 10^6 cells l^{-1} at 3 m depth and 5.2 $.10^3$ cells l^{-1} at 5 m. On July 10th, maximum phytoplankton biomass was 46.2 mg Chl *a* m^{-3}. There were no color changes on the surface. The diversity index (H') values ranged from 3.84 to 0.91, with the lowest values in places where *Pyrodinium* was dominant.

Toxicity: Saxitoxin concentration reached values of 26 to 2,092 µg STX eq. 100 g^{-1}, the mean value of 30 oyster samples collected in different localities from July 8th to August 5th was high: 639.05 µg STX eq. 100 g^{-1}. Always the highest concentration occurred in local violet oyster (*Chama mexicana*) near Puerto Marques Bay, therefore a ban was established.

Accompanying species

Among the dinoflagellates *Pyrodinium* was dominant (88%), although few *Prorocentrum (P. gracile, P. triestinum*) were found, also some naked dinoflagellates (*Karenia mikimotoi, K. brevisulcata, Gymnodinium catenatum* and *Gyrodinium fusus*) were present. Small diatoms in chains were present: *Chaetoceros curvisetus, Skeletonema* sp. (very narrow) and *Pseudo-nitzschia* spp. The latter were important reaching densities of 1.04. 10^6 cells l^{-1}.

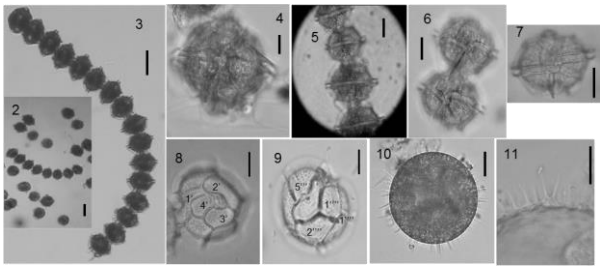

Fig. 2-11. *Pyrodinium bahamense* var. *compressum* from Acapulco Bay. 2. Bloom with solitary cells and chains. 3. Chain with 16 cells. 4. Ventral view of biggest cell. 5. Large and small cells in the same chain. 6. Cells with a long antapical spine. 7. Ventral view. 8. Apical internal view. 9. Antapical view, showing the posterior attachment pore (Pa). 10. Full cyst. 11. Typical bifurcate spines of *Pyrodinium* cysts. Scales: Figs. 2-3 = 50 µm. Figs. 5-10 = 20 µm. Figs. 4, 11 = 10 µm.

Physico-chemical Parameters

Temperature ranged from 28.9 to 29.1 ° C. Mean temperature increased one degree from June to July (27.7-28.7°). Before the heavy rainfall the salinity was 30 psu; and then decreased by two units due to the effect of the rainy season. The maximum concentrations of the nutrients appeared to be related to the formation of the HAB, especially for phosphates, whose concentrations varied from 0.02 to 5.7, Average = 3.34 mM L-1; ammonium 0.03-26.4, Average = 1.9 mM L-1, and nitrates 0.03-17.73, Average = 2.43 mM

L-1, concentrations were higher in the previous months. Using monthly averages of daily images from Modis-Aqua, it was observed that the HAB apparently started in May, 2010, in the Costa Rica Dome, moving to the Tehuantepec Gulf in June, and in July arrived to the Guerrero coasts, were Acapulco Bay is located. Finally it disappeared in August.

There exist strong correlations between the ENSO (El Niño-Southern Oscillation Events) and the *Pyrodinium* HAB in the W Pacific (Maclean, 1989) and in the first part of 2010 there was an El Niño event.In conclusion, the HAB observed was moderate compared to those that occurred in 1995-1996 and 2001-2002. It came from the oceanic area, and then picked up strength in the Acapulco Bay. During the HAB large and small cells occurred, but all were of compressed form. Since different sizes were found in the same chain, we could consider the formation of gametes or zygotes. The HAB was possibly favored by rains that increased nutrients, as cysts were noted since May but not vegetative cells. The finding of the HAB of *Pyrodinium bahamense* var. *compressum* in the Bay of Acapulco was interesting, coexisting mainly with diatoms, mostly of the genus *Pseudo-nitzschia* and *Chaetoceros*.

Acknowledgements

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Monitoring Program for Harmful Algal Blooms in Salvadoran Waters: Report of *Pyrodinium bahamense* from November 2009 to June 2010.

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Abstract

At the end of November 2009, several red patches were observed seven miles from the EL Salvador coast. This bloom was caused by *Pyrodinium bahamense* var. *bahamense* and var. *compressum*. Samples were collected at 72 sites over the continental shelf at the beginning of December 2009. In addition, four sites along the coast were selected for monitoring throughout the event. Tissue samples of bivalves (mouse bioassay) and 2 dying turtles (HPLC) were analyzed for saxitoxin. Cell concentrations of *Pyrodinium* were determined by the Utermöhl method. Maximum value was 15,3. 10^6 cells L^{-1} 60 km from the coast. In contrast only 22,000 cells L^{-1} were found at the coastal sites. The concentrations of saxitoxin ranged from 151 to 1427 STX eq/100 g in shellfish from samples collected from Salvadoran beaches. Toxicity was low in turtle tissues (brain and muscle) and faeces. This is the first time that local population has not been reported intoxicated during this event, thanks to the monitoring program and the response of local administration through shellfish industry closures.

Introduction

The Pacific coast of Central America has been impacted by blooming toxic species, mainly *Pyrodinium bahamense* var. *compressum* and several *Alexandrium* species, thus forcing a closure of the oyster industry (Mata *et al*.,1990; Vargas-Montero and Freer, 2003; Chow *et al* 2010). At the El Salvador coast there have been sporadic observations during notable blooms (Barraza *et al*., 2004; Licea *et al*, 2008). Recently a phytoplankton monitoring program was established in El Salvador in 2005 two months before a heavy PSP outbreak of *Pyrodinium bahamense* var. *compressum* in November 2005. This study reports the presence of two varieties of *Pyrodinium bahamense* and data on saxitoxin in some molluscs as well as 2 dying turtles along the coast of El Salvador from November 2009 to June 2010.

Material and Methods

Water samples were collected along the coast every 2 weeks from November 2009 to August 2010 (Fig. 1) as part of an established monitoring program, as well as tissues of shellfish were collected and kept frozen (-20°C) until analysis by mouse bioassays. In addition tissues of two dying turtles (*Eretmochelys imbricate*) found on the beach near Jiquilisco Bay in June 6th and July 13th, 2010 were analyzed by HPLC. Incidentally during an oceanographic survey on the R/V Miguel Oliver a water discoloration was observed at 68 km from the coast from December 3 to 13 in 2009. Water samples were collected for cell counting by the Utermöhl method (Edler and Elbrächter 2010). Results of the two varieties of *Pyrodinium bahamense* are given on Fig 3.HPLC analyses were carried out for each turtle tissue (about 2 grs) for toxin extraction in the HPLC system (HP 1100). Chromatography was performed as indicated by Hummert *et al.* (1997) and Yu *et al.* (1998). Toxicity factors to transform the analogs to saxitoxin equivalents were those given by Oshima (1995).

Results

Cell counts .Figure 2 shows the cell concentration of both varieties of *Pyrodinium bahamense*. The ranges found were from total

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absence of cells up to 40. 10^6 L^{-1} at Mizata. Jiquilisco and The Cobanos also had high numbers of cells. Figure 3 shows the cell concentration of both *Pyrodinium* varieties found during a water discoloration occurred at 68 km away the coast. The ranges found were from total absence to 9.7. 10^6 cells L^{-1}. The proportion of both varieties was about the same.

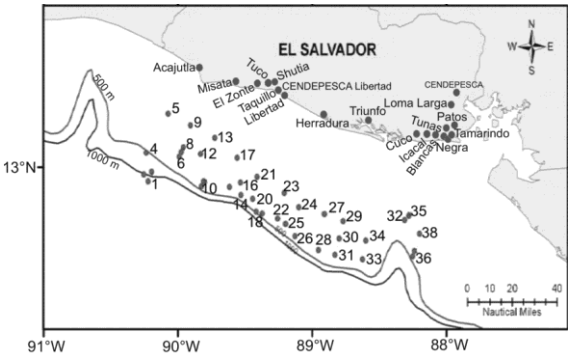

Fig.1. Sampling stations.

Mouse bioassay. The results show that PSP toxin concentrations in some of the shellfish samples were higher than the maximum permissible level established by USFDA for human consumption according to Anderson *et al.* (2001); that is 400 mouse units STX/100 g of shellfish wet weight. The ranges found between November 2009 to August 2010 were from 170 to 527 MU STX/100 g of shellfish wet weight (Table 1). As a result there was a forced closure of the shellfish industry.

Toxin in turtle tissues. Brains from one turtle (collected in July 13th, 2010) was the organ with the highest contentration of toxins (97.0 µg STX eq/80 g wet tissue) which is higher than the level permissible for human consumption. Other tissues (liver and muscle) as well as faeces contained no or relatively low levels of toxins (< 1 µg STX eq/80 g wet tissue, data not shown).

Discussion and conclusions

*Historical events.*Studies of harmful algae on Central America and in the southern Pacific of Mexico are very scarse, however there have been several anonymous reports of red tides at the Pacific coasts of Guatemala, El Salvador, Nicaragua and Costa Rica by institutions of Public Health of those countries, some of them associated to human intoxications. In the case of El Salvador, Fig. 2 was made using data from Licea *et al* (2008) to illustrate that the level of saxitoxins by *Pyrodinium bahamense* var. *compressum* have exceeded several times between 2006 and seems to be the cause of sea turtle mortalities in January 2006.

Table 1.

Localities	date	µg STX eq /80 g tissue	Shellfish
Los Cobanos	10/05/2010	139.38	*Crassostrea iridescens*
Mizata	10/05/2010	144.81	*Crassostrea iridescens*
Taquillo	10/05/2010	89.99	*Crassostrea iridescens*
Los Cobanos	10/05/2010	139.38	*Crassostrea iridescens*
El Zonte	10/05/2010	69.09	*Crassostrea iridescens*
El Zonte	10/05/2010	2.06	*Crassostrea iridescens*
Bahía Jiquilisco	13/05/2010	2.05	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	0.58	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	0.54	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	3.25	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	7.1	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	2.7	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	2.9	*Anadara granosa*
Bahía Jiquilisco	08/06/2010	6.7	*Anadara granosa*
La Palmita	22/07/2010	715.7	*Crassostrea sp.*
La Rana	24/07/2010	621.4	*Crassostrea sp.*

In Costa Rica blooms of *Pyrodinium* occurred in August 1987 extended to El Salvador and Guatemala (Mata *et al.* 1990). Vargas-Montero and Freer (2003) reported for the first time the occurrence of the morphotypes *P. bahamense* var *compressum* and var. *bahamense* during a PSP case occurred in December 2000. Recently, Chow *et al* (2010) reported a bloom associated to *P. bahamense* in November 2005 in Nicaragua with high concentrations of PSP; these authors mentioned historical blooms since 1987 with a total of 126 deaths. In the southern Mexican Pacific several toxic blooms of *Pyrodinium bahamense* var. *compressum* occurred in December 1985; December 1989 (Cortes-Altamirano et al. 1993); November 1992; November 1996; August 2001 to February 2002 (Sierra-Beltrán et al., 2004) and March 2006 and recently in June 2010 (unpublished

reports). During the bloom of 2001, rock oysters (*Crassostrea iridiscens)* contained 331 µg STX/100 g of flesh, which resulted in prohibition of their consumption by the sanitary control authorities who reported ten human intoxications and three child deaths.

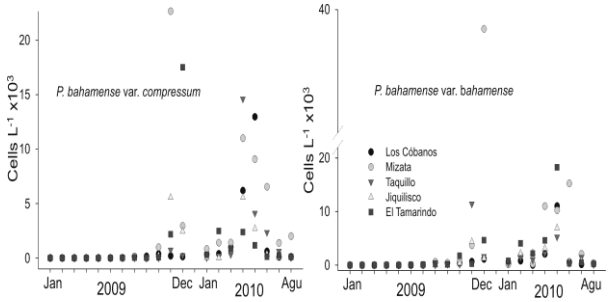

Fig.2. Cell concentrations of the two Pyrodinium bahamense varieties.

Toxins in turtles. Only one turtle had significant toxin concentration in the brain but both turtles had the same symptoms of diarrhea, erratic swimming, disorientation, and low movements. To our knowledge behavior of turtles poisoned by PSP is unknown therefore we cannot associate the observed symptoms with the presence of STX. By comparing toxin content in the same species of turtle during the 1996 event in the area, the toxin concentration in brain was almost 10x higher in 1995 (Licea *et al*., 2008). However, it is important to note that the turtle examined in 1995 was dead. A plausible hypothesis is that low levels of STX provoke the described symptoms and do not permit the turtle to feed and stay in the oceans and therefore are entrained in currents that allows for their onshore transport. Other compounds or toxins can also be acting. It can be concluded that there are favorable factors for the development of toxic blooms and harmful algae in Central America and that the influence of smaller scale oceanographic phenomena, such as oceanic gyres, upwelling and local conditions play an important role. Observed blooms of *Pyrodinium* and other toxic species may be explained by the oceanographic conditions in the Pacific of Central America where high values of chlorophyll-*a* extending from Costa Rica to the eastern part of the Gulf of Tehuantepec have been found (Chl-*a* images obtained through NASA.

Acknowledgments

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First report of *Heterosigma akashiwo*, *Fibrocapsa japonica* and *Chattonella marina var. antiqua* in Uruguay

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Abstract

Raphidophyceans had been reported worldwide associated with massive fish mortalities. The killing mechanism of raphidophyte blooms includes physical clogging of fish gills by mucus excretion and gill damage by haemolytic toxins. There is evidence of the production of superoxide and hydroxyl radicals, and some species and strains may contain brevetoxin-like neurotoxins and free polyunsaturated fatty acids. Massive mortalities of fish, often accompanying abrupt salinity changes are frequent along the estuarine and marine coastal waters of Uruguay. Considering these episodes and the possible harmful effects of raphidophyceans, which are difficult to identify in preserved samples, the National Harmful Phytoplankton Monitoring Programme has since 2007, included analysis of live samples. This led to the first report of *Heterosigma akashiwo*, *Fibrocapsa japonica* and *Chattonella marina* var. *antiqua* in Uruguayan waters. These species were observed in coastal unpreserved samples in summer-fall (January to May 2010), when water temperatures from 17 to 22º C and salinities of 24.7 to 33.4 occurred. The presence should be taken into account as a risk factor of fish mortality in these waters. Further research efforts are needed to study the accumulation of toxins produced by Raphidophyceae in fish and potentially harmful or toxic effect in fish consumers.

Introduction

Species within the class Raphidophyceae were associated with fish kills in Japanese (Toriumi and Takano 1973; Yoshimatsu 1987), Canadian (Taylor 1992), European (Billard 1992; Vrieling et al. 1995; Rademaker et al. 1998), U.S. and Chilean (Fuica et al. 2006) coastal waters. The killing mechanism of raphidophycean blooms includes physical clogging of fish gills by mucus excretion and gill damage by haemolytic substances (Shimada et al.1983; Chang et al. 1990). There is evidence of the production of superoxide and hydroxyl radicals as a mechanism of fish mortality by *Chattonella* and *Heterosigma* (Marshall et al. 2002, 2005; Twiner et al. 2004;Yang et al. 1995). Some species and strains may contain brevetoxin-like neurotoxins (Khan et al. 1996; Bridgers et al. 2002) and free polyunsaturated fatty acids **(**Marshall et al. 2002). In South American coastal waters both *Chattonella* sp. *cf. C. antiqua* and *Fibrocapsa japonica* were reported for the Patos Lagoon in Southern Brazil (Odebrecht and Abreu 1995), and *Heterosigma* akashiwo was reported in Chile (Parra et al. 1991) where it caused mortalities of important cultured salmon (Fuica et al. 2006). In the Atlantic side *H. akashiwo* has been observed as causing water discolourations in Argentine (Negri, pers. comm.) and in Brazil (Proença et al. 2004), where it was reported to be associated with mortality of benthic fish (Proença and Fernandes 2004). Massive fish mortalities are frequent along the estuarine and marine coastal waters of Uruguay particularly where salinity abruptly changes**.** This is the first report of three fish killing raphidophyceans for Uruguayan waters where fish mortalities are common.

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Materials and Methods

Raphidophyte flagellates are extremely fragile and generally best studied in unpreserved water samples. Considering the difficulties to identify raphidophytes in preserved samples, live unpreserved samples were added in the 2007 National Harmful Phytoplankton Monitoring Programme. Unpreserved water samples were taken weekly from coastal stations along the Uruguayan Atlantic coast from Jan 2007 to March 2010. Salinity and temperature were measured with a YSI (Mod 30) handheld System**.** Cells were observed with a Leitz Inverted Microscope equipped with Evolution LC digital camera. Single cells were isolated and grown in Guillard's F2 culture medium, in an incubator having 12/12 hr dark/light cycle and temperature of 20ºC.

Results and Discussion

Raphidophyceans were reported at the stations with oceanic influence (Fig. 1, St. 3 and 4).

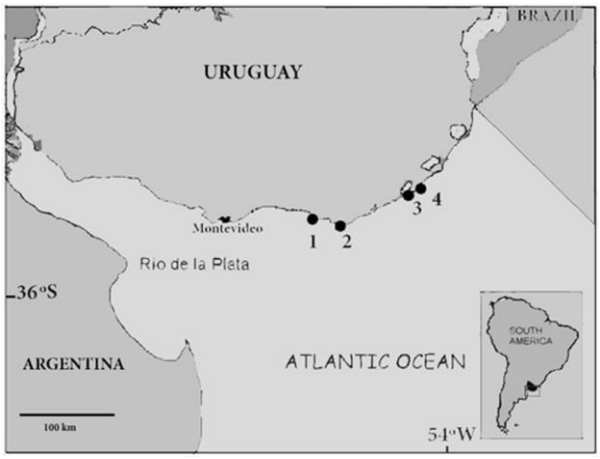

Fig. 1. Sampling Stations along the coast.

Chattonella marina var. *antiqua* (Hada) (Fig. 2A)

According to the recent taxonomic revision of *Chattonella* species (Demura et al. 2009), *Chattonella antiqua* has a new status as *Chattonella marina* var. *antiqua*. This species was observed at two occasions in the Uruguayan coast: St. 4 (Jan 16, 2007) and St. 3 (April 7, 2010), at salinities of 32/24.7 and temperature 20ºC, respectively. Morphological characters are coincident with Hallegraeff and Hara (2004). Cell dimensions: 73-78.5 µm in length and 28.5-38.5 µm in width, with a prominent posterior tail. A diplontic life cycle was described by Yamaguchi and Imai (1994), with cyst formation occurring after meiosis in vegetative cells. Cysts were not observed at field samples. Massive fish kills are known from Japan, Korea, the Netherlands, USA and Brazil. The toxic *C. marina* is capable to affect fish osmoregulatory homeostasis in vivo, possibly by triggering hyperactive excretion of Na^+ and Cl^- in gill chloride cells (Tang et al. 2007).

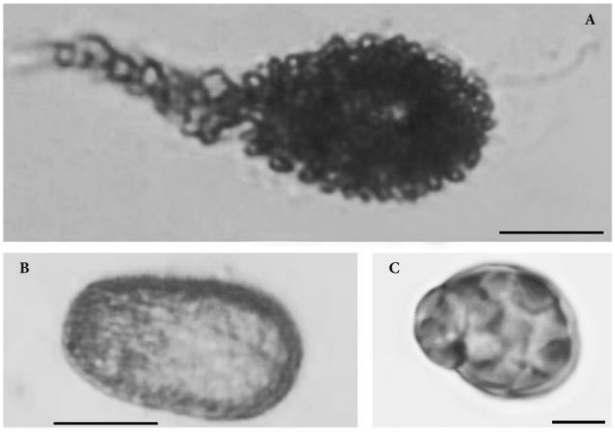

Fig. 2. (A) *Chattonella marina* var. *antiqua*, (B) *Fibrocapsa japonica*, (C) *Heterosigma akashiwo*.Scale bar A-B = 20µm, C = 10µm.

Fibrocapsa japonica Toriumi et Takano (Fig. 2B)

Fibrocapsa japonica was observed in coastal waters of Arachania (St. 4) on 20 January and 29 March 2010 when salinity of 29.8/33.4 and temperatures of 20/22ºC occurred respectively. Cells sizes were 30-37.8 µm length and 20-25 µm wide, slightly larger than the described by Toriumi and Takano (1973). All morphological characters corresponded to the description by Hallegraeff and Hara (2004). Rod-shaped conspicuous mucocysts, particularly in the posterior end of the cell, were observed to eject long threads**.** The nucleus was centrally located. *F. japonica* has worldwide distribution in temperate regions (Boer et al. 2005). *F. japonica* caused damage to coastal fisheries in Japan. According to Fua et al. (2004), the mechanism behind the toxicity of *F. japonica* is still under debate. Oda and co-workers (1997) showed the production of reactive oxygen species by a Japanese *F. japonica* strain that could damage fish gills. Polyether neurotoxins were found in an European strain and identified as brevetoxins-like on the basis of their chromatographic behaviour in HPLC (Khan et al. 1996), and brevetoxins were also recently detected using ELISA technique in American strains (Bridgers et al. 2002).

Heterosigma akashiwo(Hada) Hada (Fig. 2C)

Heterosigma akashiwo was found in coastal waters at St. 4 at Arachania beach on 29 March 2010, where a salinity of 29.8 and temperature 22º C were measured. Cell shape, chloroplasts, pyrenoid, nucleus, flagella and swimming pattern corresponded to the description by Hallegraeff and Hara (2004). Mucocysts were similar to those of *Chattonella* and *Fibrocapsa*. Cysts were not observed in field samples. Cell dimensions: 11.2-15.6 x 10-11.8 µm (N=9). *H. akashiwo* appeared to be a superior competitor at low phosphate, ammonium and nitrate levels, with somewhat higher maximum growth when nutrients are not limiting (Zhang et al. 2006). *H. akashiwo* produces organic compounds that can alter the metabolic activity of mammalian cells (Twiner et al. 2004). It is well known that *H. akashiwo* produce haemolytic activity and its presence during summer in Chile in 1988, caused the death of more than 2000 tons of salmonids (Fuica et al. 2006).

Ecological aspects

The report of these species is an important indicator suggesting a potential risk factor that now has to be taken into account in the Uruguayan massive fish mortalities. Further research is needed to define the accumulation of toxins produced by Raphidophyceae in fish and potentially their harmful or toxic effects on the ecosystem, including mammals. Along the entire sampling interval comprising more than three years, raphidophycean species were detected only during summer and fall period. Considering that temperature and salinity in this area during the study period was 10-24ºC, and 7.9-34.6 respectively, these three species were registered during the warmest period and at medium and highest salinities which reflect the estuarine environment with dominance of oceanic water over the freshwater discharge of the Río de la Plata.

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Observations on *Chattonella globosa* in Norwegian coastal waters Are *Chattonella globosa* and *Dictyocha fibula* one species?

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Abstract

Chattonella globosa Y. Hara & Chihara was observed for the first time in Norway in September 2007 where it appeared in eastern Skagerrak and later on at the west coast of Norway, presumably transported there by the Norwegian Coastal Current. The highest density of 15,000 cells/L was observed in inner Oslofjorden at low light conditions in November 2007. It reoccurred in September 2008 in Skagerrak and became the dominant autumn bloom species at our monitoring station in southern Norway (Arendal st.2, E08.8167, N58.3833 (EUREF89-WGS84)), where it constituted 54% of the total algal carbon. As in the previous year, *C. globosa* spread to the west coast reaching densities of 53,000 cells/L in early November co-occurring with *Pseudochattonella* sp. and *Heterosigma akashiwo*. During this bloom 100 tons of caged salmon died. A special observation was made during this blooming event, where cells of *C. globosa* were observed containing *Dictyocha fibula*-like skeletons in different stages of development. This observation was confirmed for at least 11 different localities along the west coast. Our findings suggest that *C. globosa* and *D. fibula* are related and may be stages in a common life cycle.

Introduction

Chattonella globosa Y. Hara & Chihara (cell diameter 30-55 micron) had not been reported from Norwegian coastal waters until cells identified as *C. globosa* were registered during the autumn of 2007 in Skagerrak and Oslofjorden. *Chattonella globosa* is known from eutrophicated coastal waters of Japan, South East Asia, Australia, Canada, the British Isles and Greece (e.g. Hallegraeff & Hara 2003, Ignatiades & Gotsis-Skretas 2010). Blooms in these areas have caused discoloration of the water and have been associated with fish kills. *Chattonella globosa*, earlier described as a Raphidophyte, has now been transferred to Dictyochophyceae based on molecular phylogenetic studies (Takano et al. 2007, Demura et al. 2009). The genus *Dictyocha* Ehrenberg is a habitual member of the Dictyochophyceae in Norwegian coastal waters. The most common and numerous species is *D. speculum* Ehrenberg, generally occurring throughout the whole year, occasionally forming blooms in Norwegian fjords. The less common *Dictyocha fibula* Ehrenberg (cell diameter 10-45µm) occurs in the autumn, but generally only in low numbers.

Material and methods

Water and net (20µm mesh) samples were collected at different depths in surface waters (upper 6 m) at stations along the southern part of Norway and in Skagerrak. The samples were observed live and/or preserved (formaldehyde and Lugol's solution). Cell counts were made using the Utermöhl technique (Utermöhl 1958).

Results

Chattonella globosa (Figure 1.1-2) has been detected in Norwegian coastal waters in autumn and early winter when light intensities are low, during 2007, 2008 and 2009 (Table 1) often co-occurring with *Heterosigma akashiwo* and *Pseudochattonella* sp. During the periods when *C. globosa* occurred, temperatures ranged from 6.0-15.8°C and salinities varied from 26.4-32.6. Only one year after it was

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reported for the first time, *C. globosa* was the dominant species during the autumn bloom of September 2008 at the monitoring station (Arendal st.2, E08.8167, N58.3833 (EUREF89-WGS84)), (Figure 2), reaching cell density of 36,800 cells/L and constituting 54 % of the total algal carbon. Two months later *C. globosa* appeared in a new bloom together with *Pseudochattonella* sp. in Rogaland at the south-west coast, where 115 metric tons of caged salmon died (Bergtun, Marine Harvest,

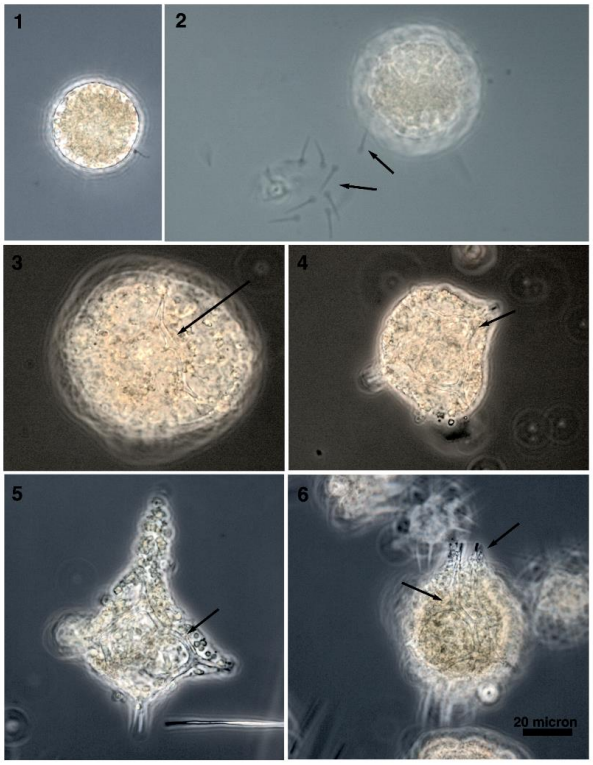

Fig. 1. *Chattonella globosa*. 1.1 with intact flagella. 1.2 releasing typical nail shaped bodies (arrows). 1.3 with skeleton in development (arrow). 1.4 with a skeleton outline of *Dictyocha fibula* inside (arrow). 1.5 A nearly fully developed skeleton of *Dictyocha fibula* inside the membrane of *C. globosa* (arrow). 1.6 cell with two skeletons inside (arrows).

pers. com.). Water samples collected during the fish-kill event showed cell density of 21,000 cells/L, but maximum concentration in the vicinity during the bloom was 52,000 cells/L. *Pseudochattonella* sp. was the main co-occurring species with a maximum registration of 780,000 cells/L. Death of caged salmon was also reported further north in Sunnhordland, but there are no corresponding algal data from that area. During the 2008 bloom at the south-west coast cells of *Chattonella globosa* containing *Dictyocha fibula* skeletons at various stages of development were observed (Figure 1.3-6). This observation was confirmed for at least at 11 localities from Rogaland to Sogn og Fjordane suggesting that *C. globosa* and *D. fibula* are related and may be stages in a common life cycle. Normally *D. fibula* occurs in low numbers (<100 cells/L), but during this blooming event of *C. globosa* cell densities of *D. fibula* reached unusually high level, 7,200 cells/L.

Table 1. Maximum cell numbers of *Chattonella globosa* registered in Norwegian coastal waters during 2007, 2008 and 2009.

Chattonella globosa	**2007**	**2008**	**2009**
Inner Oslofjord Observed	Sept-Dec	Sept-Dec	Oct
Max number cells/L	Oct. 18,600	Oct 12,300	Oct 80
South Coast Observed	Sept-Dec	Sept Nov	Oct-Dec
Max number cells/L	Sept 16,200	Sept 36,800	Oct 1,840
Skagerrak Observed	Sept-Nov	Sept-Oct	-
Max number cells/L	Oct 10,350	Oct 34,500	-
West Coast Observed	Sept-Oct	Sept-Nov	Nov
Max number cells/L	Oct 2,720	Nov 52,800	Nov 40

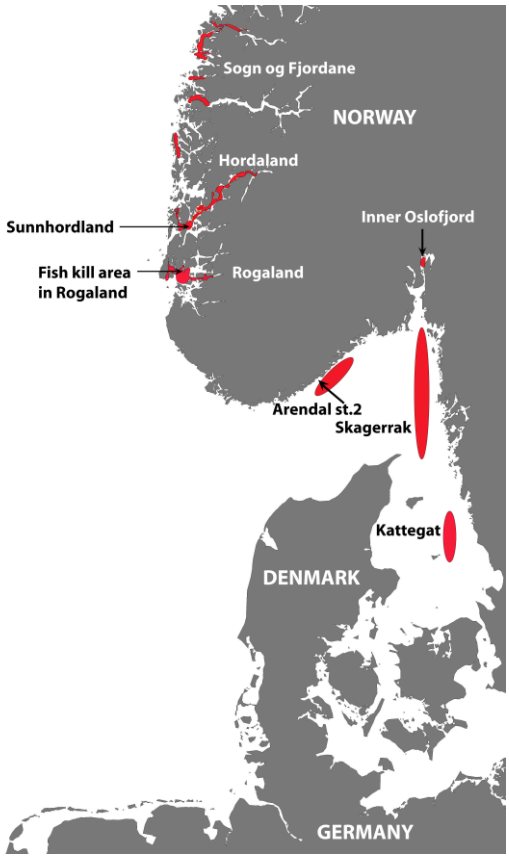

Fig. 2. Map of the coastal waters of the southern part of Norway. Red areas indicate occurrence of *Chattonella globosa*.

Discussion

Norwegian waters have been monitored routinely since 1992 and *Dictyocha fibula* is commonly recorded in the autumn, though in low numbers. *Chattonella globosa*, however, was not registered prior to 2007. The last decade has been part of a long period of increasing sea temperature in Norwegian waters. Especially during the last 10 years we have experienced an increasing number of episodes when plankton species like e.g. *Neocalyptrella robusta*, *Pseudosolenia calcar-avis*, *Dinophysis caudata*, *Dinophysis tripos*, *Peridinium quinquecorne* normally found in warmer waters have been registered during autumn.

We speculate that the milder climate along the Norwegian coast have produced more favourable growth conditions for the *Chattonella globosa* stage. Our findings of *Dictyocha fibula* skeletons in development inside *Chattonella globosa* indicate that *C. globosa* may be a skeleton lacking motile stage in the life cycle of *D. fibula*. This is not surprising as the closely related *Dictyocha speculum* has a similar skeleton lacking stage (Moestrup & Thomsen 1990) commonly occurring in our waters. Further evidence for *Chattonella globosa* belonging to the genus *Dictyocha* has been recently published by Hoe Chang et al. (2012). *Chattonella globosa* has been associated with fish kills in several areas of the world (Hallegraeff & Hara 2003) and the fish kills in Rogaland was probably caused by *Chattonella globosa* and the co-occurring *Pseudochattonella sp*.

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Blooms of *Cochlodinium polykrikoides* along the coast of Oman and their effects

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Abstract

Intensive blooms of *Cochlodinium polykrikoides* caused massive fish kills in nature and aquaculture farms and affected tourism along the coasts of Oman bordering the Arabian Gulf, Oman and Arabian seas during 2008-2009. Water quality parameters were estimated for microbes, heavy metals, toxicity and organic and inorganic compounds. Fish and shellfish samples were collected during the bloom period and heavy metals, microbes, Parasite infection and toxicity were analyzed. Phytoplankton species were identified using light and scanning electron microscopy. The histopathological alterations in normal and affected fish gills were studied using light and electron microscopy. The mechanism of how *C. polykrikoides* could cause massive marine organisms kill is discussed.

Introduction

Cochlodinium polykrikoides Margalef, an unarmored harmful dinoflagellate formed large blooms accompanied with serious damage to aquaculture in the past two decades particularly in Japan and Korea (Kim, 2006). This species was first reported from the Caribbean Sea along the coast of Puerto Reco (Margalef, 1961). It has since been reported in northern Atlantic waters along the American east coast and is widely distributed in Northwestern Pacific waters along the coast of Japan and Korea. *C. polykrikoides* appeared in August 2008 in the Oman Sea, extended into the Arabian Gulf and formed a large-scale red-tide of long duration from August 2008 to April 2009 accompanied with huge mass mortalities of marine nekton and benthic organisms. Despite the widespread occurrence of this species in the Pacific, Atlantic, and Indian Oceans (Matsuoka et al., 2008), only a few studies of its ecophysiology have been conducted in the Oman Sea and the Persian Gulf. This study provides an overview of the massive fish kills caused by many blooms of *C. polykrikoides* in Arabian and Oman Sea, the causative factors of these blooms and their effects.

Materials and Methods

During *C. polykrikoides* blooms, all data of bloom date, site, species of fish and shellfish killed were identified and amount of fish or shellfish killed determined. Phytoplankton samples were collected using a Bongo net (80 µm mesh) and Niskin bottles, and preserved with 4% formaldehyde and identified under light microscopy. Phytoplankton were also identified using scanning electron microscopy. A total of 24 fish were collected and analyzed for microbiology, heavy metals and parasites. Total Plate count (TPC), Total Coliforms, *Escherichia coli*, *Staphylococcus*, *Salmonella*, and *Vibrio* bacteria were determined during blooms. 25g of fish were added aseptically into 225ml of buffered peptone water, homogenized and followed by serial dilution. Then, 1ml of serial dilution added to Petri plates, in duplicate;. 15ml added of molten Total plate count medium and mixed. Plates were incubated at 30 C° for 48 to 72 hrs.

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Colonies were counted and calculated. The same samples and serial dilutions prepared for TPC were used for *Escherichia coli, Staphylococcus, Salmonella,* and *Vibrio* except changed in molten medium. Heavy metals (total mercury, lead and cadmium) were analyzed. GBC 932 plus Atomic Absorption Spectrometer with auto-sampler SDS-270 were used to analyse total mercury. Inductively Coupled Plasma- OES Varian, Vista-MPX was used for lead and cadmium. In addition to the 24 different fish species from Oman collected, 11 specimens of Indian oil sardine (*Sardinella longiceps)* were examined for parasites or abnormalities. Among the 11 specimens, 5 were bought from the fish market and 6 collected at a red tide site. The surface of fish was observed with naked eye for external parasites or abnormalities. Abnormal cavities of the fish were cut open to search for internal parasites. All viscera were removed to be examined on petri dishes by dissecting microscope for parasites embedded in the tissues of visceral organs. Next, the gills were excised and separated and observed under the microscope for gill flukes. Finally, the flesh was filleted to observe parasites embedded in the flesh. During *C. polykrikoides* blooms, drinking water samples were collected from different sites in Oman and Arabian Seas and analysed for organic and inorganic compounds. For volatile organic compound (VOC), Gas chromatography/ Mass GC/ MS /MS (purge and trap) method EPA VOC 524.2 analyses were used. The analyses of semivolatile organic compounds were done by Gas Chromatography/MS EPA method 8270C (sample preparation by extraction with methylene chloride). Inorganic compounds were analyzed by Ion chromatography, Titration and UV spectroscopy. Ten specimens of Indian oil sardine (*Sardinella longiceps)* examined for parasites were also used to examine the effect of *C. polykrikoides* blooms on the gills by light and scanning electron microscopy. Five Indian oil sardine fish and two bottles of seawater were sent to Dept of Chemistry in Napoli University, Italy for toxicity tests. The presence of PSP toxins was investigated by mouse bio-assay and a combination of hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-MS)^2 .

Results & Discussion

Blooms of *C. polykrikoides* started in the east Arabian Sea and north Oman Sea in August 2008. The blooms reached the Oman North coast in September 2008, causing discoloration of Musandam and north Al Batinah coast but no fish kills were recorded. On 7th October 2008 about 1 ton of dead fish was observed on Shinas coast, north Al Batinah coast. Between 12 - 20 October 2008, about 5 tons of fish were killed by the same blooms in Saham and Soaik, on Al Batinah coast. On 27th October, the blooms reached Muscat coast and lasted 2 weeks, killing a few hundred wild fish. After 2 weeks, on 14th November 2008, another *C. polykrikoides* bloom caused 5 tons of fish losses in Muscat. On 23rd November 2008, the blooms reached aquaculture farms in Qurayat, south Muscat and killed all caged goldlined sea bream (70 tons), as well as wild fish (10 tons). On 28th November 2008, the bloom reached North Arabian Sea and killed 5 tons of wild fish in Sur. In January, February, March and April 2009, many fish were killed in Sharqiah region including Sur and Al Ashkarah coasts. In the same period, *C. polykrikoides* blooms were observed causing tons of fish kills in Masirah and Al Dokom in Al Wasta coast. On the Dhofar Coast, fish kills started in Shoymaiah by end of January 2009 and lasted until March in Hasik coast. However, on 14th November 2008, the blooms again affected Al Batinah coast in Sohar and killed a few tons of wild fish. During April 2009, massive shellfish kills were observed along north coast of Al Batinah (70 tons). The total fish and shellfish lost were about 200 tons, comprising 70 tons of caged fish, 70 tons of shellfish and 60 tons of wild fish.

During the blooms, strong odours (methyl sulfide compound) were produced by *C. polykrikoides,* which affected all Arabian and Oman Seas coasts. There were no records of effects on human health. Some schools near

the sea in Muscat closed during strong odour of the blooms.The blooms also affected the osmosis membrane of desalinations plant and many stopped drinking water supply. Tourists canceled booking in near beach hotels .

Phytoplankton samples were identified by light and confirmed by electron microscopy. The dominant species found in water samples during all blooms was *C. polykrikoides*. The confirmation of species taxonomy in Arabian Gulf and Oman Sea were also done by other scientists (Matsuoka et al., Unpublished; Richlen et al., 2010). The 24 fish specimens which tested for Total Plate count (TPC), Total Coliforms, *Escherbia coli, Staphylococcus, Salmonella*, and *Vibrio* bacteria, did not present any abnormal levels. The same fish specimens were tested also for heavy metals contamination (total mercury, lead and cadmium). As for the microbiology tests, no abnormal levels of heavy metals were found. The parasites tests on the 24 fish specimens and 10 Indian oil sardine, showed no infection for the Oman sea species, but 5 Indian oil sardines were normal, whereas 5 were affected by *C. polykrikoides*. No effect of blooms on parasite quantity was observed in all tests and no variation was found between normal and bloom affected sardine.All tests of water quality including organic and inorganic compounds for samples during blooms did not show any abnormal levels. The lamellar thickness and interlamellar distance measured in normal Indian oil sardine (*Sardinella longiceps)* fish gill and effected by *C. polykrikoides* bloom fish gill filaments were reported.

For normal fish, the secondary lamellae of *Sardinella longiceps* were regularly-spaced, long and appeared normal. The surface area of lamellae was 84.9%. For fish gill affected by blooms, the lamellae appeared damaged in some fish and surface area was reduced by 33% (down to 51.9%). Scanning electron microscopy showed proliferation of mucus which could clog the gill during the blooms.

The results of the mouse test according to AOAC procedure for PSP showed that all three mice injected with seawater extracts survived. Extracts from flesh as well as heads/guts from 5 Indian oil sardine fish were combined to generate Sample A and Sample B, respectively. For sample A, the first mouse died within 6h while the second and third mice did not die. For sample B, the first mouse died within 1h, the second mousse within 7h, while the third mouse survived. With regard to detection of PSP toxin by multiple reaction monitoring mode (MRM) for hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-MS), STX and NEO toxin was detected at high level but. other toxins; GTX2, GTX3, GTX1, GTX4 and GTX5 were within or below normal levels.

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Annual dynamics of toxic phytoplankton in Novorossiysk Bay

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Abstract

This work presents the results of research conducted between 2005 and 2007 into the seasonal dynamics of the qualitative structure and quantitative development of toxic phytoplankton in Novorossiysk Bay. The network of stations for monthly monitoring of the community of phytoplankton covered areas with various hydrological and chemical characteristics as well as ballast dumping locations, most suited to biological invasion. Twenty-three toxic species with a concentration of up to $3x10^9$ cell/m^3 were recorded. The dominant species were Bacillariophyta *Pseudonitzschia* genus, Dinophyta *Prorocentrum* genus, *Dinophysis, Scrippsiella, Ceratium*, Chrysophyta - *Chrysochromulina pontica*. The future development of the most toxic species in the Black Sea, of the *Alexandrium* genus is discussed.

Introduction

Cases of harmful algal blooms (HABs) as a result of development of toxic phytoplankton species in the Black Sea have become considerably more frequent and are directly related to the receipt of auto- and allochthonous organic matter in coastal zones (Nesterova, 1979; Zernova, 1983; Vershinin, Kamnev, 2001; Vershinin, Moruchkov, 2003; Terenko, Terenko, 2005). In recent years, in the North-east part of the Black Sea, "red tides" caused by development of Dinophyta toxic species, have begun to be observed (Vershinin and all, 2005; Yasakova, Berdnikov, 2008). Novorossiysk Bay and the adjoining areas of the Black Sea are subject to pollution from city effluent and from port industrial discharges. This therefore requires constant control to allow the forecasting of negative impacts from the development of toxic algal species. Besides, in recent times, the greatest concern has been the possibility that an especially toxic species of phytoplankton could invade the Black Sea ecosystem from other regions by means of tankers' water ballast. The Novorossiysk port is the largest port on the north-east part of Black Sea, and is most subject to the risk of biological invasions. The volume of water ballast arriving at the Novorossiysk port makes up $37x10^6$ tons per year. The object of this research is the monitoring of toxic phytoplankton species' development in and around Novorossiysk Bay, including HAB species which could be delivered with ballast water.

Materials and methods

More than 300 samples of phytoplankton were collected monthly from March 2005 until December 2007 from the Novorossiysk Bay water area. The sampling points' distribution (Fig.1) is dictated by different levels of human- load on these areas. Novorossiysk Port (I) is a small water area under maximum anthropogenic load. The mid part of the bay (II) is also subject to considerable human-caused pollution from the shipyard, cement works, and oil harbor as well as storm water discharge and residential sewage. An open part of the bay (III) is the healthiest area with good water exchange. Samples of phytoplankton were collected from the marine surface during day time using a special vessel. Concentration of phytoplankton samples was carried out using a back filtration method. An initial 1.5 liter volume of sea water was concentrated by means of filters (aperture size 1 micron) up to 20-50 ml and then fixed by formalin to final concentration of 1 %. Identification of species, calculation of cell count and biomass of the phytoplankton was conducted by means of a MIKMED-2 microscope with magnifications of x200 and x400. Standard manuals (Dodge, 1982; Carmelo, 1997) were used for the identification of species.

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Results

The 23 species of toxic phytoplankton identified include: Dinophyta (17 species), Bacillariophyta (3 species) and Chrysophyta (3 species). Among Bacillariophyta there were the potentially toxic species: *Pseudonitzschia pseudodelicatissima* (Hasle) Hasle, P *pungens* (Grunow ex Cleve) Hasle, *P. seriata* (Cleve) H. Peragallo. The maximum concentration of these algae (up to $2.82.10^9$ cells/m^3) has been noted in an open part of the Bay and in the port area during spring (April-May) 2007. That makes up 90 % of the total amount of phytoplankton. A small increase in these species in the port water area (728.10^6 cells/m^3) was noted in August 2006. At other times the concentration did not exceed 100.10^6 cells/m^3.

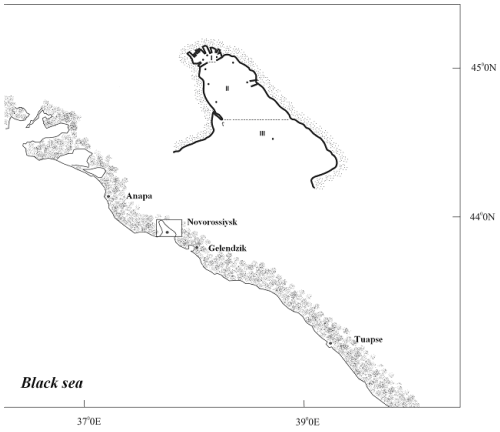

Fig.1 Sampling points' of phytoplankton in Novorossiysk Bay: I - Port, II – mid part of the bay, III – open part of the bay

The highest concentration was found among potentially toxic species of the *Prorocentrum* genus. The peak of the *P. cordatum* (Ostf.) Dodge development ($9.2\text{-}16.7.10^6$ cells/m^3) was in the port area and the mid part of the Bay during the summer period 2006-07 (fig.2). Abundant development of *P. compressum* (Bailey) Abé ex Dodge was observed in April, 2006 in the port area ($18.6.10^6$ cells/m^3). During the rest of the period, the concentration of this species did not exceed $5.6.10^6$ cells/m^3. The maximum concentration of *P. micans* Ehr. was noted during the spring and summer period (April-June), 2006-07 ($5.6\text{-}14.10^6$ cells/m^3). Species of the *Dinophysis* genus: *D. rotundata* Clap. et Lach., *D. caudata* Saville-Kent, *D. acuminata* Clap. et Lach., *D. fortii* Pavillard and *D. acuta* Ehr. developed successfully in Novorossiysk Bay. Their concentration during the summer period reached $2.5.10^6$ cells/m^3, biomass – 30 mg/m^3. The density of *Dinophysis* species was close to the threshold (3.10^6 cells/m^3) at which they are capable of causing human shellfish poisoning from (Hallegraeff et al., 2003). Peaks of *Scrippsiella trochoidea* (Stein) Loeblich concentrations in the mid part and in the open part of the Bay occurred in spring (March, May) and summer (August) 2005-06 ($2.4\text{-}8.6.10^6$ cells/m^3). In 2007 the concentration of this species reached $1.2.10^6$ cells/m^3. Species of the *Ceratium* genus also formed high biomass (up to 1.2 g/m3). In the February – June period of 2005-06 the concentration of *C. tripos* (O.F. Muller) Nitzsch. reached $3.2\text{-}6.8.10^6$ cells/m^3 in the Bay. In 2007 a small peak of *C. tripos* was noted in June-July ($0.3.10^6$ cells/m^3). Seasonal development of *C. fusus* (Ehr.) Dujardin was characterized by multiple peaks: in the port area and in the open part of the Bay the maximum development of this species was in March and June, 2006 (up to 5.10^6 cells/m^3). High concentrations of this species ($2.4 - 2.8.10^6$ cells/m^3) were noted in the centre of the Bay in May 2005 and in the port water area in October, 2007. For the whole year, in the open part of the bay, there were species of *Chrysophyta - Distephanus speculum* Her., with a maximum in September, 2007 (11.10^6 cells/m^3). During the warm period (July-September), the development of *Gymnodinium sanguineum* Hirasaka (up to $1.2.10^6$ cells/m^3) was noted there, increased concentration occurred in June, 2006 ($2.6.10^6$ cells/m^3). Amongst toxic algae in the open part of the Novorossiysk Bay, the invasive species *Alexandrium ostenfeldii* (Paulsen) Balech et Tangen (200.10^6 cells/m^3) was found in 2004. The new for this region species, *Chrysochromulina pontica* Rouch was registered in 2006 in the port water area ($20\text{-}160.10^6$ cells/m^3). Earlier these species had not been recorded in the Bay. They have, however,

been found in the ballast water of some tankers coming for loading to Novorossiysk port.

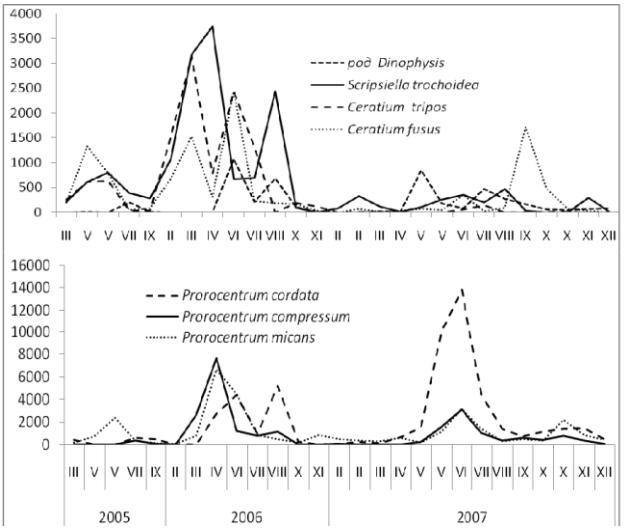

Fig.2 Seasonal dynamics of abundance (cell/m^3) of toxic Dinophyta in Novorossiysk Bay

Discussion

"Red tides", caused by development of *P. cordatum, P. compressum, G. sanguineum* have been observed in the North-west part of the Black Sea between 1973 and 2000 (Nesterova, 1979; Terenko, Terenko, 2005). Until 2005 the "red tide" phenomenon in the North-east part of the Sea was observed only once: in 2001 in Utrish Bay *Cochlodinium polykrikoides* Margalef. developed (69.10^6 cells/m^3) (Vershinin, 2005). Until 2008 in Novorossiysk Bay only *Pseudonitzschia* developed up to bloom level among toxic species. However, as early as March, 2008, the "red tide" caused by the development of Dinophyte *S. trochoidea* (350.10^6 cells/m^3 and 3.14 g/m^3) has been noted within a considerable area of the North-east shelf of the Black Sea. (Yasakova, Berdnikov, 2008). The ecosystem of the Black Sea, especially its coastal areas have been weakened as a result of anthropogenic eutrophication, and for this reason the threats of toxic blooms in sea water cause alarm.

Conclusions

1. In Novorossiysk Bay 23 toxic algae species were identified, comprising up 90% of total abundance of phytoplankton.

2. Abundant development (up 3.10^9 cells/m^3) where noted of potentially toxic species of *Pseudonitzschia*.

3. Most abundant among potentially harmful Dinophyta were *Prorocentrum*, *Dinophysis*, and *Scrippsiella*.

4. The maximum biomass was noted for *Ceratium* (up to 1.2 g/m^3).

5. Among new species of phytoplankton the potentially toxic *Alexandrium* genus was found, requiring special control.

6. Operational information concerning taxonomic, spatial and seasonal structure of toxic algae, dynamics of their distribution and development, based on satellite system can be used in the strategy of state local environmental monitoring.

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Potentially Harmful Microalgae from the Southern-Central Coast of Cuba

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Abstract

Benthic and planktic toxic microalgae from the southern-central region of Cuba were surveyed intermittently between 2007 and 2009, mainly in the estuarine Cienfuegos Bay and its adjacent coast. The highest abundance of both benthic and planktic species were recorded in dry season and at the beginning of the rainy period which is directly correlated with a higher incidence of ciguatera and other episodes associated to harmful algal blooms. *Ostreopsis lenticularis* and *Gambierdiscus caribaeus* were the dominant benthic species in the eastern coast. After heavy rain, *Ostreopsis ovata* and *Prorocentrum lima* were dominant. Moderate abundance of the potentially ciguatotoxic *Prorocentrum concavum*, *P.rathymum* and the dermatotoxic cyanophyte *Lyngbya majuscula* were registered on blooms of macroalgae. *Gymnodinium catenatum*, *Pyrodinium bahamense* and *Dinophysis ovum* were first reported in the region. Blooms of the diatom *Pseudo-nitzschia multistriata* have been observed in eutrophic zones. A small episode of dead fish was associated to blooms of *Heterocapsa circularisquama*. Other toxic or noxious species have been seen accompanying such as *Cochlodinium polykrikoides*, *Dinophysis caudata*, *Gonyaulax polygramma*, *G. spinifera* and *Prorocentrum minimum*. High abundance of the cyanobacteria *Planktothrix isothrix*, *Pseudoanabaena* sp., *Dolichospermum solitarium* and *Microcystis aeruginosa* were registered in the bay after hurricanes.

Introduction

Coastal Cienfuegos Province in the southern-central part of Cuba (Fig. 1) is very diverse, with bays, lagoons, mangroves, coral reefs, and brackish rivers. Cienfuegos Bay is a semienclosed embayment of estuarine characteristics that represents the most important natural resource in the province, due to fishing activities, maritime transport, tourism industry, and natural parks. The northern part, with sewage input from the city of Cienfuegos (106 504 inhabitants), industrial waste and the inflow from Damují and Salado rivers, is under stronger anthropogenic impact than the southern part. Blooms of toxic and nontoxic dinoflagellates and diatoms occur near domestic waste discharged from Cienfuegos city, mainly at the end of the dry season and beginning of the rainy season, when temperatures increase and potential flushing is low. This province, like other regions in Cuba, is affected by ciguatera episodes. However, until 2005, there had only been one study of the taxonomy and ecology of potentially benthic toxic dinoflagellates in one station of the bay. This paper describes recent occurrence of benthic and planktonic toxic microalgae at this region in Cuba.

Materials and methods

Planktonic species were sampled from 16 selected stations in Cienfuegos Bay (Fig. 1) every 3 months during 2009, as part of a monitoring program for water quality control. Plankton samples were collected by submerging a 5-L Niskin bottle below the surface and were preserved with modified Lugol solution. For the determination of densities, aliquots of concentrated samples were determined under a microscope in a Rigosha counting cell. For taxonomic purposes, samples were taken with a 20 µm mesh Nytal net. Benthic dinoflagellates were surveyed using a mixture of the most abundant macroalgae as substrate in Cienfuegos Bay and its adjacent coast between 2007 and 2009.

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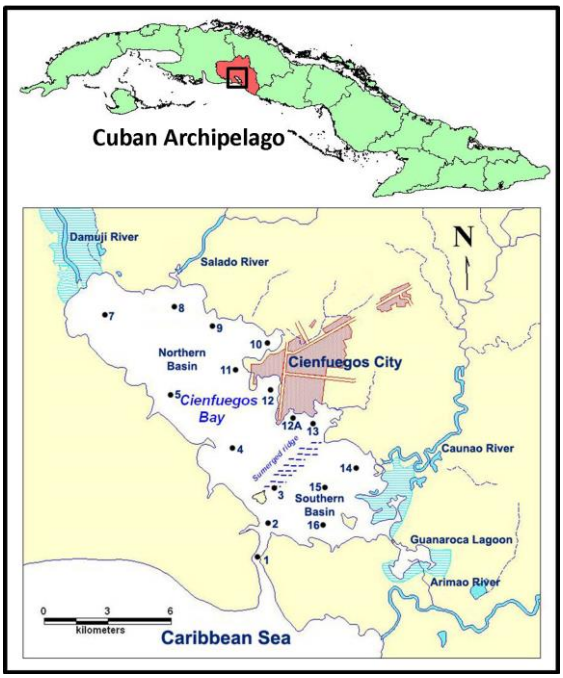

Fig. 1. Sampling stations in Cienfuegos Bay, Cuba

Results

Planktonic species

Gymnodinium catenatum, potential producer of PSP toxins, was reported for the first time in the southern part of Cuba. It was mainly found in the southern cleaner waters of the Bay. The maximum concentration was detected at station 14 (3.3 x 10^4 cells L^{-1}). Four-celled chains were predominant, but pairs, triplets and 6 to 8 celled chains were also observed (Fig. 2). In the Caribbean Sea, *G.catenatum* associated with PSP outbreaks was first reported in Venezuela (La Barbera 1993). The potential PSP producer *Pyrodinium bahamense* was first reported in Cuba. Cells were singular (Fig. 2) and not in chains, the latter a characteristic of the non-toxic *Pyrodinium var. bahamense*. It was observed off the Bay (concentrations up to 6.3 x 10^3 cells L^{-1}) in the eastern littoral in the beginning of the dry season (Nov. 2008).

Among the potential DSP-toxin producers, the most frequently observed species (concentrations up to 10^3 cells L^{-1}) were *Dinophysis caudata* and *D. ovum* (Fig. 2), which was more abundant in areas subjected to anthropogenic and riverine inputs. This is the first report for Cuban waters of *D. ovum* that has recently been associated with a toxic outbreak in Texas (Gulf of Mexico).

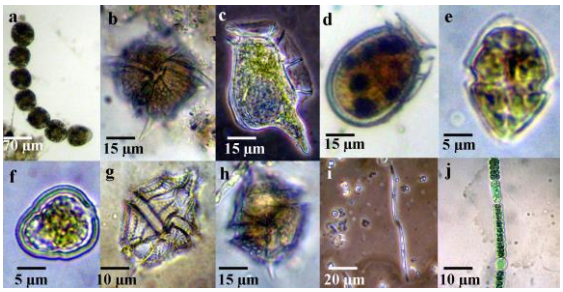

Fig. 2. Some potentially planktic toxic species: a. *Gymnodinium catenatum*, b. *Pyrodinium bahamense*, c. *Dinophysis caudata*, d. *D. ovum*, e. *Heterocapsa circularisquama*, f. *Prorocentrum minimum*, g. *Gonyaulax polygramma*, h. *G. spinifera*, i. *Pseudo-nitzschia multistriata*, j. *Dolichospermum solitarium*.

At the beginning of the rainy season, July 2009, about 50 to 100 dead fish were observed near domestic waste discharged from Cienfuegos city, near station 12, coinciding with blooms of *Heterocapsa*. Cell shape and the position of the nucleus and pyrenoid coincided with the description of *H. circularisquama* (Horiguchi 1995). Cells of this dinoflagellate were abundant in the fish's gills, but cells of the benthic *Amphidinium* cf. *carterae*, which produce haemolytic ichthyotoxins, were also found. Other toxic or noxious species have been seen accompanying blooms such as *Cochlodinium* cf. *polykrikoides*, *Dinophysis caudata*, *Prorocentrum minimum*, *Gonyaulax polygramma* and *G. spinifera* (Fig. 2).

The presence of a high concentration of the potentially amnesic toxic diatom *Pseudo-nitzschia* cf. *multistriata* was detected in a bloom of diatoms at station 10, with a density of 1.1 .10^6 cells L^{-1} during the late dry period. Blooms of other *Pseudo-nitzschia* (*delicatissima* group) and the presence of *Pseudo-nitzschia* cf. *pungens* have been reported. Potentially toxic freshwater cyanobacteria were detected in high concentration in the bay after tropical storms and hurricanes (e.g. Dennis Hurricane in 2005), including *Planktothrix isothrix*,

Pseudoanabaena sp., *Dolichospermum solitarium* and *Microcystis aeruginosa* (Fig. 2).

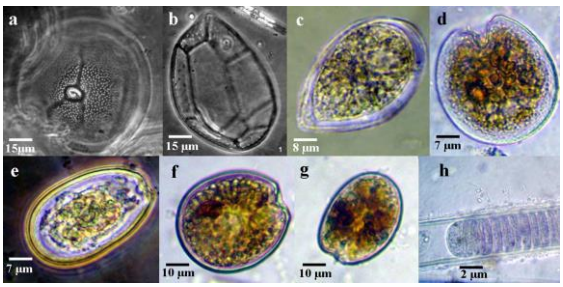

Fig. 3. Some potentially benthic toxic species: a. *Gambierdiscus caribaeus*, b. *Ostreopsis lenticularis*, c. *O. ovata*, d. *Prorocentrum emarginatum*, e. *P. lima*, f. *P. concavum*, g. *P. rathymum*, h. *Lyngbya majuscula*.

Benthic species

During the dry season of 2007 in an embayment off Cienfuegos Bay, *Ostreopsis lenticularis* (101 cells g^{-1}wet weight) and *Gambierdiscus caribaeus* (53 cells g^{-1}) were dominant. The 2007 rainy season has been one of the wettest in recent years, and seems to have affected the benthic vegetation in the embayment, as witnessed by lower diversity and abundance of the macroalgae and an increase in suspended particles. After the rainy period, the structure of the benthic dinoflagellate community changed radically, and *Ostreopsis ovata* (203 cells g^{-1}) and *Prorocentrum lima* (94 cells g^{-1}) were the dominant (Fig 3).

In July 2008 in the western part of the province, *Prorocentrum concavum* (150.5 cells g^{-1}) was dominant, together with *P. emarginatum* (8.2 cells g^{-1}). At the same date in Cienfuegos Bay, *P. concavum (*3.3 $.10^3$ cells g^{-1}), *P. rathymum* (3.1 x 10^3 cells g^{-1}), *P. lima* ($2.1.10^2$ cells g^{-1}) and *Lyngbya majuscula* ($3.0.10^3$ cells g^{-1}) were recorded on blooms of macroalgae (Fig 3). *Gambierdiscus caribaeus* was only found in the canal which links the bay to the Caribbean Sea.

These are the first records of *Ostreopsis lenticularis* , *O. ovata* and *Prorocentrum emarginatum* in Cuba. The presence of this wide variety of potentially toxic benthic species may be linked to outbreaks of ciguatera in the region, and refute the belief that the south of Cuba is unaffected by this syndrome. Despite the need to increase these data, the results of this survey combined with epidemiological studies provide evidence of a high incidence of ciguatera in the southern central part of the island during the dry season (November– April) (Maya 2007).

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Potentially harmful dinoflagellates (Dinophyceae) from the coast of Pakistan

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Abstract

A bimonthly interval (May 2002-July 2003) plankton survey was initiated at two separate sites along the Manora Channel, Karachi Pakistan. Using both light and electron microscopy a large number of dinoflagellates was identified, most of them first records for the Northern Indian Ocean. Potentially harmful, bloomforming non-toxic dinoflagellate genera include *Prorocentrum* (11 species), *Ceratium* (21), *Scrippsiella* (2), *Gyrodinium* (2), *Katodinium* (1), *Noctiluca* (1), *Gymnodinium* (2) , *Akashiwo* (1) and *Gonyaulax* (5). A number of known toxin producers were also observed during the sampling period including the ichthyotoxic dinoflagellate *Cochlodinium* (1*)*, the okadaic acid producing *Dinophysis* (8), and the saxitoxin producing *Alexandrium* (3). A number of typically benthic dinoflagellates were also observed including okadaic acid and hepatotoxin producing *Prorocentrum* (4) and the palytoxin producer *Ostreopsis* (1).

Introduction

Harmful algal blooms (HABs) in coastal areas often cause shellfish, finfish, mammal mortality, and can damage aquaculture farms (Banner *et al*. 1960; Smayda 1990; Shumway 1990; Taylor 1993; Landsberg and Steidinger 1997; Richlen *et al*. 2010). The occurrence of high biomass non-toxic dinoflagellates can initiate harmful algal blooms, known as red tides, brown tides, green tides (discoloration of water) and cause oxygen depletion (Smayda 1990; Hallegraeff 1993; Landsberg 2002), affecting the aquatic environment. Phytoplankton studies are sporadic along the Pakistan coastline. Previous plankton surveys from the Arabian Sea have reported a number of potential bloom forming species such as *Prorocentrum micans* (Hassan and Saifullah 1971), *Gonyaulax* spp (Saifullah and Hassan 1973), *Ceratium shurunk* (Hassan and Saifullah 1974), *Gonyaulax diesing* (Chaghtai and Saifullah 2001) and *Noctiluca scintillans* (Chaghtai and Saifullah 2006). This paper describes potentially harmful dinoflagellates from the northern Arabian Sea, Manora coast of Karachi, Pakistan.

Material and Methods

Triplicate samples were collected bimonthly during May 2002-July 2003 from near shore waters of Karachi harbor: St. I (24˚49.77'N 66˚57.85'E) and Mouth of Manora Channel St. II (24˚47.93'N 66˚58.87'E) Karachi. Niskin bottle samples were collected at 1m depth and fixed with Lugol. Cells were enumerated using the Utermohl technique. For identification, epifluoresence microscopy (BX 51, Olympus, Japan) and scanning electron microscopy (JEOL, 5600Lv) were used. The epifluoresence microscope was equipped with a magnafire digital color camera (Olympus, Tokyo, Japan), in order to examine cells using differential interference contrast (DIC) microscope. Cells were stained with 1% calcofluor white MR2 (Sigma, St Louis, MO) to visualize the thecal plates and thecate species identification was done according to Kofoidian plate tabulation. For scanning electron microscopy (SEM), cells were desalted using a 10% step gradient of seawater to freshwater and dehydrated by using a series of acetone. After dehydration, samples were coated with 1.5 nm of gold-platium using a Denton sputter-edge coater (Moorestown, NJ) and examined with a JEOL 5600LV (Tokyo, Japan) scanning electron microscope.

Results.

A total of 98 species were identified including 61 species which were first reports from Pakistani waters. Among them 66 species were blooms forming non-toxic harmful species including *Prorocentrum*, *Ceratium*, *Scrippsiella*, *Gyrodinium*, *Akashiwo*, *Heterocapsa cf. circularisquama*, *Katodinium*, *Noctiluca*, *Gymnodinium*, and *Protoperidinium* (Table 1). A total of 28 species were known toxin producing dinoflagellates including *Gymnodinium*, *Gonyaulax*, *Lingulodinium*, *Dinophysis*, *Alexandrium*, and *Cochlodinium*. A number of

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known toxic epiphytic dinoflagellates, including the genus *Prorocentum,* and *Ostreopsis*, were also observed in planktonic samples (Table 2). Our results indicated that maximum cells densities per litre of *Alexandrium ostenfeldii, Prorocentrum minimum, P. dongaiense, Scrippsiella trochoidea* was > 1000-6000 cells/L has great diversity throughout the year and these were most dominant species found from both stations (Tables 1 & 2). *Dinophysis caudata, Ceratium furca, Prorocentrum micans* were frequent occurring species (Tables 1 & 2) and number of toxic species found rare from St. A for example, *Lingulodinium polyedricum, Protoceratium reticulatum, Gymnodinium catenatum, Alexandrium minutum, D. acuta, D. miles* and *D. fortii, D. rotundata, D.infundibula* etc from St. B (Table 2).

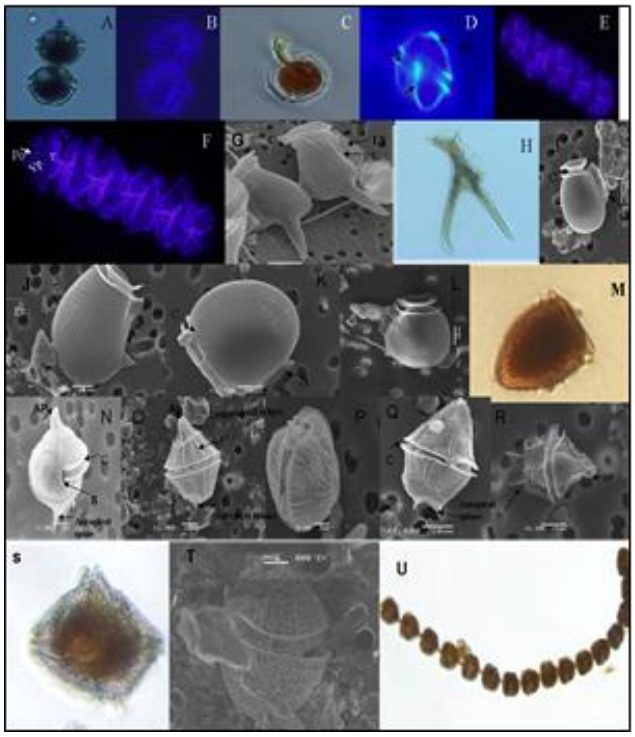

Fig. 1. Potentially toxic dinoflagellates from Pakistan. A, B *Alexandrium ostenfeldii;* C. *A. minutum;* D. *A. tamarense;* E-F. *A. tamiyavan- ichi;* G. *Dinophysis caudata;* H. *D.miles; I. D.acuminata; J. D.fortii;* K. *D. rotundata;* L. *D. infundibulus;* M. *D.acuta;* N. *Gonyaulax spinifera;* O. *G. polygramma;* Q-R. *G. verior;* S. *Lingulodinium polyedricum;* T. *Protoceratium reticulatum;* U. *Gymnodinium catenatum.*

Discussion

The coastal area of Karachi is influenced by anthropogenic activities, whereas industrial, agricultural and domestic waste from Layari river, adversely influence water quality of Manora Channel. During this survey a number of both toxic and non-toxic dinoflagellates were recorded along the coast of Pakistan, showing the potential for future HAB events. A number of these non-toxic species such as *Prorocentrum minimum*, *P. donghiense*, and *Ceratium fusus* have been linked to coastal eutrophication worldwide. Due to the presence of toxin producing dinoflagellates, the coast of Pakistan is at risk from shellfish and finfish toxic events from saxitoxin and okadaic acid-like toxins, spirolides, yessotoxin, palytoxin, hepatotoxin and ciguatoxin. Most of these toxic species have not been observed in the northern Arabian Sea previously. The morphological characters of the toxic dinoflagellates have been described (Fig 1). However additional studies are required to determine if toxins are present in the coastal area of Pakistan.

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Table 1. Important red-tide species of dinoflagellates from St. A and St. B, Karachi coast ; Dominant (*****), Abundant (****), Frequent (***), Rare (, *).**

Species	St. A	St. B	Fishery impacts
Akashiwo sanguinea	**	**	Redtide
Ceratium furca	*	**	Fish killer, redtide
C. lineatum	*	**	Fish killer, redtide
C. fusus	*	***	Fish killer, redtide
Heterocapsa cf. circularisquama	*	***	Fish killer, redtide
Prorocentrum micans	***	****	Fish killer, redtide
P. dongaiense	****	*****	Fish killer, redtide
Scrippsiella trochoidea	****	****	Fish killer, redtide
Pyrophacus steinii	**	***	Fish killer, redtide
Protoperidinium divergens	**	**	Fish killer, redtide

Table 2. Potential toxin producing dinoflagellates from St. A and St. B, Pakistan Dominant (*****), Abundant (****), Frequent (***) and Rare (,*)**

Species	St. A	St. B	Toxins
Alexandrium concavum	*	Absent	Saxitoxin and Congeners
A. ostenfeldii	****	*****	Saxitoxin and/or spirolides (some strains)
A.tamarense	***	Absent	Saxitoxin and Congeners
A. tamiyavanichi	**	**	Saxitoxin and Congeners
A. minutum	*	Absent	Saxitoxin and Congeners
Cochlodinium cf. fulvescens	**	***	Unidentified icthyotoxic
Dinophysis acuminata	*	*	Okadaic acid and congeners
D. acuta	*	Absent	Okadaic acid and congeners
D. caudata	**	**	Okadaic acid and congeners
D. fortii	Absent	*	Okadaic acid and congeners
D. rotundata	Absent	*	Okadaic acid and congeners
D.infundibula	Absent	*	Okadaic acid and congeners
D. miles	*	Absent	Okadaic acid and congeners
D. mitra	*	Absent	Okadaic acid and congeners
Gymnodinium catenatum	*	Absent	Saxitoxin and Congeners
Gonyaulax spinifera	**	***	Yessotoxins (some strains only)
G. digitalis	*	**	Yessotoxins (some strains only)
G. polygramma	*	Absent	Yessotoxins (some strains only)
G. verior	*	*	Yessotoxins (some strains only)
Ostreopsis cf ovata	*	**	Palytoxin like compound
Protoceratium reticulatum	*	Absent	Yessotoxins
Lingulodinium polyedricum	*	Absent	Homoyessotoxins
Prorocentrum balticum	**	**	Unidentified hepatotoxins (some strains)
P. faustiae	*	Absent	Okadaic acid and congeners
P. lima	*	Absent	Okadaic acid and congeners
P. minimum	****	*****	Unidentified hepatotoxins (some strains)

BIOLOGICAL INTERACTIONS

The role of allelopathy in the succession of harmful algae in the East China Sea: culture experiments of harmful algal species in algal bloom water

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Abstract

Using algal bloom filtrate water collected during the succession of algal bloom in the East China Sea from April to May 2007, we cultured the main red tide species, *Prorocentrum donghaiense, Alexandrum tamarense* and *Skeletonema costatum* in the laboratory in order to observe if allelopathy plays some role in the algal blooming succession in the East China Sea. In spring 2007, there was a short *Synechococcus* bloom between diatom and dinoflagellate blooms. The filtrate of *Synechococcus* bloom inhibited the growth of *P. donghaiense, A. tamarense* and *S. costatum*. The inhibitory action on *S. costatum* in *Synechococcus* blooming gestation period was stronger than in the bloom period; the opposite was true on *P. donghaiense*. The culture experiments essentially agreed with the *in-situ* algal bloom succession.

Introduction

Besides physical and chemical environmental factors and nutritional status, biological factors play an important role in the process of red tide occurrence and succession. Allelopathy is one of the important biological factors to influence phytoplankton structure. Previous studies have shown that allelopathy can affect interspecies competition in red tides, and thus affect community succession（Levino, 1964; Vance, 1965; Keating, 1978. Although the importance of allelopathy is under debate, the subject attracts increasing attention of marine scientists. Allelopathy between red tide algae is probably a key factors in obtaining advantage during competition. The studies of allelopathy between marine algae will provide new ideas on clarifying occurrence, succession mechanism of red tide and ecological regulation and control. The filtrate culture method is the classic method of allelopathy studies (Arzul, 1994). Using filtrate to culture red tide algae one can study the effect of the secretion into the medium on the algal growth under conditions which eliminate cells in direct contact and nutrient competition. This study used typical red tide water collected from the Zhejiang coast of the East China Sea during April - May, 2007 to culture *Prorocentrum donghaiense*, *Alexandrium tamarense* and *Skeletonema costatum*, in order to reveal the effects of various red tide water on algae growth.

Material and methods

Collection of red tide water

The surface red tide water samples in the Zhejiang coast of the East China Sea was collected in the period from diatom blooms to *Prorocentrum donghaiense* blooms from April to May, 2007. The properties of the samples are listed in Table 1 and sample stations shown in Figure 1. The water was filtered through 0.45μm acetate filters and preserved in plastic cask at -20℃, which was used to culture microalgae in the laboratory.

Table 1. Red tide condition of the samples.

Sample No.	Station	Sampling time	Red tide condition
za4-427	za4	27-04-2007	The dense *Synechococcus*, mixed with small amount of diatom and dinoflagellate
zb10-502	zb10	02-05-2007	*Synechococcus* blooming
rb10-513	rb10	13-05-2007	Phytoplankton dominated by *P. donghaiense* and diatom, but didn't reach bloom density
zb7-521	zb7	21-05-2007	*P. donghaiense* bloom
Normal seawater	zb12	27-04-2007	Non algae bloom near open waters

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The red tide algae

Skeletonema costatum, Prorocentrum donghaiense and *Alexandrium tamarense* derived from the algae seed bank in the Laboratory of Environmental and Ecology Sciences, Institute of Oceanology, Chinese Academy of Sciences.

Cultivation of microalgae

The water sample was thawed at room temperature. 200mL samples in transparent triangular flaskwere used to culture *Skeletonema costatum*, *Prorocentrum donghaiense* and *Alexandrium tamarense*, in the light incubator. The normal water from open sea aged about half a year was used as control. The nutrient, trace element and vitamin in the culture medium were unified to the f/10 level. Each culture was grown at similar temperature, 20 ± 1 ℃; illumination, 2700~3000 lux; light: dark, 12 h: 12h. OD of the algal liquid was determined every 24 hrs to survey algal growth. The calibration equation between algae density and OD value is listed in Table 2.

Table 2. Calibration between algae density and OD

Algae species	The calibration equation between algae density and OD value	R^2	n
P. donghaiense	y=57.259x-0.2619（10^6cell/L）	0.9997	6
S. costatum	y=293.99x-1.0699（10^6cell/L）	0.9995	6
A. tamarens	y=65.551x-0.1241（10^6cell/L）	0.9998	6

The algae cell relative growth rates were calculated according to the following equation: relative growth rate constant: K=log (Nt/N0) / T， in which Nt and N0 are cell number at day t and 0.Mean doubling time: G=0.30l /K.

Results and discussion

Rb10-513 and zb7-521 water affect weakly the three algae growth. The *Prorocentrum donghaiense* blooming water zb7-51 shows a little stimulative effect on the two dinoflagellates, no effect on the *Skeletonema costatum*. The mix water of *Prorocentrum donghaiense* and diatom Rb10-513 presents a feeble stimulative effect to the *Skeletonema costatum*, but no effect on the two species of dinoflagellate. Both the two Synechococcus water za4-427 and zb10-502 display the significantly inhibitory action on the three algae The four kinds of seawater, za4-427,

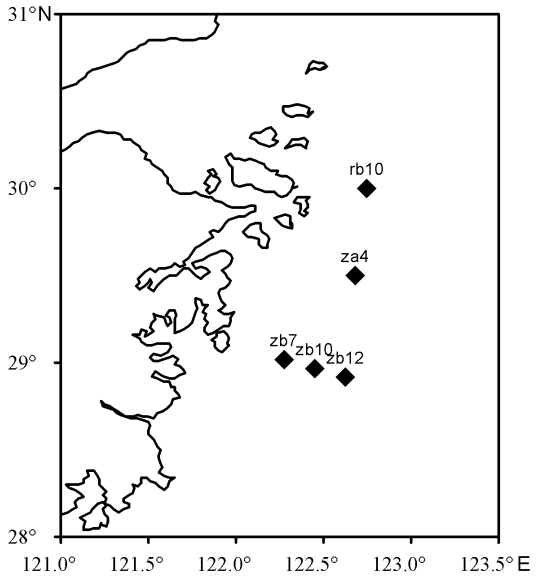

Fig. 1 Sampling stations

zb10-502, rb10-513 and zb7-521 were collected in the period of diatom change to *Synechococcus* bloom and then dinoflagellate bloom, which can represent the various stages of red tide succession. Za4-427 water was in the diatom bloom dispersion period and *Synechococcus* blooming gestation period. Zb10-502 water was in the *Synechococcus* bloom period. The results show that both waters exhibited inhibitory action on *P. donghaiense*, *A. tamarenes* and *S. costatum* (Figure 2). On 27th April *Synechococcus* density increased, *Prorocentrum* density changed slightly and diatom density reduced. In 2nd May, *Synechococcus* proliferated to the high density, *P. donghaiense* blooming is in the gestation period, diatom and *P. donghaiense* remain in the same density. After the diatom blooming, the inorganic nutrients were reduced in the water. Diatom blooming dispersion produced abundant tyrosine-like matter (Zhuo and Zhao, 2009), which suit Synechococcus on the nutritional requirements. *Synechococcus* proliferated quickly, which produced substances to inhibit *P. donghaiense*, *A. tamarense* and *S. costatum* propagation and the Synechococcus became dominant species. Table 3 showed that the inhibitory action of zb10-502 on *P. donghaiense*, *A. tamarense* is stronger than

Table 3. Algal growth parameters in the various red tide waters

Culture water	K_t			G		
	P. donghaiense	*A. tamarense*	*S. costatum*	*P. donghaiense*	*A. tamarense*	*S. costatum*
Normal seawater	0.118	0.044	0.162	2.551	6.888	1.863
za4-427	0.039	0.010	0.040	7.650	30.93	7.589
zb10-502	0.023	0.008	0.100	13.030	39.51	3.013
rb10-513	0.114	0.038	0.170	2.637	7.98	1.767
zb7-521	0.122	0.038	0.163	2.472	7.841	1.846

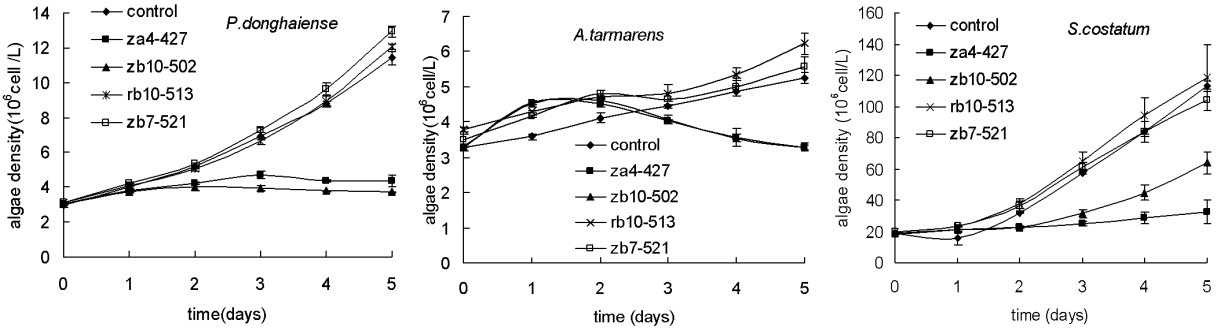

Fig 2. Growth curves of *P. donghaiense*, *A. tamarense* and *S. costatum* cultivated in various red tide waters.

za4-427, but weaker on *S. costatum*, which may be one of the factors about diatom proliferating in advance of the dinoflagellate. At the time sequence from 27th April, 2nd May, 13th May to 21st May, the action of seawater on the *P. donghaiense* and *A. tamareneds* changed from inhibitory to promotive and the strongest inhibitory action occurred on 2nd May. The action on *S. costatum* varied from inhibitory to promotion and the strongest inhibiting effect occurred on 27th April, the strongest facilitation presented on 13th May and then weakened. In the exclusion of nutrients and temperature and other factors, the various effect produced by different red tide water acting on the same algae may be due to some substances in the water to promote or inhibit the algae growth. Namely, allelochemical has played an important role in the phytoplankton growth process. The results agreed with the on-site phenomena.

Conclusion

Using the on-site red tide water to culture the red tide algae, it indicated that in the East China Sea, the seawater in the end of the diatom dispersion, Synechococcus blooming gestation period and the Synechococcus blooming period, have a certain inhibitory action on the *P. donghaiense*, *A. tamarense* and *S. costatum*. The inhibitory action on *S. costatum* in Synechococcus blooming gestation period is stronger than in the Synechococcus blooming period, the opposite is true on the *P. donghaiense*. The results basically agree with the on-site red tide succession process and indicates that allelochemicals play a role in the red tide succession process of the East China Sea.

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The nature of biocidal effects between cyanobacteria and arthropods

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Abstract

Cyanobacterial responses were close to activity of terrestrial plants producing allelochemicals for defense Diverse and multilateral cyanobacteria-arthropod relationships lead to different interpretations. We evaluate cyanobacterial activity by using terrestrial arthropods as the test objects. Insects - lackey moth, fall webworm and Colorado potato beetle - were exposed to cyanobacterial samples (*Microcystis aeruginosa* - 70-100%) from Dnieper basin. Leaves for insect feeding were sprayed with cyanobacteria. Nutrition, growth, metamorphosis, viability and histopathology were examined. Cyanobacteria exhibiting general inhibitory effects, can be defined as including deterrent, metatoxic and larvicidal actions; inhibition of larval growth and disorder of metamorphosis. Larval nutrition and growth was inhibited dependent on dietary habits; anatomical, biological and ecological characteristics of insects, pupation and imagination fell; teratogenesis was observed, and reproduction and fecundity of next generation reduced.. However histopathology was found in the digestive tract and fat body. Insect mortality was recorded at several life-history stages, not only due to toxic activity, but also regression of trophic function and growth. Cyanobacterial biocidal characteristics are more likely to be a deterrent than toxic.

Introduction

Cyanobacterial biologically active substances are important in aqueous environments, and produce different interpretations as to their ecological nature. Long-range, diverse and multilateral symbiotic, antagonistic and parasitic connections exist between cyanobacteria and herbivorous arthropods ("grazers") dependent on complex biochemical interrelations. Recent studies provide evidence of the defensive role of cyanobacterial secondary metabolites, distinct from known biotoxins affecting warm-blooded animals and aquatic organisms during water blooms (Amsler 2008). These compounds influence vital functions of competitors and/or herbivorous organisms, including stress, repellent and deterrent action (but not elimination!) (Jüttner *et al*. 2001; Shaw *et al*. 1997). Cyanobacterial responses are close to inhibitory activity of microalgae and macrophytes to herbivorous consumers (Hay *et al*. 1987; Paul *et al*. 2007), or terrestrial plant-producers of allelochemicals for the defense from plants or microbial pathogens (Gol'din 1995, Gol'din and Gol'dina 2002, 2004). Cyanobacterial metabolites thus may be a source of agricultural and medical preparations for biological control of harmful organisms. Our experiments confirmed the theoretical background for these investigations: antibacterial (Gol'din 2003), antihelminthic (Gol'din & Mendzhul 1996), deterrent, metatoxic and insecticidal (Gol'din 1982, 2004; Gol'din & Gol'dina 2001) characteristics of cyanobacteria were revealed. Cyanobacterial preparations were successfully used in laboratory and field testing as the agents for plant protection (Gol'din & Sirenko, 1998). Here we evaluate cyanobacterial activity in terrestrial arthropods as the test objects, and provide experimental data concerning inhibition of vital functions by cyanobacteria and their derivatives.

Methods and materials

Cyanobacterial samples were collected in the Dnieper basin during summer-autumn "water blooms" in collaboration with Institute of Hydrobiology of t National Academy of Sciences of Ukraine. *Microcystis aeruginosa*. dominated (70-100%); and co-occurred with *Microcystis* spp., *Anabaena variabilis Aphanizomenon flos-aquae*

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,Phormidium mucicola. and *Phormidium* spp.. Cyanobacterial biomass was purified and treated by heating (37°C), lyophilic or acetone drying and milled by laboratory grinding to produce powder. The aqueous suspension (0.5 and 1%) of dry powder forms were used for spraying of leaves for insect feeding. Herbivorous insects with different nutrition - lackey moth *Malacosoma neustria*., fall webworm *Hyphantria cunea* and Colorado potato beetle *Leptinotarsa decemlineata* - at several life-history stages (eggs, different instar larvae and imago) were exposed to cyanobacterial preparations. Insects were from natural populations and kept in glass containers of 1 l volume, 10-15 specimens in each. In model tests leaves of most typical host-plants (potato for Colorado potato beetle, apricot for lackey moth, and ash-leaved maple for fall webworm) were treated via syringe and fed to insects to estimate biocidal activity of cyanobacteria, deterrent and insecticidal characteristics. Each experiment included 3-5 replications. Insect nutrition (% of consumed leave surface per individual), feeding behavior, growth, metamorphosis, and viability were observed during 10-20 d, and histopathology of test-objects examined.

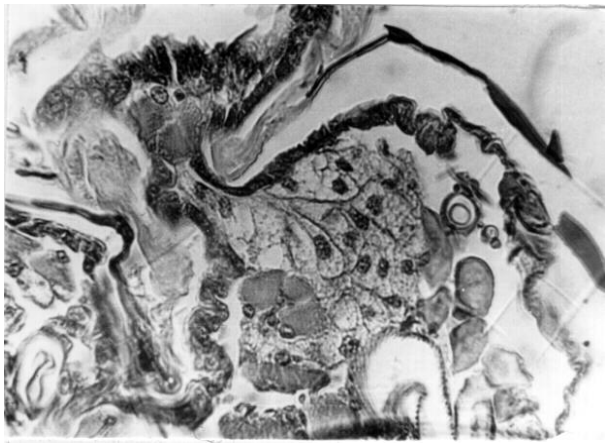

Fig.1. Fat body of Colorado potato beetle during starvation. Cytoplasm of fat body cells is foamy and "hollow", some cells deprived of nuclei, but midgut has no pathological characteristics during 5 d experiment, 16x6.3, hematoxylin - eosin stain.

Results and discussion

Cyanobacteria demonstrated general inhibitory effects to a number of insect functions, including nutrition, fat synthesis, metamorphosis, growth, reproduction and viability (especially in junior instars). After-effects were observed at later life stages (inhibition of pupation, forming of imago and fecundity, and appearance of larval, pupal and imaginal teratogenesis). As a result, insect survival was very low. This complex cyanobacterial impact can be defined as biocidal activity with several components: (1) Deterrent action: larval nutrition inhibited dependent on dietary habits, anatomical, biological and ecological characteristics of insects.

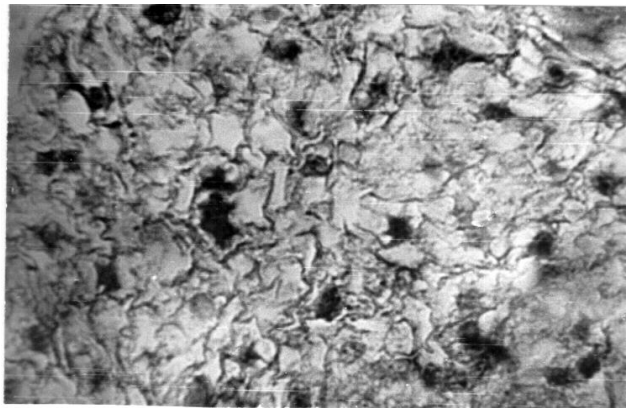

Fig.2. Fat body of fall webworm. Pathological situation, 5th d after infection. “Anucleate cell fields” and foci of patholysis and structural degradation; 16x6.3, Van Gieson stain.

Visual observations and data of leave surface measurement can evidence depression of trophic function (Table 1; Fig. 1). (2) Inhibition of fat synthesis and delay of larval growth: lackey moth – in 50% and more, fall webworm - in 29%, Colorado potato beetle – in 49-64% (larvae) and 15-16% (imago); (3) Disorder of metamorphosis: imago formation in Colorado potato beetle fell in 2.0-4.4 times; formation of pupae and imago were accompanied by significant morphological deflections, such as teratogenesis (non-viable individuals with bullate cuticle in the pupae of fall webworm; reduced wing covers in imago of Colorado potato beetle). (4) Metatoxic activity: reproduction and fecundity of next generation reduced; (5) Toxic action: histopathology in digestive tract and fat body (Fig. 2). In the long run, insect mortality was recorded at several life-history stages; caused not only by toxic/insecticidal activity, but also regression of trophic function and growth. Thus, cyanobacterial biocidal characteristics can be determined to be deterrent rather than toxic. Susceptibility of insect species to cyanobacterial effects depends on the spectrum of nutrition and dietary habits, anatomical, biological and ecological characteristics. Comparison and analysis of the indices of inhibitory processes in feeding, growth and metamorphosis and lethal termination in different cyanobacterial

preparations reflects % *M. aeruginosa* domination as the key factor in the manifestation of biocidal activity. It is possible to trace the direct relation between concentrations of aqueous slurries of primary preparations and their inhibitory and lethal effects. Our study demonstrated that the biocidal effect is due to ingestion of the treated food by herbivorous insects or their direct treatment. Combination of both ways of treatment increases biocidal effects (Table 2) and leads to degradation of all systems in insects. Biocidal activity of cyanobacteria is similar to plant and microbial insecticides in their spectrum and mechanism of action.

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Table 1. Deterrent activity of cyanobacterial samples (leaves treated by 0.5% suspension; 150 larvae each)

N of samples	Way of treatment of cyanobacteria	Consumption of treated leaves during 10 days, % of control					
		Colorado potato beetle			Fall webworm		
		2nd instar	3rd instar	4th instar	2nd instar	3rd instar	4th instar
1	Roller drying	42.8±4.0	40.8±5.5	-	11.1±0.2	-	19.6±3.5
2	Lyophil drying	18.7±3.2	55.0±7.5	-	1.5±0.2	2.0±0.5	42.0±7.7
	Thermal drying	18.0±4.6	49.8±7.0	74.4±3.2	0	12.0±2.6	32.2±10.6
	Aqueous filtrate of biomass (1:1)	7.1±2.2	35.7±6.1	79.1±1.9	12.0±1.0	29.5±9.7	32.5±8.3
3	Thermal drying	5.8±0.6	33.9±4.8	68.3±3.2	12.2±0.4	11.5±1.9	27.8±3.2
Control		100.0	100.0	100.0	100.0	100.0	100.0

Table 2. Cyanobacterial activity (*M. aeruginosa* 98%) and ways of treatment; larval phase, 2nd instar, 30 individuals

Version	Mortality, %									
	Colorado potato beetle					Fall webworm				
	3 day	5 day	7 day	10 day	15 day	3 day	5 day	7 day	10 day	15 day
Treatment of feeding	10.0± 3.1	40.0± 6.8	73.3± 3.4	96.7± 3.4	96.7± 3.4	3.3	20.0±6 .8	20.0± 6.8	66.7± 3.4	100.0
Treatment of insects	0	13.3± 6.8	20.0± 6.8	40.0± 3.4	76.7± 10.2	46.7± 6.8	56.7± 10.2	56.7± 10.2	63.3± 3.4	80.0± 6.8
Complex feeding and insects	16.7± 3.4	53.3± 13.6	76.7± 13.6	93.3± 6.8	93.3± 6.8	40.0± 17.0	46.7±3 .4	50.0	76.7± 10.2	100.0
Control: water	0	0	3.3	13.3± 3.4	13.3± 3.4	0	0	0	0	0

Phytoplankton community composition and diversity effects on the growth of marine *Vibrio* bacteria

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Abstract

Marine autochthonous bacteria of the genus *Vibrio* contain several species of utmost importance to human health. An environmental study indicated that several diversity factors, such as species richness, were major contributors to *Vibrio* growth. In a follow-up lab experiment we found that there was a significant positive effect of increasing phytoplankton species richness on *Vibrio* growth. Thus, this study suggests that healthy phytoplankton cells can serve as a carbon source for *Vibrio* spp. and that increased species richness can positively affect abundance of vibrios. This knowledge should be considered when assessing algal blooms and their affect on pathogenic bacteria.

Introduction

The ecology of bacteria of the genus *Vibrio* is of great importance to humans because it contains several pathogenic species (Conejero and Hedreyda 2004). Most notably is *V. cholerae* which is the causative agent of the lethal, and occasionally pandemic, disease cholera. Previous research has found that vibrios can proliferate together with a number of different phytoplankton taxa (Islam 2002; Rehnstam-Holm et al. 2010). However, it is unknown which phytoplankton community factors regulate variations in *Vibrio* spp. abundance. This project aimed to explore how community factors (such as phytoplankton diversity) contribute to the variation of particle associated *Vibrio* spp. in an oligotrophic tropical marine area of the south-west coast of India. Furthermore, a lab experiment explored the effects of increased phytoplankton species richness on the growth of *Vibrio* spp, in a controlled experimental environment.

Methods

Environmental study

Samples were taken outside the old port of Mangalore, India. Net and surface water samples were collected from a boat at < 1 m depth. One hundred µl from the net samples in different dilutions were spread onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar plates, which for these tropical areas tend to give a good approximation of the abundance of *Vibrio* (Bolinches et al. 1988). Biovolumes of each recorded phytoplankton species were estimated according to formulae in Sun and Liu (2003). Several phytoplankton community factors were calculated. Shannon diversity indices (Shannon 2001) for each sample were calculated based on the number of individuals per ml (HN') and the biovolume per ml (HB'). Also the total phytoplankton biomass per sample, species richness and total number of plankton individuals per sample were calculated. Separate 2-tailed Pearson correlations between Log(x) transformed *Vibrio* spp. data and species richness, HB', HN' and crustacean zooplankton were made in SPSS 16 (SPSS Inc, 2007)

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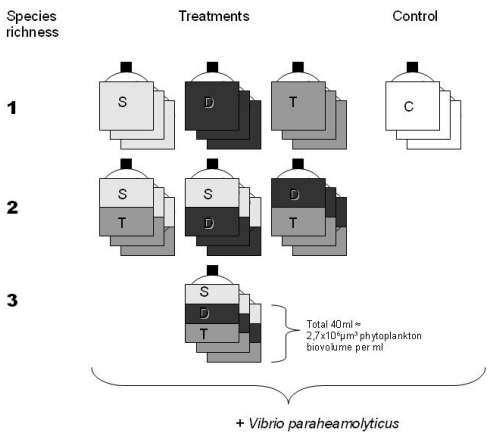

Fig 1. Experimental set-up of 7 different treatments in 3 levels of species richness and 1 control without phytoplankton.

Lab experiment

The experimental set-up contained three treatments with monocultures of the diatoms *Skeletonema tropicum* (S), *Ditylum brightwellii* (D) or *Thalassiosira pseudonana* (T). An additional three treatments contained two-species cultures with the following species combinations: S+T, S+D or D+T. One final treatment contained all three species in equal biovolume, S+D+T. One control treatment consisted of only f/2 medium. All treatments and the control were in triplicate. All experimental flasks contained equal total phytoplankton biovolumes of phytoplankton cells (Fig1). A culture of *Vibrio paraheamolyticus* Kvp4 (provided by the Sven Lovén Centre for Marine Sciences, Sweden) was grown in Lysogeny Broth at room temperature (21ºC). At the experiment start, 100 µl of bacterial solution in exponential phase was added to each of the seven treatments, and to the controls. After 12 hours, 100 µl from each replicate was sampled, spread on a TCBS plate and incubated. The number of colonies on each plate was counted and the effect of the phytoplankton species, and species combinations, on the number of CFUs ml^{-1} was tested against each other with two separate one-way analysis of variance (ANOVA). Secondly, the effect of phytoplankton was tested against the control with a t-test. Finally, the effect of species richness on *V. parahaemolyticus* growth was tested with a one-way ANOVA. To decrease the risk of a type-1 error due to the four tests done on one set of data, the significant p-value was adjusted according to Bonferroni correction (Rice 1989).

Results

Environmental studies

Pearson correlations between *Vibrio* abundances and the different diversity factors showed significant correlations with the number of phytoplankton species (Fig 2a) and HN' (Fig 2b). Pearson correlation between *Vibrio* abundances and HB' was non-significant (Fig 2c). Pearson correlation between *Vibrio* abundance and the abundance of crustacean zooplankton was significant (Fig 2d).

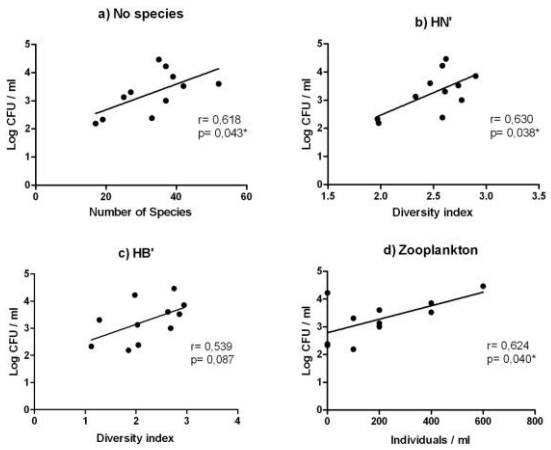

Fig 2. Pearson correlation between environmental *Vibrio* spp. abundances and a) number of phytoplankton species, b) Shannon diversity index based on phytoplankton abundance, c) Shannon index based on phytoplankton biovolumes and d) zooplankton abundance. * indicate significant correlations.

Lab experiments

The lab experiment failed to find any difference between the effects of the three different species (F=0,5800 p=0,5884, Fig 3a) or species combinations (F=1,797 p=0,2447, Fig 3b) on the growth of *V. parahaemolyticus.* This indicates that the observed differences in bacterial growth were not due to single phytoplankton species effects. Secondly, the study indicated that there was a significant effect of adding algae to the bacteria, compared

to growth in f/2 media only (t=3,519 df=22 p=0,0019, fig 3c). Consequently, the added *Vibrio parahaemolyticus* were favoured by exponentially growing algae. Thirdly, the experiment showed a significant positive effect when species richness was increased >1 (F=7,143 p=0,0052, fig 3d).

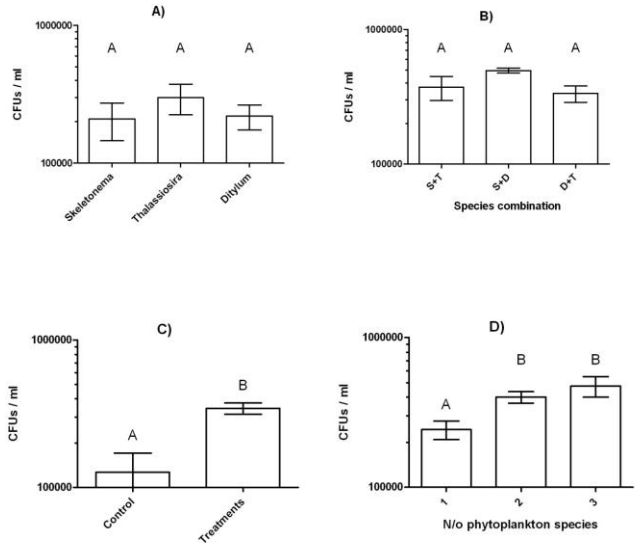

Fig 3. Results from the lab experiment.: a) Monocultures ; b) Combinations from two-species treatments c) Effect of algae; d) Effect of increased species richness. Letters (A or B) indicate significantly distinct groups.

Discussion

This study showed that healthy phytoplankton in exponential phase can serve as an important carbon source for *Vibrio* spp. bacteria. Furthermore, increased species richness of phytoplankton did positively affect the abundance of vibrios, both in the lab experiment but also in the environmental study. Consequently, diversity of phytoplankton may have an effect on the bacteria, which lies beyond the effect of the included individual species. The effect of single phytoplankton species on the growth of *Vibrio spp.* may change with environmental conditions. However, phytoplankton diversity may be a more general contributing factor to *Vibrio* growth. This knowledge is important during assessments of *Vibrio* epidemiology.

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Isolation of *Microcystis*-killer bacterium *Agrobacterium vitis* from the biofilm on the surface of the water plant *Egeria densa*

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Abstract

Microorganisms such as bacteria appear to be promising control agents against cyanobacterial blooms of toxic species such as *Microcystis aeruginosa*. We investigated cyanobacteria-killer and -inhibitor bacteria in the biofilm on the surface of water plants such as *Egeria densa* and *Ceratophyllum demersum* in Lake Biwa, Japan in 2008. Bacteria were isolated using nutrient agar plates, and the killing ability was examined for *M. aeruginosa* by co-culture experiments. We isolated *Microcystis*-killer and growth-inhibiting bacteria from the surface of these water plants. The most powerful bacterial strain (identified as *Agrobacterium vitis*) entirely killed *M. aeruginosa* culture cells within two days after the initiation of co-culture experiment when bacterial cells were added as agglomerations of cells (colony cake) at equal densities of about 10^5 and 10^6 cells/ml. However, killing of *M. aeruginosa* was not observed but growth inhibition was observed, when bacterial cells were inoculated as a cell suspension after the complete dispersion of colony. These results suggest a quorum sensing for killing mechanism of *M. aeruginosa* by the bacterium.

Introduction

Toxic blooms of cyanobacteria have been observed with increasing frequency in lakes, ponds and drinking water reservoirs in almost every part of the world (Cronberg and Annadotter 2006). *Microcystis* is well known as one of the most harmful bloom-forming cyanobacteria causing toxicity and deterioration of water quality to give negative effects on animals and human beings, and decreasing aesthetic value of affected water. Therefore, there is an urgent and compelling need for the development of mitigation strategies for reducing negative impacts of toxic cyanobacterial blooms. As environmentally friendly tools for biological control of toxic cyanobacterial blooms, algicidal bacteria have received attention to be promising agents against the blooms since they are abundant in aquatic ecosystems, proliferate rapidly, and are sometimes prey-specific (Salomon and Imai 2006). It was newly discovered that huge numbers of algicidal bacteria are attached to the surface (in biofilm) of seaweeds and seagrass in coastal seas (Imai et al. 2006, 2008). In the present study, we investigated algicidal bacteria in a water plant bed in Lake Biwa, the biggest lake in Japan, and we newly isolated effective algicidal bacteria (strains of *Agrobacterium vitis*) from the surface (in biofilm) of the water plant *Egeria densa*. We here report on algicidal activity of the isolated bacterial strain against *M. aeruginosa*.

Materials and Methods

Samples of water plants (*Egeria densa* and *Ceratophyllum demersum*) were collected using 250-ml sterilized bottles at the water plant zone of Ogoto Port (Fig. 1; 35.09°N, 135.90°E), facing to Lake Biwa, on May 26, June 9, 16, 30, July 14, August 29 and September 29, 2008. Samples were chilled and transported to the laboratory within 2hrs. Autoclaved water (200ml) was added to the bottle containing each collected water plant, and the bottles were shaken 600 times by hand to release microorganisms from the surface of water plants. After taking out the plants, the water with detached microorganisms was appropriately diluted and smeared to the nutrient agar plate (Ishida et al. 1986) to count and isolate viable bacteria after colony formation. Each bacterial colony was transferred to a new agar plate to grow again, and each bacterial clone culture was established. The screening of algicidal activity for each bacterial clone was carried out by co-culture experiments using axenic culture of *M. aeruginosa*. Small piece of bacterial colony was picked up with sterilized toothpick and put into 0.5-ml culture of *M. aeruginosa* (ca. 10^5 cells/ml) in each well of 48-wells microplate (bacterial cell density of about

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10^5 cells/ml), and observed the changes of cyanobacterial cells every day using an inverted microscope.Bacterial strains (O-0805a15, O-0805a19 and O-0805b11) were obtained as killer bacteria with highly effective activity from the water plant *E. densa* (Fig. 1) collected on May 26, and identified according to information on 16S rDNA sequences. The difference of algicidal activity was examined by changing the manner of inoculation of bacteria, i.e. free-floating suspension or colony cake. For free-floating experiment, the colony of bacterium was collected and put into 1ml CT medium in a 1.5-ml microtube and completely dispersed and prepared bacterial cell suspension at the density of about 10^8 cells/ml. Bacterial suspension was then inoculated on day 2 at a final density of about 10^6 cells/ml into 5-ml *M. aeruginosa* culture (ca 10^5 cells/ml) in triplicate test tubes. For colony cake experiment, a piece of colony was put into the same *M. aeruginosa* culture. Control was by no addition of bacteria to *M. aeruginosa* culture. Changes of *M. aeruginosa* (*in vivo* fluorescence) were monitored every day with a fluorometer. Bacterial cell densities were also measured by sampling 0.1-ml from the test tubes every day and by counting with epifluorescence microscopy after DAPI-staining.

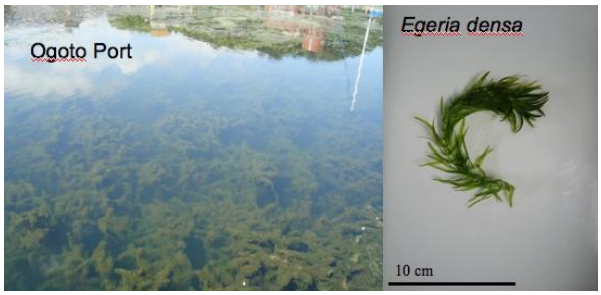

Fig 1. Sampling site of water plant zone in Ogoto Port, south basin of Lake Biwa, and the water plant *Egeria densa*.

Results and Discussion

As a result of screening of algicidal activity for each bacterial clone, a total of 37 strains were obtained from water plants as killer bacteria and growth-inhibiting bacteria against *M. aeruginosa*. Three strains (O-0805a15, O-0805a19 and O-0805b11) were highly effective algicidal bacteria that entirely killed *M. aeruginosa* within two days after the addition of cells with density of about 10^5 cells/ml (Fig. 2). These three strains were isolated from the water plant *E. densa* collected on May 26, and identified to be *Agrobacterium vitis*.

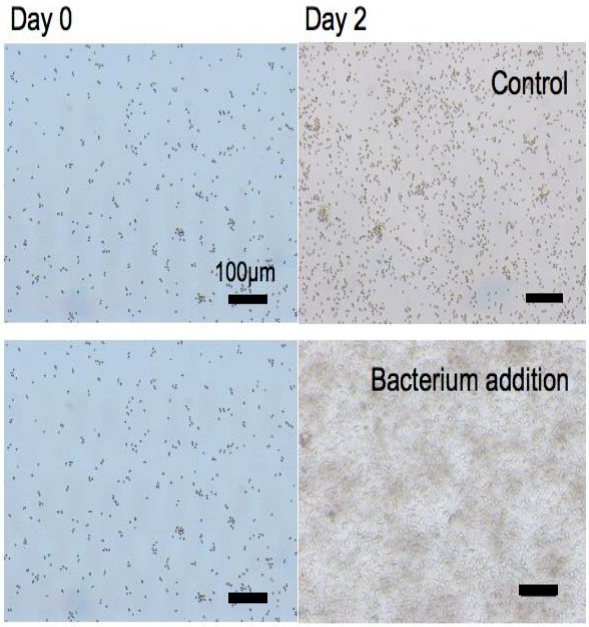

Fig 2. Photomicrographs of co-cultures for screenings of algicidal bacteria against *Microcystis aeruginosa*. Bacteria were added as small colony cake at equivalent density of about 10^5 cells/ml. Control, no addition of bacteria; Bacterium addition, with algicidal bacterium *Agrobacterium vitis* strain O-0805a15.

As *Microcystis*-killer bacteria, different groups were reported such as gliding bacteria (*Lysobacter, Alcaligenes, Flavobacterium and Cytophaga*), *Pseudomonas* and *Bdellovibrio* (Daft *et al*. 1975, Caiola and Pellegrini 1984, Mitsutani *et al*. 1987, Yamamoto *et al*. 1993, Manage *et al*. 2000). These were all isolated from water. The bacterium *A. vitis* killed *M. aeruginosa* more effectively when inoculated in the state of agglomeration of cells (colony cake) than floating cell suspension (Fig. 3). This result suggests a possibility of involvement of quorum sensing in the algicidal procedure. *A. vitis* is reported as a pathogen of crown gall of grape (Burr *et al*. 1998), and quorum sensing is involved in the disease (Whitehead *et al*. 2001). Quorum sensing is also reported in the regulation of algicidal activity against marine harmful microalgal species (Skeratt *et al*. 2002, Nakashima *et al*. 2006). Therefore, it is much interesting to further investigate the algicidal mechanisms of *A. vitis*. In the south basin of Lake Biwa, the area of water plant zone has increased from 1994, and it currently occupy about 70 - 80% of the bottom area of the south basin (Hamabata and Kobayashi 2002). Interestingly,

occurrence incidents of *M. aeruginosa* blooms have decreased in accordance with increase of water plants. We here present a hypothesis that the decrease of *M. aeruginosa* blooms attribute to the algicidal activity of bacteria released from the surface of water plants (in biofilm), although Nakai *et al*. (1999) reported the growth inhibition of cyanobacteria by allelopathic effects by macrophytes themselves. Comparative investigations are needed on dynamics of bloom-forming cyanobacteria and algicidal bacteria in biofilm of water plants and in waters in plant zone and offshore areas.

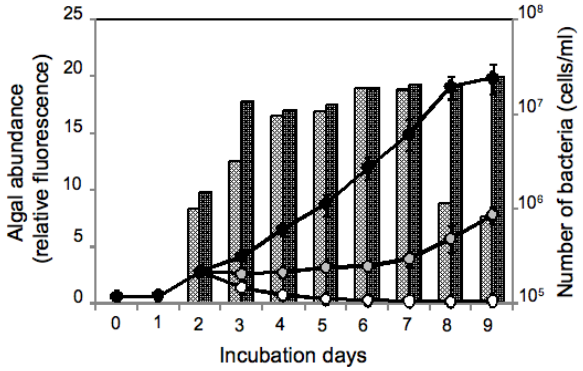

Fig 3. Effects of the bacterium *Agrobacterium vitis* strain O-0805a15 on the growth and death of *Microcystis aeruginosa* (black circle: growth of *M. aeruginosa* with no addition of bacteria; grayish circle: cyanobacterial growth with completely dispersed and suspended bacteria; white circle: cyanobacterial growth with agglomeration (colony cake) of bacteria). Bacteria were inoculated on day 2 of incubation of *M. aeruginoa*. Checked bar: cell density in tubes with addition of bacterial agglomeration; Dark bar: cell density in tubes with addition of dispersed and suspended bacteria.

Acknowledgements

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IMPACTS ON FOODWEBS

Thalassorheology, algal blooms and vertical carbon flux

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Abstract

Many natural waters are functionally non-Newtonian. Thalassorheology (TR) is the study of the Rheology of seawater and other natural waters, while Rheology is the study of the deformation of non-Newtonian liquids, whose properties lie between those of an ideal liquid and an ideal solid. The state of the art of TR is reviewed to provide the context encompassing current TR research in China. This context includes control of vertical carbon flux and pycnocline dynamics by TR properties, due to plankton-produced exopolymeric substances. In particular, TR properties of phytoplankton and bacterioplankton cultures are being investigated as a function of length scale. A capillary-tube viscometer with pressure transducer has been devised, where an explicit length scale is given by tube diameter.

Thalassorheology

Rheology is the study of the deformation of materials whose properties lie between those of ideal liquids and ideal solids. Thalassorheology is the rheology of natural waters, that Jenkinson (2010) and Jenkinson & Sun (2010) have reviewed, particularly in relation to algal blooms. Rheological measurements have shown that the viscosity of seawater consists of a Newtonian component due to water and salts plus a non-Newtonian "excess" component due mainly to algal and bacterial exopolymeric substances (EPS). This excess viscosity shows relationships with shear rate (usually negative), phytoplankton abundance (usually positive) and probably length scale (negative) (Jenkinson & Sun 2010, in press). Total seawater viscosity,

$$\eta = \eta_W + \eta_E$$
$$\eta_E = k.\dot{\gamma}^P.Phyto^Q.L^d$$

where η_W is the component of it due to water and salts, and η_E that due to EPS. k is an empirically measured coefficient, $\dot{\gamma}$ is shear rate, *Phyto* is phytoplankton biomass and L is length scale. P, Q and d are power-law exponents that have to be determined case by case. The length-scale exponent d is due to the lumpiness of the EPS, where a value of zero would correspond to perfectly dispersed EPS, while increasingly negative values of d correspond to increasing fractal lumpiness. EPS also adds elasticity to the seawater.

Vertical carbon flux

CO_2 is removed from the surface layers of the oceans as fixed organic and inorganic carbon by vertical flux consisting of a physical pump and a biological pump (BP). Both pumps deliver this fixed carbon to deeper layers, where the organic fraction is partially respired, causing benthic and deep-water deoxygenation and even dead zones (Wei *et al* 2006; Hansell *et al.* 2009). The BP is determined by: 1) phytoplankton secretion and aggregation; 2) zooplankton grazing and repackaging; 3) aggregation due collisions from both differential shearing and differential settling and collision; 4) aggregate fragmentation at depth (Burd & Jackson 2008). Rheological properties (particularly strength) and density of phytoplankton exopolymers (EPS) are part of a suite of properties determining the processes mentioned above (Jenkinson *et al.* 1991). Eutrophication, by increasing phytoplankton production and standing stocks, also tends to increase the BP, and so is a parameter in models of both CO_2 drawdown and deep-water deoxygenation (Emerson *et al.* 2001).

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Pycnoclines and thin layers

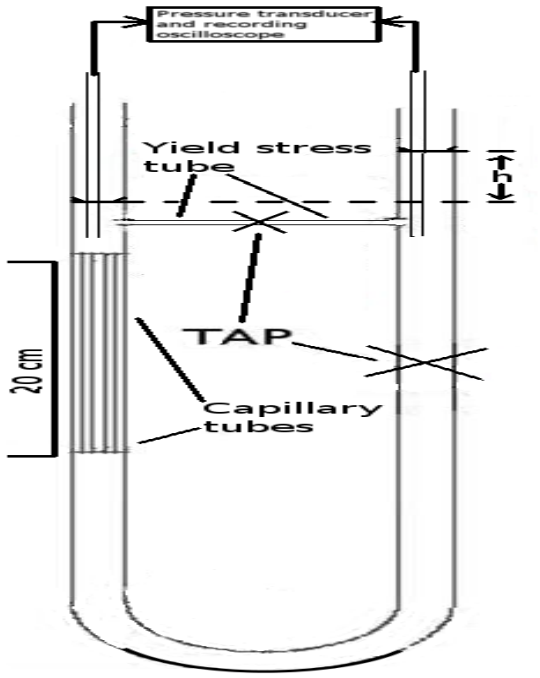

Fig 1. Sketch (not to scale) of the capillary viscometer. h indicates the height (pressure difference) between the water levels in each of the two arms. This is measured by a differential pressure probe and recorded as the test material flows from the right arm to the left one through a battery of capillary tubes.

Thin layers of high concentrations of phytoplankton occur frequently associated with pycnoclines. Although rheological measurements have not yet been made in water from such thin layers, their generally enhanced amounts of organic aggregates, both in total biovolume and in mean volume per aggregate (Alldredge *et al*. 2002) suggests that they may be zones of markedly increased viscosity. Thin layers of abundant plankton occur also in the central Yellow Sea (YS), associated with the summer thermocline, and off the Changjiang (Yangtze) estuary, where summer stratification is thermohaline (Tsunogai *et al.* 2003; Wei.*et al.* 2007).

Biological and rheological control of pycnocline dynamics

The biorheological effect of blooms of the harmful dinoflagellate *Karenia mikimotoi* on pycnocline stability has been recently modeled (Jenkinson & Sun, in press), incorporating control of pycnocline thickness, by a critical Richardson number. It uses published values of shear-rate-related viscosity, measured at a length scale of 0.5 mm (Jenkinson 1993). The model indicates that whether high phytoplankton biomass will control pycnocline dynamics, particularly thickness. This will depend strongly on the length-scale exponent *d*, explained in the Thalassorheology section above.

Turbulence and food-web structure

The effect of turbulence on controlling food-web structure has long been recognised (Margalef *et al*. 1980), and this has recently been studied using a validated model of turbulence in a shelf sea, made using the General Ocean Turbulence Model (GOTM) framework (Allen *et al*. 2004). If biological control of pycnocline dynamics is significant, for example through differential heating (Murtugudde *et al*. 2002) and/or rheological modification, some phytoplankton species and genes will be able to engineer (i.e. manage) water-column structure to suit their evolutionary advantage (Jenkinson & Wyatt, 1995; Wyatt & Ribera d'Alcalà, 2006). In the GOTM community, discussions are underway for incorporating data on thalassorheology as a turbulence modifier. Like EPS, fluid mud is non-Newtonian. We are therefore following GEOHAB (in press) recommendations by co-ordinating with fluid mud workers to establish modules that incorporate Thalassorheology data into GOTM.

China Seas

In the decades up to 2005, increases in the phytoplankton of the Changjiang estuary, have been associated with eutrophication due to increased nutrient input (Zhou *et al.* 2008), in common with many coastal waters worldwide. Here, as in other areas, eutrophication-fuelled vertical flux is contributing to hypoxic "dead zones" (Wei *et al*. 2006). In the central YS and the northern East China Sea, a strong summer thermocline develops, which caps Cold Yellow Sea Bottom Water (Liu *et al*. 2009). In China there is concern to understand the BP

better, partly as a component in global CO_2 and ocean pH dynamics and partly with respect to ocean O_2 dynamics.

Rheological measurements in China

We are measuring the effect of length scale on the viscosity of phytoplankton and marine bacterial cultures. The primary object is to validate the model of Jenkinson & Sun (in press), and derive values for *d*. The apparatus (Fig. 1) is derived from previous ichthyoviscometers (Jenkinson *et al*. 2007a, 2007b), but the fish gills have been replaced by modules of parallel capillary tubes, 0.7 to 3 mm in diameter, arranged vertically as in an Ostwald or Ubbelohde viscometer. Test fluid is added to the right-hand tube, so as to flow through the capillaries, while a pressure transducer and recording oscilloscope measure the change in hydrostatic pressure. By carrying out measurements with capillaries with a range of diameters, viscosity is measured as a function of both pressure (stress) and an explicit length scale (tube diameter).

Acknowledgements

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Sodium Channel Variant in the Softshell Clam *Mya arenaria* Offers Natural Resistance to Toxic Algal Blooms

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Abstract

Paralytic shellfish poisoning (PSP), caused by human consumption of bivalves that feed on toxic dinoflagellates, *Alexandrium spp.,* is a recurrent problem in the Gulf of Maine, USA. Softshell clams, *Mya arenaria*, are native to areas affected by PSP toxins, requiring annual harvesting closures that result in significant economic losses. A mutation of the Na^+ channel gene (Domain II pore region) in resistant *M. arenaria* individuals markedly decreases the affinity at the saxitoxin-binding site, allowing these individuals to achieve much higher toxicities than those of sensitive clams without this mutation. Prior work discovered that the incidence of this Na^+ channel variant is higher in softshell clam populations that experience frequent, intense red tides, compared to those unaffected by PSP. We surveyed clam populations throughout the Atlantic coast of North America that varied in their history of PSP. Sequencing of the Na^+ channel pore region (Domain II) allowed comparison of the percent of homozygous (resistant or sensitive) and heterozygous individuals. Genotypic composition was related to the clams' PSP history and to softshell seed planting practices. The resistant genotype is prevalent in clam populations affected by PSP, including those that are geographically distant, suggesting that this variant has occurred multiple times in separate populations.

Introduction

We have previously identified a mutation in Domain II of the pore region of the sodium (Na^+) channel in *Mya arenaria* that appears to grant a natural "resistance" to the effects of paralytic shellfish toxins (PSTs) produced by the harmful dinoflagellates *Alexandrium spp.* This mutation is the result of a single amino acid substitution, where glutamic acid is replaced with aspartic acid (Bricelj *et al*, 2005) (Table 1). This change ultimately results in an alteration of the saxitoxin binding site which drastically decreases binding affinity, thus conferring the observed resistance. The latter leads to increased survival and toxin accumulation during Alexandrium blooms. Previous work has shown that this Na^+ channel mutation follows a simple autosomal Mendelian inheritance pattern (Hamilton 2009), which results in three possible genotypes: one that is homozygous for the "resistant" allele (RR), one that is homozygous for the "sensitive" gene (SS), and one that is heterozygous (RS). Heterozygotes appear to have intermediate nerve resistance between SS and RR clams (Connell *et al*, 2007). Laboratory experiments have demonstrated that a simulated *Alexandrium* bloom strongly selects individuals carrying the resistant (R) allele in a population (Bricelj *et al*, 2010), which raises the following questions: Do costs associated with resistance under conditions of low or undetectable PSTs result in a genetic pool that increases the percent of the sensitive (S) allele in the population? Do populations that have had a continual new influx of the R allele maintain the genotypic composition of the source population under non-selective conditions? To investigate these questions we conducted surveys of the genotypic composition of clam populations in four regions of the NW Atlantic coast (Canada and the USA), comparing these results against the known incidence of *Alexandrium* blooms in those regions. The eastern Maine/Bay of

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Fundy (EM/BoF) region experiences annual, intense bloom events, and clams in this region are frequently exposed to high levels of PSTs. The Mt. Desert Island (MDI) and western Maine regions have fewer and less intense blooms. The fourth region, in southern New England and New York (Southern NE/NY) historically does not experience *Alexandrium* blooms, although one site within that region (Northport Harbor, NY) has a very recent history of low toxicity red tides. Since PST exposure strongly selects for the R allele, we expect to see a correlation between the frequency and intensity of *Alexandrium* blooms and the proportion of resistant clams.

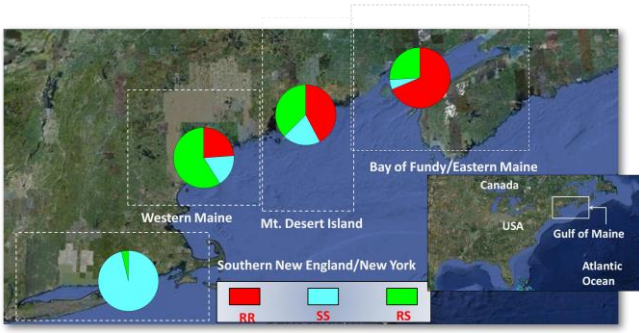

Figure 1. Proportion of homozygote resistant (red), homozygote sensitive (blue), and heterozygous (green) individuals at each sampling site along the Atlantic coast of North America. Sites were sampled over a period of time from 2007 to 2010.

Methods

Adult *Mya arenaria* were collected from sites throughout the four regions of interest: EM/BoF, MDI, Western ME, and Southern NE/NY. The number of clams collected at each site ranged from 21 to 100, averaging 43 clams per site. Hemolymph was drawn from the anterior adductor muscle of each clam using a syringe, and was used to obtain DNA for sequencing Domain II of the pore region of the Na^+ channel. DNA extraction and sequencing followed the methods described by Connell *et al* (2007) and Hamilton (2009). Each hemolymph sample was centrifuged to produce a cell pellet, which was suspended in Tris-HCl buffer and stored at -20°C. A 172bp fragment containing the allele of interest was amplified using PCR, and the resulting product was purified using the Promega Wizard SV gel and PCR clean-up system. Sequencing was carried out by the University of Maine DNA Sequencing Facility, and the sequences were analyzed using Sequencher 4.8. The DNA sequences were examined to determine the genotypic composition of the population at each site, and to ascertain the percentage of RR, RS, and SS clams. These results were then compared to the known incidences of *Alexandrium* blooms in these regions, as well as *M. arenaria* juvenile planting records in the Gulf of Maine.

Results

The EM/BoF region (8 sample sites) was comprised predominantly of clams carrying the R allele (averaging 68.7% RR or 26.0% RS) with few SS clams present (5.3%). The MDI region (n = 7 sample sites) had a larger percentage of heterozygote (RS) (37.4%) and SS individuals (20.3%) than the EM/BoF region, but was still dominated by RR individuals (42.3%). The Western ME region (n = 3 sites) had an intermediate distribution of genotypes dominated by RS clams (23.8% RR, 58.9% RS, and 17.2% SS). The Southern NE/NY region (n = 3 sites) was comprised entirely of clams carrying the S allele, with only a few heterozygotes, and no resistant clams at any of the sample sites in that region (0.0% RR, 3.7% RS, and 96.3% SS).

Genotype	Nucleotide	Amino acid
SS	AA	EE
RR	CC, TT or CT	DD
RS	AC or AT	ED

E (glutamic acid) ➡ D (aspartic acid)

Table 1. Three identified genotypes in *M. arenaria* based on the pore region (DII) sequence of the Na^+ channel gene, and the corresponding amino acid in the encoded protein. A = adenine, C = cytosine; T = thymidine. SS = homozygote sensitive; RR = homozygote resistant; RS = heterozygote of intermediate nerve resistance.

Discussion.

As predicted, we found that the frequency of the R allele in each region reflected the history of PST exposure. The highest percentages of the R allele were found in the EM/BoF region where the highest exposure to PSTs occurs. As selective pressure for the R allele decreases with decreasing PST exposure in the MDI and Western ME regions, the percentages of resistant clams decreased as well.

An important factor that must be considered when relating the incidence of the R allele with PSP history is the ongoing practice of field plantings of *Mya arenaria* juveniles in the Gulf of Maine. This practice has been occurring for decades (Beal, 2004), and the source population for these juveniles is in the EM/BoF region. Thus, in interpreting results it must be kept in mind that clams from this region have been propagated from north to south throughout the Gulf of Maine, introducing the R allele into geographically distant clam populations. However, despite this repeated introduction of the R allele into the MDI and Western ME

regions, the incidence of the RR genotype in those areas remains lower than that of the source population in the EM/BoF region (Figure 1). This has led us to hypothesize that there is some cost associated with resistance to PSTs that only makes resistance beneficial in areas that are frequently exposed to high levels of PSTs. In turn, when resistant individuals are introduced to a population under non-selective conditions they may be at a disadvantage compared to sensitive clams. Future work will investigate this potential cost of resistance.

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Red tides and anoxia: an example from the southern Benguela current system

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Abstract

The oxygen regime in St Helena Bay was investigated in relation to the development and decay of red tide leading to an anoxia-induced mass mortality in April 1998. Sampling of three transects in the St Helena Bay region at the time of the mortality was informative with respect to the mechanisms of formation and scales of anoxia within the Bay. Anoxia was shown to be confined to shallow nearshore environments where the decay of red tide was advanced through the inaccessibility of subthermocline nutrients. The challenge of monitoring and predicting events of anoxia in St Helena Bay is exacerbated by their episodic and localized character.

Introduction

The expansion of hypoxia and anoxia represent major perturbations to the diversity, structure and functioning of marine ecosystems. In the Benguela Current Large Marine Ecosystem low oxygen events have a pronounced impact on living marine resources (van der Lingen et al. 2006). In particular the rock lobster *Jasus lalandii* has been subject to large mortalities in the St Helena Bay region, following anoxia linked to the decay of red tides (Cockcroft et al. 2000, 2008; Monteiro et al. 2006). Despite their dramatic impact such events of anoxia have been inadequately sampled and therefore remain poorly parameterized. This paper reports on the oxygen regime in St Helena Bay and its linkage to red tide following reports of mortalities on the south eastern shores of the Bay in April 1998.

Data Collection

Phytoplankton samples collected daily at Elands Bay (Figure 1) from 1 July 1997 – 30 June 1998 were enumerated using the Utermöhl method (Hasle 1978). Following reports of marine mortalities in the region of Dwarskersbos, three transects were conducted in the greater St Helena Bay region (Figure 1) – off Lambert's Bay (22 April 1998), Elands Bay (23 April 1998), and Dwarskersbos (24 April 1998). Profiles of hydrographic and biological parameters were obtained with a Seabird CTD (with pH and oxygen probes) and a Chelsea Instruments Aquapack fluorometer.

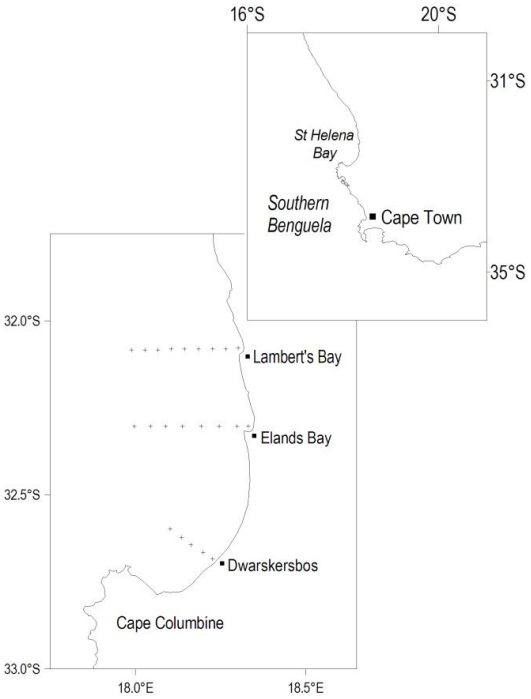

Fig. 1: A map depicting the positions of three transects conducted out of Lambert's Bay, Elands Bay and Dwarskersbos in April 1998.

Results and Discussion

Shore-based sampling of the phytoplankton at Elands Bay revealed the presence of red tide in summer and autumn (Figure 2). A mixed dinoflagellate bloom, dominated initially by *Prorocentrum micans* and later by *Ceratium*

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furca, was evident in December and early January. The bloom returned to the nearshore environment in April at which time it was dominated by *Ceratium lineatum* and *Ceratium balechii*. The anoxic event, leading to mortalities in the region of Dwarskersbos in late April, was considered an outcome of the inshore accumulation, southward transport and ultimate decay of this bloom.

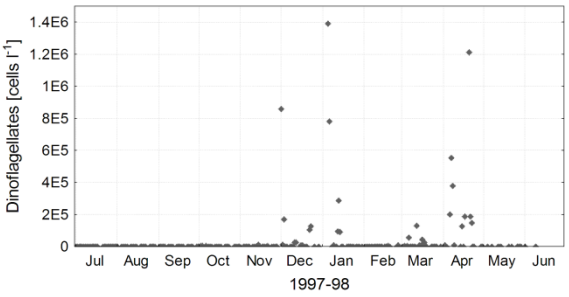

Fig 2: Daily time series of dinoflagellate concentrations at Elands Bay from 1 July 1997 to 30 June 1998.

The sampling of 3 transects in the St Helena Bay region at the time of the mortality provided valuable insight into the mechanisms of formation and scales of anoxia within the Bay (Figure 3). The Lambert's Bay and Elands Bay transects were characterized by a strong thermocline over much of their extent. Low oxygen (<1 ml l^{-1}) was found offshore of the 50 m depth contour in cold bottom water, with greater depletion (<0.5 ml l^{-1}) off Elands Bay. Good correspondence of inshore fluorescence and oxygen maxima indicated the presence of a healthy high biomass dinoflagellate bloom. The high inshore fluorescence demarcated a band of red tide approximately 5 km wide. The fluorescence maximum offshore of the marked colour front was subsurface.

The Dwarskersbos transect (Figure 3) revealed downwelling toward the shoreline. In contrast to the other sections, the inshore fluorescence maximum off Dwarskersbos coincided with a zone of severe oxygen depletion providing evidence of bloom decay. The band of severe oxygen depletion extended to a depth of approximately 10 m. At the shallow (3 m) inshore station oxygen concentrations of approximately 0.1 ml l^{-1} were measured throughout the water column. Inshore levels of pH were also exceptionally low (6.6) confirming high rates of heterotrophic respiration and the oxidation of organic material. The very high oxygen demand leading to anoxia in the shallow nearshore environment off Dwarskersbos was largely attributable to the exceptional carbon load contributed by the red tide in a relatively small volume of water. The decay of the bloom was attributed to the inaccessibility of subthermocline nutrients to the inshore bloom under conditions of persistent downwelling. An intense front established by the inshore oxygen demand indicated limited lateral exchange with offshore oxygenated waters. Biogeochemical processes appear to establish the oxygen front independent of any density gradient. Such events of shallow water anoxia are likely to be maintained only under benign weather conditions while wind-induced mixing or wave action is likely to lead to their termination.

Conclusion

Events of anoxia in St Helena Bay are likely to demonstrate a transient character owing to the extreme variability of much of the Bay shoreline governed by synoptic variations in the wind. The resulting episodic and localized scale of anoxia within the Bay will contribute to the challenge of monitoring and predicting these events.

Acknowledgements

We thank Rick Harding, Stewart Bernard and Desiree Calder for assistance in sampling and analysis.

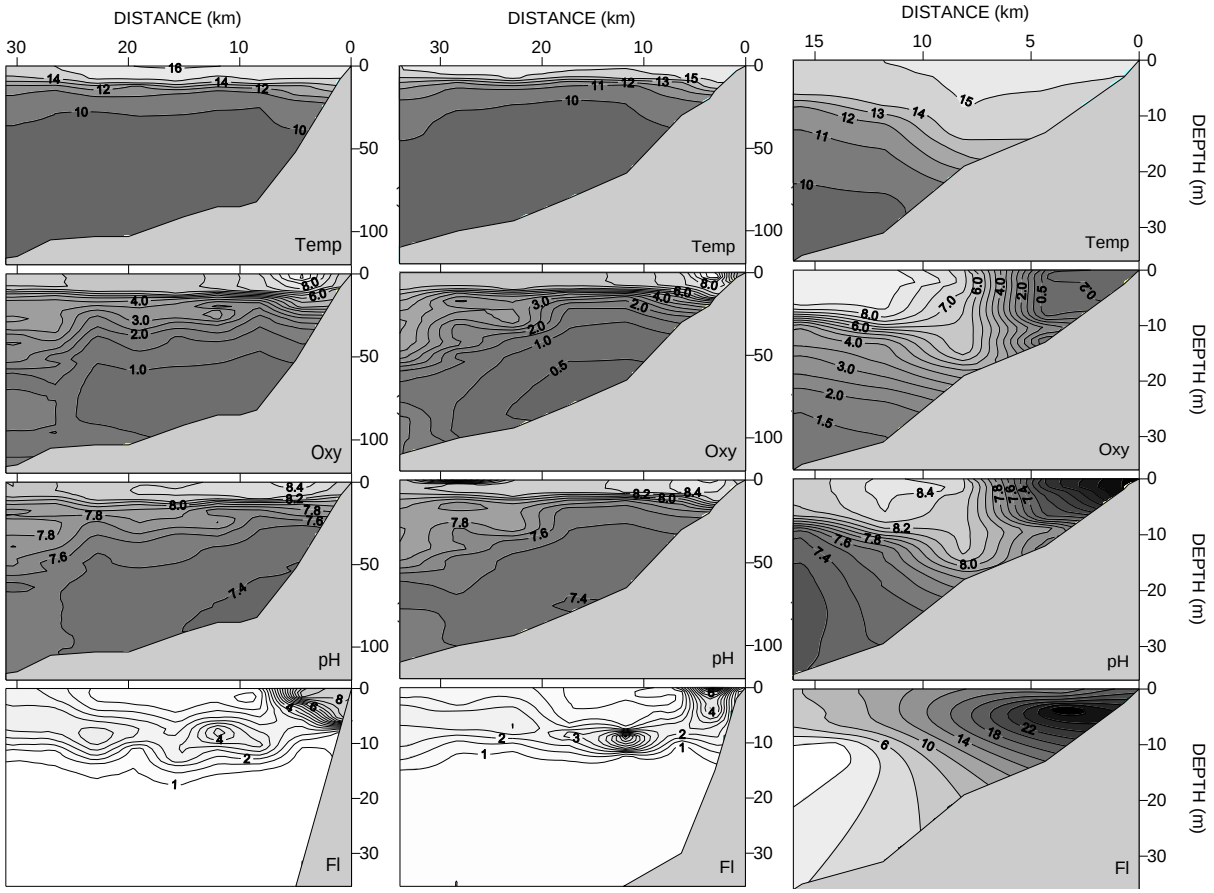

Fig 3: Sections of temperature (°C), oxygen (ml l^{-1}), pH and fluorescence obtained from transects conducted out of Lambert's Bay (left), Elands Bay (middle) and off Dwarskersbos (right).

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Toxin accumulation in benthic populations under blooms of *Dinophysis acuminata* and *Pseudo-nitzschia multiseries*

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Abstract

The present study summarizes the results of toxin accumulation in macroinvertebrates during *Dinophysis acuminata* and *Pseudo-nitzschia multiseries* blooms, in Thermaikos Gulf and Ria de Vigo respectively. Toxin accumulation was investigated in cultured mussels and in benthic populations sampled under and outside the mussel culture area. Okadaic acid (OA) contamination in Thermaikos Gulf was higher in *Mytilus galloprovincialis*. Among the natural populations the highest levels of OA have been found in *Modiolus barbatus*, while scallops, clams, and polychaetes contained only traces or undetectable values of OA. Domoic acid (DA) contaminated all the natural populations in Ria de Vigo, while *M. galloprovincialis* showed undetectable or minimal toxicity. Among the benthic populations *Nassarius reticulatus* presented the highest toxin concentration. Toxin accumulation in benthic populations seems to be a process related to species-specific properties, while DA is a potent food web transferred toxin, entering the benthic compartment.

Introduction

Harmful algal blooms cause severe economical losses to aquaculture and adversely impact human health. Although the direct effects of HAB toxins on humans are known, it is not as well known what pathways different HAB toxins take in the food web during and after a HAB event. The aim of the present study is to investigate how HAB toxins are accumulated in benthic organisms. Field surveys during *Dinophysis acuminata* blooms in Thermaikos Gulf and *Pseudo-nitzschia multiseries* blooms in Ria de Vigo were performed, in order to investigate how HAB toxins are accumulated among individual shellfish; how HAB toxins are accumulated in and between different trophic levels in benthic populations.

Materials and methods

The two sampling areas were Thermaikos Gulf in Greece and Ria de Vigo in Spain. Two surveys were carried out in Thermaikos Gulf (Fig.1) during *Dinophysis acuminata blooms* in May 2003 and March 2004. The macroinvertebrates sampled belonged to the filter-feeding trophic group: the cultured mussel *Mytilus galloprovincialis* and harvest benthic populations of *Modiolus barbatus*, *Flexopecten proteus*, *Chlamys varia*, *Venus verrucosa* and *Sabella spallanzanii*.

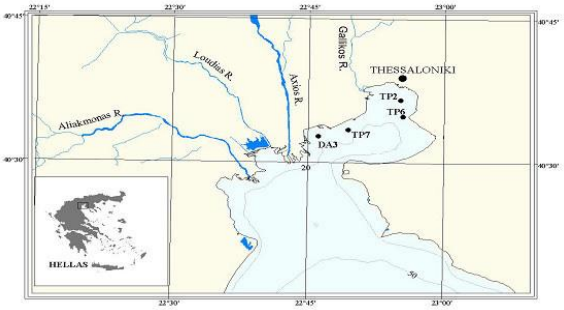

Fig. 1 Sampling stations in Thermaikos Gulf

In Ria de Vigo (Fig.2) the survey was performed in the mussel culture area during a *Pseudo-nitzschia multiseries* bloom in July 2003. During this survey cultured mussels (*M. galloprovincialis*) were collected and six benthic species belonging to three trophic groups: the filter feeders *Cucumaria* sp. and *Ascidiella* sp, the deposit feeders *Amphiura chiajei*, *Ophiura ophiura* and *Aporrhais pespelecani*, and the scavenger *Nassarius reticulatus*. Pooled samples of each species were prepared, weighed and stored at 20ºC for whole tissue toxin analyses. Okadaic acid and domoic acid analyses were performed with high performance liquid chromatography (HPLC) (Zhou et al. 1999**;** Furey et al. 2001**)**.

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Fig.2 Sampling area in Ria de Vigo

Results

Two successive DSP outbreaks with different intensity were reported in Thermaikos Gulf. The first was observed in May 2003 where *D. acuminata* cell densities ranged from 500 cells l^{-1} to 2200 cells l^{-1}. The second toxic bloom has been recorded in March 2004 and it was more intense, with *D. acuminata* cell densities varying from 600 cells l^{-1} to 10,700 cells l^{-1} (Reizopoulou et al. 2008). The severe toxic bloom of March 2004 in Thermaikos Gulf was associated with an increased OA accumulation in animal tissues and also a higher number of filter-feeding species found to be contaminated.

Table 1. OA concentration in macrofauna in Thermaikos Gulf during both blooming periods.

Species	OA (ng g^{-1}
Mytilus	75 - 2123
Modiolus barbatus	8 – 648
Flexopecten proteus	nd – 149
Chlamys varia	nd – 80
Venus verrucosa	nd – 38
Sabella spallanzanii	nd – 37

nd=not detected

Within the trophic group of filter feeders a marked interspecific variation in OA accumulation was observed (Reizopoulou et al. 2008). The highest concentration of OA was recorded in *M. galloprovincialis*. Among the harvest populations *M. barbatus* was the most contaminated while the rest of species accumulated much less OA, and only during the intense toxic bloom of 2004 (Table 1).
In the second investigation in Ria de Vigo the cell densities of *P. multiseries* reached over 650,000 cells l^{-1} during July 2003. Cultured mussels (*M. galloprovincialis)* were found to contain only traces of DA, ranging from undetectable values to 186 ng g^{-1} dw. In the opposite all the rest of the benthic species were found to
be contaminated with maxima in the gastropod *N. reticulatus* (Table2).

Table 2 DA concentration in macrofauna in Ria de Vigo.

Species	DA (ng g^{-1} dw)
Mytilus	nd -186
Cucumaria sp.	161-1.376
Ascidiella sp.	477 - 629
Amphiura chiajei	442 - 836
Ophiura ophiura	36 - 405
Aporrhais pespelecani	108 - 2173
Nassarius reticulatus	308 - 41052

nd=not detected

Among all trophic groups DA was higher in the scavenger (*N. reticulatus*) while filter and deposit feeders presented lower toxin amounts (Fig.3). Among the filter feeders DA was highest in the echinoderm *Cucumaria* sp., and among the deposit feeders DA was highest in the gastropod *A. pespelecani* (Fig.3).

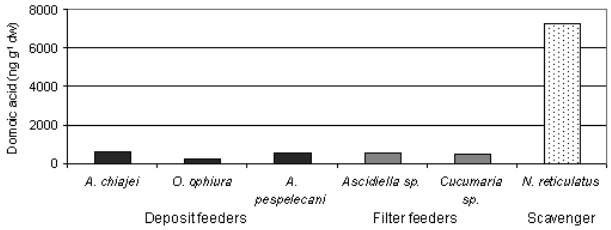

Fig. 3 Mean DA concentrations in benthic trophic groups.

Discussion

The investigation on *D. acuminata* blooms in Thermaikos Gulf showed that among filter feeders there was a marked interspecific variation in OA accumulation. The main accumulators of OA were the mussels (*M. galloprovincialis* and *M. barbatus)* exceeding the regulatory level on both sampling dates. The highest OA accumulation was found in *M. galloprovincialis* suggesting this species as the most appropriate indicator for OA contamination in Thermaikos Gulf. The higher potential of mussels to accumulate OA it is

also documented by other studies (Suzuki and Mitsuya 2001). The investigation on *P. multiseries* blooms in Ria de Vigo showed that benthic populations were all contaminated, while *M. galloprovincialis* showed minimal or undetectable toxicity values, further validating that measuring mussel toxicity only, is not an efficient indicator for DA contamination (Scholin et al. 2000)**.** DA was detected in all the trophic groups studied, with the higher DA concentration found to the secondary consumer *N. reticulatus*, a scavenger consuming decomposing prey on the bottom.Marine toxins can enter the benthic food web, zoobenthos can then act as a vector for transferring toxins to the higher trophic levels.The accumulation of the toxic algae on benthos obviously requires sedimentation of the toxic algae or particles containing the toxin to the bottom. Several studies indicate that sinking particles once they left the surface layer can rapidly reach the seabed. DA is a potent food web transferred toxin (Scholin et al. 2000; Vigilant and Silver 2007), entering the benthic compartment by the sedimenting algal cells. Flocculating diatoms can easily reach the sediments in coastal waters (Alldredge and Gotschalk 1989), delivering toxins to the benthic environment. The vertical fluxes of the DA are a substantial source of the toxin to the food webs, while DA may persist to the sediments long after the *Pseudo-nitzschia* blooms (Sekula-Wood et al. 2009).Variation of toxin accumulation in specimens could be attributed to the ecological preferences of each species and to the patchiness of the toxic bloom, influencing the degree of exposure of populations to different amounts of toxins within the various habitats. Moreover, differences in animal toxin accumulation may arise from the selective retention or elimination of individual toxins or from other transformation processes (Brijeli and Shumway 1998). Monitoring of shellfish toxin accumulation has important implications for managing bivalve populations for human consumption; however, further investigation on macroinvertebrates is crucial to determine toxin accumulation in various benthic species that could potentially act as vectors for toxin transfer in the food webs.

Acknowledgements

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Domoic acid accumulation in *Mytilus galloprovincialis*: laboratory experiments with *Pseudo-nitzschia multiseries*

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Abstract

Domoic acid (DA) accumulation was studied in *Mytilus galloprovincialis* collected from rafts in Ría de Vigo, Spain, during short-term laboratory experiments. Mussels were fed with *Pseudo-nitzschia multiseries* cultures (1900, 3800 and 6000cells/ml) and *P. multiseries* blooming in a phosphorus limited mesocosm. Toxin concentration in the mussel whole tissue was proportional to the toxin offered in food except for the lowest *Pseudo-nitzschia* cell concentration (1900 cells/ml). In that experiment, no toxin was detected in the mussel whole tissue while food quantity, measured as chlorophyll *a* concentrations, was below the minimum requirements for mussels to save energy for maintenance at limited ingestion. Domoic acid concentration in fecal material was higher than in the mussel whole tissue in all experiments. Large percentage of missing DA from the toxin offered to mussels by food was assumed to be due to toxin metabolism within mussel body, to symbiotic DA-utilizing bacteria and/or to the dissolved form of DA released in seawater as a result of food processing by the mussels.

1. Introduction

Domoic acid (DA) is a water-soluble neurotoxic amino acid, produced by the marine diatom *Pseudo-nitzschia*. Domoic acid intoxications of suspension-feeding bivalves occurred primarily via ingestion of toxic diatoms during feeding activity rather than from the dissolved fraction. Mussels, due to their ability to remove large amounts of particulate matter, can transfer algal toxins to higher trophic levels in aquatic food webs, acting as effective DA vectors (Vale & Sampayo, 2001). Mussels have flexible feeding behavior (filtration, ingestion and assimilation) which allows them to maintain a positive energy balance, even from nutritionally poor and/or concentrated suspensions (Kreeger et al, 1995).The aim of the present study was to examine DA accumulation in bivalves exposed to low levels of toxin under different quantity and quality of food. The exposure of *Mytilus galloprovincialis* to toxic *Pseudo-nitzschia multiseries* cells was investigated in short-term laboratory bioaccumulation experiments.

2. Materials and methods

Cultured mussels *M. galloprovincialis* were collected from Ría de Vigo mussel rafts (Galician NW coasts of Spain) during July 2003. Before the experiments, pooled mussel samples were examined for background toxin in their whole tissue. Individuals, from the same cultivated population with shell length of 5-6cm, were kept for acclimation to laboratory conditions in natural seawater without supplementary food. Three different food concentrations with *P. multiseries* from monocultures (1900, 3800 and 6000cells/ml) and water from *P. multiseries* in a phosphorus (P) limited mesocosm were tested to simulate real conditions in the field under potential phosphorus limitation (Table 1). Production of DA is known to be triggered by shortage of phosphorus (Pan et al, 1998). For each treatment, mussels were placed individually in 6 replicate tanks containing 2L of GF/F filtered seawater with gentle aeration, while two tanks without mussels were used as controls. Food quality has usually been measured in terms of particulate organic matter or chlorophyll content. Samples for Chl *a*, particulate organic carbon (POC), DA and phytoplankton cell density were

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taken from the cultures and mesocosm at the beginning of each experiment. Six hours (6h) after addition of food, fecal material was collected on pre-weighed Whatman GF/F filters and mussels dissected (whole tissue) for DA analysis. Samples were frozen, freeze-dried, weighed and stored at –20ºC until DA analysis with High Performance Liquid Chromatography (HPLC).

Table 1: Short-term DA accumulation with mussels *M. galloprovincialis* exposed to toxic *P. multiseries* from culture and mesocosm

exp I	1900cells/ml
exp II	3800cells/ml
exp III	6000cells/ml
exp IV	-P mesocosm water

3. Results and Discussion

A toxic *Pseudo-nitzschia* sp. bloom was in progress during July 2003 in Ría de Vigo; however, DA was not detected in mussels collected from the rafts before laboratory experiments. Toxin dilution factor within mussel whole body was assumed similar among experiments and specimens due to the same body size and dry weight of soft tissue. No mussel mortality was recorded during the experiments. When a battery of biomarkers at molecular and cellular level were investigated in the blue mussel *M. edulis* Dizer et al (2001) found that biological effects were not always significant especially at lower DA exposure. Mean values of DA in mussel whole tissue, in all experiments (Table 2), were lower than regulatory limit for public health (20 µg/g ww) (Mos, 2001). Toxin concentration in mussel whole tissue was proportional to toxin offered in food (r=0.79, p<0.05, Spearman rank correlation) except for lowest *Pseudo-nitzschia* concentration (1900 cells/ml). Differences in mussel DA among the remaining experiments (except exp I) were not statistically significant (Kruskal Wallis analysis, p<0.05, Tukey test) neither as toxin concentration (Table 3) nor as % DA offered in food per individual mussel (Table 2). Riisgard & Randløv (1981) in laboratory filtration experiments observed that phytoplankton concentrations of *Phaeodactylum tricornutum* > 3×10^4 or < 1500 cells cm^{-3} induce shell closure of *M. edulis* and thereby reduce filtration. However in the field phytoplankton represents only a fraction of particulate matter. Thompson & Bayne (1972) found that particle concentration < 300 to 200/ml was a threshold below which *M. edulis* did not filter. Phytoplankton concentrations measured by Dolmer (2000) as the minimum threshold to support mussel filtration activity in the field (approximately 1000 cells/ml) converted to 0.5mg Chl α m^{-3}, corresponding to 42 mg m^{-3} carbon (Taylor et al, 1997). Food quality, which is directly related to assimilation of organic matter, has an inverse effect on toxin assimilation in *M. galloprovincialis* exposed to PSP toxins (Moroño et al, 2001). Organisms might be forced by environmental conditions to exploit thoroughly available organic carbon sources. Under low mussel food availability gut passage time and intracellular digestion are increasing to optimize available organic matter exploitation (Allison et al, 1998). At low food concentrations (1000cells/ml or less) organic carbon assimilation efficiency was 89%, and linearly decreased as cell concentration increased (Thompson & Bayne, 1972). Although in exp I (1900 cells/ml) and exp III (6000 cells/ml), DA was equally offered (0.26 and 0.23µg DA/L respectively), DA in fecal material was higher for mussels exposed to higher cell concentration (6000cells/ml). Mussels exposed to food enriched in POC and Chl α, showed increased DA undigested and excreted in fecal material although the similar fecal production (Table 2). Domoic acid may be metabolized by bacteria (e.g. *Alteromonas* and *Pseudomonas*) present in tissue of blue mussels (*M. edulis*) (Stewart, et al, 1998). Hagstrom et al (2007) showed that most rapid degradation and/or release of DA from *P. multiseries* was observed in the presence of mussel fecal material.

Table 2: *P. multiseries* cells, POC, Chl *a* and DA in food, mussel whole tissue and fecal material

	culture			-P mesocosm
	Low	**median**	**high**	
P. multiseries cells/ml	1900	3800	6000	
POC in food (mg/L)	0.23	0.35	1.32	1.36
Chl *a* in food (µg/L)	0.14	0.59	14.34	6.95
fecal production (mg/ind dw)	13.9	16.0	15.1	16.8
DA in food (µg/L)	0.26	1.12	0.23	0.10
DA in mussels (µg/g dw)	0 (0%)	0.61 (34%)	0.15 (28%)	0.08 (25%)
DA in fecal material (µg/g dw)	2.3 (6%)	8.3 (7%)	11.0 (32%)	1.2 (5%)

Note: low concentration=exp I, median=exp II, high=exp III, -P mesocosm=exp IV; values in brackets are DA as % percentage of the toxin amount offered in food per individual

Recently Stewart (2008) demonstrated that it is possible the diatom cells consumed by the mussel *M. edulis* (essentially intact resting cells awaiting digestion) could continue to produce substantial amounts of DA in the dark (within mussel body).

Table 3: Statistical comparisons by Kruskal-Wallis analysis of DA concentrations in food, mussel whole tissue and mussel fecal material (95% Tukey HSD intervals) among experiments according to Table 1

DA in food	exp IV exp III exp I exp II ----------
DA in mussels	exp I exp IV exp III exp II ----------
DA in fecal material	exp IV exp I exp II exp III ----------

Note: experiments under the same line belong to the same homogenous group

Finally, size and shape of *Pseudo-nitzschia* frustules perhaps make them difficult to be transferred to the mouth or they are broken prior to ingestion and thus DA released into the water (Kvitek et al, 2008) which in its dissolved form was not measured in the present study. Missing DA from the toxin budget could be attributed to DA metabolism compensating low nutritional value of food (exp I) or related to the DA-utilizing bacteria due to degradation and/or generation of DA at higher *Pseudo-nitzschia* cell concentration (exp III). At conditions mimicking field exposure (exp IV) DA as % percentage of the toxin offered in food, retained by the mussels or eliminated in their fecal material, was lower than in the experiment with similar nutritional food value from *Pseudo-nitzschia* monoculture (exp III). However in all experiments part of the missing toxin could be due to the dissolved form of the DA released in seawater during the feeding procedure.

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GENOMICS

Evolution of the Dinoflagellates: from the origin of the group to their genes

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Abstract

Dinoflagellates have often been regarded as bizarre examples of evolution. They belong to one of the most strongly supported macrolineages among the protists: the superphylum/kingdom Alveolata containing three main phyla: the Dinoflagellata, Apicomplexa and Ciliata. These organisms share the features of cortical alveoli and micropores. Until the early 1990s, dinoflagellate classification of both living and fossil taxa relied almost exclusively on morphological characters, such as plate tabulation. Molecular data have supported many morphological groups, whereas others have been shown to be paraphyletic. Phylogenetic relationships within the dinoflagellates have changed as more taxa and more genes have been added. There is also much variation with the algorithms used to analyse the data. Dinoflagellates have become notorious for the variety of plastid types that they have acquired by secondary and tertiary symbiosis. They possess the most diverse array of plastids of any eukaryotic lineage and are truly the kings of symbioses. Genome rearrangements have taken place as the plastid has evolved. The genes that have been moved to the nucleus in the peridinin plastid dinoflagellates is different from all other eukaryotes, as well as the genes left in the plastid, being arranged in mini circles. As tertiary endosymbiosis has taken place, the plastid genome has become rearranged. At the species level, some, but not many, cryptic species have been uncovered. However, below the species level, microsatellites have shown immense spatial fragmentation in dinoflagellate populations. EST libraries have been constructed for several species. Initial annotation results indicated that a low percentage of the genes could be annotated. Recent advances have pushed this level to nearly 29% of the ESTs. Gene expression has been studied in relationship to stress conditions for several species. Some common responses to stress conditions have been noted. Only in EST libraries that have been screened by 454 sequencing (Illumina) have toxin genes been identified. The complete genome of *Heterocapsa circularisquama* will be done within the year.

Introduction

Most scientists who work on dinoflagellates have said themselves or heard it said that dinoflagellates break all the rules. Certainly, there are many examples, where the dinoflagellate's way of doing certain metabolic processes, from organisation of their chromatin down to acquisition of plastids has not followed the norm. This has lead to some very remarkable features being discovered in the dinoflagellates, of which I will try to review some of the more interesting points here.

Origin of the group. The dinoflagellates as a group are monophyletic with *Oxyrrhis* (pre-dinoflagellate) lying outside the core dinoflagellates and are sister to the perkinsid flagellates (Apicomplexa) with high bootstrap support to form a clade that is sister to the ciliates, again with high bootstrap support (Leander and Keeling 2004). This forms a group, termed the Alveolata, which is recognised at the superphylum/kindgom level (Adl et al. 2005) and defined morphologically by the series of cortical membranes beneath the plasmalemma and tubular cristae. A less robust sister relationship (<50%) is recovered in most trees with the stramenopiles or heterokont organisms (Leander and Keeling, 2004), which together with the cryptomonads and haptophytes form the chromalveolates. Recent re-analysis of 108 genes from the nuclear, plastid and mitochondrial genomes have failed to recover a well supported host cell lineage for the chromalveolates, which has resurrected the hypothesis of multiple secondary endosymbiosis for the chromalveolates (Baurain et al. 2010). Molecular clocks have used other eukaryotic fossil dates to date the divergence between the stramenopiles and the alveolates at 950 MA (Douzer al. 2004) and Medlin (2008) used the dinoflagellate fossil record to date the divergence of the dinoflagellates from the apicomplexans at 650MA. The presence of triaromatic dinosteroids in fossils of pre-Carboniferous age (Moldowan et al. (1996), which have been assumed to be dinoflagellates, were likely in the last common ancestor (LCA) of the apicomplexans and the dinoflagellates and likely in the LCA of this clade and the ciliates.

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Evolution within the dinoflagellates. Saunders et al. (1997) have reviewed the morphological steps that have evolved leading to a final radiation of the free-living autotrophic dinoflagellates (Fig 1). Peduncle feeding (myzotosis) evolved early in the dinoflagellates. Noctilucales are basal to the core dinoflagellates. Once the core dinoflagellates radiate, there are three different theories of evolution to explain the separation of thecate and non-thecate genera (Bujak and Williams 1971). These are the plate increase, the plate reduction and the plate fragmentation models, with the first and third models placing the Gymnodiniales in a derived position. The peridinioid taxa are in a different evolutionary position in each of the three models. It is interesting to assess new molecular trees in light of these evolutionary schemes based on morphology. However, there are few molecular trees that sample the entire range of the dinoflagellates, with most molecular trees concentrating on a group of species or closely related genera. Saldarriaga et al. (2004) have produced one of the earliest trees that sampled across most major core dinoflagellates. Using ciliates as an outgroup, the parasitic and atypical taxa diverge in exactly the sequence predicted by their morphological features until the divergence of the core dinoflagellates. After that clades of gymnodinioid dinoflagellates alter in divergence with clades of peridinioid dinoflagellates. Each clade consists of monophyletic well-supported genera, with the exception of *Gymnodinium*, which is paraphyletic. However relationships between the clades are not supported. There is a final divergence of the Gonyaulacales, but the Prorocentrales are paraphyletic; thus no real support for any of the morphological models. Adding more taxa to the tree has not really improved the situation but has added a few new surprises. Fig.2 shows a tree of 1246 dinoflagellates from a maximum likelihood analysis of the 18S rRNA gene. Divergences from the ciliates to the core dinoflagellates follow a similar pattern as seen in the other trees. The core dinoflagellates diverge simultaneously into four major clades. The first major clade contains a mixture of gymnodinioid and peridinioid taxa with *Amphidinium* often occuring as a basal divergence in a peridinioid clade. Dinophysiales are a basal divergence and the Prorocentrales are split into benthic and planktonic clades not too distantly related. The second major but smaller clade is a gymnodinioid clade. The third major clade contains a mixture of gymnodinioid and peridinioid taxa. However, Noctilucales are embedded in this clade, a position also recovered in the heat shock protein tree by Hoppenrath and Leander (2010). Gonyaulacales are a final divergence. The fourth major clade is primarily a naked clade with the Suessiales as a final divergence. However, there is one smaller clade in which there is a mixture of thecate and non-thecate taxa in the same clade. This is an unusual feature in this tree as most clades are either one or the other, the only exception being the basal position of *Amphidinium* spp. at the base of some of the peridinioid clades in the first major clade. Zhang et al. (2007a) used three genes with a reduced taxon sampling and recovered other significant relationships. With a three gene concatenated data set, *Amphidinium* was at the base of the lineage. The major well-supported divergence in this tree was between endosymbiotic taxa and free-living taxa with the Gonyaulacales being monophyletic with one exception. Prorocentrales were now a monophyletic group, which was also recovered in the heat shock protein tree of Hoppenrath and Leander (2010).

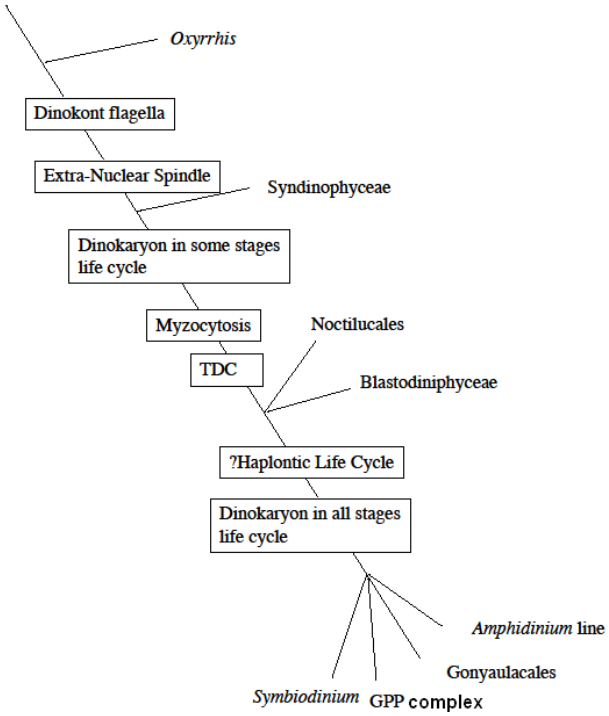

Fig 1. Scheme of the morphological steps in the evolution of the dinoflagellates

Evolution at the species level (cryptic diversity). Molecular analyses have wreaked havoc at the species level in nearly all groups and the dinoflagellates are no exception. As expected in the non-thecate taxa, relationships are difficult to ascertain morphologically and

many cryptic species have been discovered molecularly. The best example can be found in the Suessiales where 8 clades of symbiotic dinoflagellates have been recovered (Pouchon et al. 2006) where originally only one species had been described from corals. The *Alexandrium tamarense/fundyense/catenella* species complex has shown that these taxa are related by their geographic origin and not by their morphological features (Scholin et al. 1994, John et al. 2003). Using a molecular clock John et al. (2003) proposed a scenario for the historical biogeographic history of this complex from a single global ancestor. The later divergences of the non-toxic Western European clade from the toxic Temperate Asian could be dated to the closing of the Tethys Sea, which likely explains presence of relict cells of this clade in the Mediterranean. The separation of the non-toxic Mediterranean Clade from the toxic North American clade was dated to the rising of the Isthmus of Panama. With time, the Mediterranean clade went extinct in the western tropical Atlantic waters and it thrives today in the Mediterranean where it is now endemic. Cryptic diversity at the species level is usually recovered first with sequence data from ribosomal genes and then reconfirmed with data from other genes. Piganeau et al. (2011) have analyzed the rate of evolution in protein coding genes v. 18S ribosomal genes using whole genomes. They found that the rate of evolution between them is different among unicellular organisms and multicellular organisms. In unicells, the rate of evolution in the 18S rRNA gene is ca. 5x faster than the rate of evolution in the protein genes as compared to multicellular organisms. They attribute this to differences in their effective population sizes with the smaller population sizes having a faster rate. If this is the case, then in the dinoflagellates, among the Suessiales are two different endosymbiotic lineages with two different rates of evolution (Shaked and de Vargas 2006) occur in two lineages with different effective population sizes. The endosymbionts within planktonic forams have an effective larger population size and therefore evolve much slower than the endosymbionts within the corals with a very much smaller effective population size with some lineages being endemic to a single coral head. Piganeau et al. (2011) feel that a single base difference in the 18S rRNA gene denotes a different species.

Plastid Evolution. Photosynthetic organisms have arisen through a series of endosymbiotic events. The primary endosymbiosis arose from a heterotrophic ancestor which engulfed and transformed a cyanobacterium into a plastid. This resulted in the red, green and glaucophyte algae (see reviews in Archibald and Keeling 2002, Sanchez-Puerta and Delwich 2008). These host and plastid lineages are both monophyletic supporting that this event happened only once. A secondary event took place between another heterotrophic organism and either a red or green alga from the first endosymbiosis. Counting the number of membranes around a plastid can determine whether the alga was derived from a primary endosymbiosis (2 membranes) or a secondary endosymbiosis (3-4 membranes). Recent evidence from whole genome and EST analyses has uncovered an unusual sequence of events leading to the chromalveolates lineages (Moustafa et al. 2008). Traces of green genes can be found in this lineage, which, today, is a red algal plastid lineage. Current opinion is that there was originally a green gene in the lineage, which was replaced by a red one. Falkowski et al. (2004 have hypothesized that the red algal plastid only had an adaptive advantage after the Permium Triassic extinction when the ocean became anoxic and trace metal chemistry changed and Fe is abundant and needed for cytochrome 6 in the plastid but Zn and Cu are depleted, which is needed for plastocyanin in the green plastid. Medlin (2011) constructed a molecular clock to determine if the radiations in the different chromalveolates lineages corresponded to 250 MA, the timing of the P/T extinction to provide supporting evidence that the green plastid was replaced by the red one and found that the P/T boundary corresponded to different taxonomic levels of radiation in the various lineages. If it is correct that there were multiple secondary endosymbioses (Baurain et al. 2010), then each lineage could have acquired a red algal plastid at a different time and dumped the green one atdifferent time points in their radiation. In the dinoflagellates, there are several different types of plastids: the

predominate peridinin plastid, which is believed to have been derived from the red algal secondary endosymbiosis and cryptophyte, prasinophyte and haptophyte plastids derived from a so-called tertiary endosymbiosis when the peridinin plastid was replaced by another from either of these three lineages (see review in Saldarriaga et al. 2001). The perplexing observation about the peridinin plastid is that none of the plastid gene trees show the peridinin lineage as an independent lineage as are the stramenopiles, cryptophytes and haptophytes (Yoon et al. 2005, Verbruggen 2011, Moustafa, pers.comm.); instead the peridinin lineage is embedded in the stramenopiles lineage either sister to the diatoms (Yoon et al. 2005) or with better taxon sampling sister to the chrysophytes /synurophyte lineage, which is sister to the diatoms (Vergegroen 2011) or its closest sister in the green lineage. This implies that the dinoflagellates did not have a red plastid that was transformed into a peridinin plastid and that all of the plastids in the dinoflagellates are tertiary plastids and it was the green plastid that was eliminated at time of the four tertiary endosymbiosis. This hypothesis fits using a molecular clock to place the timing of the P/T extension over the dinoflagellate tree, which corresponds to generic level radiation in the dinoflagellates and to phylum level radiation in the stramenopiles (Medlin 2011). Generic level radiation of the extant dinoflagellates also occurred after this time according to their fossil record (Fensome et al. 1997). After the P/T extinction, the dinoflagellates likely underwent several tertiary endosymbioses of several different algal groups to evolve from a heterotrophic lineage to an autotrophic or mixotrophic lineage. One puzzling point is why they would have re-engulfed a prasinophyte algae if the green algal plastid was a disadvantage at this time. It is more parsimonious to predict that the dinoflagellate/ prasinophyte lineage is a relict of the green plastid that was originally present in all the alveolate lineage because there is evidence that the LCA of the dinoflagellates, apicomplexans and ciliates likely had a green plastid, because there are traces of green genes in all of these host lineages (Takishita et al. 2003, Patron et al. 2006, Hackett et al. 2004, Moustafa et al. 2008). The presence of only 3 membranes around the dinoflagellate plastid is likely the results of peduncle feeding where the host cell membrane was left behind after the dinoflagellate finished sucking out its contents. Still even more interesting is the transformation of the heterokont plastid into the peridinin plastid after it was engulfed. There is a massive transfer of genes from the plastid into the nucleus, leaving behind about 12 genes coding only for plastid function, which form minicircles of genes of different sizes in different species of the same genus but sharing nearly identical spacer regions between the genes within a genus (Zhang et al. 2002). This did not happen in the haptophyte and cryptophyte plastid bearing dinoflagellates, adding further evidence that the tertiary endosymbioses are independent events and that the red algal plastid in each of these lineages resulted from different, independent secondary endosymbioses. The genes encoded by the plastid possess another different feature. When their rRNAs are converted to cDNA, the normal poly A tail is replaced by a poly T tail (Wang and Morris 2006), so plastid-encoding ESTs from dinoflagellates can be differentially separated from other plastids.

Mitochondrial Evolution. Within the alveolate lineage, the ciliates have a normal sized circular genome, both the apicomplexans and the dinoflagellates have a reduced genome with only three genes and after transcription the mRNAs can be modified to change the coding region into another protein (Lukes et al. 2009).

Evolution below the Species Level (Genetic Diversity). Diversity below the species level is most robustly measured by fingerprinting methods of which microsatellites are the most computationally intensive. MS are short repeated sequences of 1-6 nucleotides in length, e.g., (GT)n. MS are codominantly inherited markers that provide very high levels of heterozygosity and the ability to measure gene flow from one population to another. Microsatellites have been established in a number of dinoflagellates and each study has shown distinct population structure and reduced gene flow between close areas (Table 1). Population structure in two clades of

Symbiodinium is different depending on the inheritance type of the endosymbiont (Thornhill et al. 2009). In clade B, which has horizontal inheritance (coral larvae take endosymbionts from the plankton), has host, within host and even reef endemism and strong population structure and are temporally stable. In contrast, Clade C with vertical inheritance (endosymbionts in the coral larvae) has no population structure. The endemism in Clade B could also be the result of the host coral expelling other genotypes to maintain a specific population and its density.

Table 1. Summary of microsatellite studies on dinoflagellates

Species	Source
Alexandrium tamarense/ fundyense/catenella NA	Nagai et al. 2004, Alpermann 2009
Alexandrium tamarense/ fundyense/catenella TA	Nagai et al. 2004, Nishitani et al. 2007a
Alexandrium minutum	Nagai et al. 2006
Cochlodinium polykrikoides	Nishitani et al. 2007b
Heterocapsa circularisquama	Nagai et al. 2007
Symbiodinium sp. Clades B & C	Bay et al. 2009, Santos & Coffroth 2003
Karenia brevis	Renshaw et al. 2006
Lingulodinium polyedrum	Frommlet & Iglesias-Rodriguez 2008

Microsatellites have clarified the origin of the Temperate Asian of the *Alexandrium tamarense/fundyense/caten-ella* species complex. Lilly et al. (2002), comparing Mediterranean and Asian strains with LSU rRNA sequence data, concluded that the presence of TA strains in the Mediterranean was a clear case of ballast water introduction. Penna et al. (2005), using ITS sequence data concluded that it was likely a case of ballast water introduction. Masseret et al. (2009), using micro-satellites, showed the Mediterranean populations to be clearly distinct and distant from the Asian strains but could not offer any reasonable explanation for their occurrence in the Mediterranean. However, referring back to the historical biogeography of the species complex by John et al. (2003), the closing of the Tethys Sea, which is the vicariant event separating the West European Strains from the Temperate Asian strains, the presence of the TA genotypes in the Mediterranean is likely a relict population from when the two groups were once joined and their presence in the French Lagoons is likely caused by a change in environmental conditions that have caused them to bloom and be noticed. The first hierarchical study of genetic diversity has been conducted on populations of *Alexandrium tamarense* (NA clade) in the Orkney Islands, UK (Alpermann 2009). On a global scale, the Orkney Island populations are more closely related to Pacific Isolates from Japan than to populations on the east coast of North America, which suggests that these populations were introduced to the region by cells directly coming across the Arctic Ocean in contrast to the hypothesis put forward by Medlin et al. (1998) that these populations entered from the Pacific and moved along coastal pathways of the eastern side of North America until they reached the Gulf Stream, which carried them across to the Orkney Islands. Moving to the diversity of local populations, at one site in the Orkney Islands, four populations were discovered that could interbreed and these populations were determined to be different year classes that had hatched from local cyst beds.

Evolution of their genes and their transcription. The dinoflagellates have long been known to possess an unusual genome structure (Hackett & Bhattacharya 2006). The chromosomes are permanently condensed. The DNA content of the nucleus contains 2-200 pg/cell as compared to 0.5 pg/cell in other algae with many copies of each gene with long stretches of non-coding regions, which may not be so different in length to other organisms (Triplett et al. 1993).

All of the copies of each gene are transcribed on a single mRNA. What is not known is if the long stretches of non-coding regions are in between each multiple copy of a gene or between clusters of multiple copies of a gene. If the former, then crossing over during meiosis will likely be more difficult as will the transcription of multiple genes into a single mRNA. In contrast, the plastid and the mitochondrial genomes are dramatically reduced. These questions, among others, will likely be answered when the first genome of a dinoflagellate is completed. Its immense size has daunted many labs to avoid the dinoflagellates and even isolating a single chromosome for sequencing has resulted in so much non-coding data that contig overlaps were impossible (John pers. comm.). *Heterocapsa circularisquama* has been targeted for genome sequencing by the Bhattacharya lab at Rutgers University USA primarily because its virus has had

Table 2. Summary of EST libraries made to date and the most interesting genes recovered.

Author	Species	EST % identified	Most interesting gene identified
John et al. 2005	*Alex tamarense (NA)*	9% of 2500 ESTs	Polyketides genes
Lidie et al. 2005	*Karenia brevis*	29% of 7001 ESTs	Microarray generated SNPs in multiple gene copies
Hackett et al. 2005	*Alex tamarense (NA)*	20% of 6723 ESTs	Histone genes
Yang et al. 2010	*Alex minutum*	28% of 3000 ESTs	4 stress libraries, toxic and non-toxic strains express different genes
Toulza et al. 2010	*Alex catenella (TA)*	24% of 21236 ESTs	Alveolin protein genes
Lin et al. 2010	3 SL environmental clone libra ries	20-27% of 1000 clones	Nucleosome histone core, modification and assembly genes, Rhodopsin

its genome completely sequenced (Nagasaki et al. 2005).

A comparison of expressed sequence tags (ESTs) from dinoflagellate has shown that they possess a splice leader sequence that is unique to all mRNAs of the dinoflagellates (Zhang et al. 2007, Lidie and Van Dolah 2007). Because of this, it is possible to retrieve all mRNAs from dinoflagellates separated from all other mRNAs in an environmental sample. Environmental clone libraries have now been made using exactly this technique and have discovered the expression of genes not known to exist in dinoflagellates, e.g., nucleosome core histones and rhodopsin, the latter gene implicated in non-photosynthetic solar energy capture (Lin et al. 2010). As sequencing capacities have increased and more whole genomes have been edited, the annotation of ESTs has improved (Table 2). Recently 454 sequencing of EST libraries has recovered the entire operon for the saxitoxin genes (Hackett et al. 2010). Of the eight genes involved, three are of cyanobacterial origin, one is of eukaryotic origin and four are of unknown origin, so there has not been a lateral gene transfer from the Cyanobacteria into the dinoflagellates. Interestingly, these same genes have not been recovered in lower sequencing capacity EST clone libraries, suggesting some unique control over the production of toxins. In *Karenia*, type 1 polyketide genes for brevetoxins have been recovered from EST libraries but with a type 2 polyketide protein complex (Snyder et al. 2003, Monroe and van Dolah 2008).

In summary the dinoflagellates have evolved many unique features but a comparison of other eukaryotic cells also reveals similar features in the Euglenozoa (Lukes et al. 2009). Both groups share flagella with paraflagellar rod, large nucleolus and permanently condensed chromosomes, cell walls composed of "proteinaceous/cellulosic" strips or plates, mucocysts or trichocysts ejected through pores, thylakoids with 3 lamellae, CER composed of 3 membranes. Is this a strange case of convergent evolution? Dinoflagellates only have 3 genes in the mitochondrion; euglenids have mini circles with three genes (?just the next evolutionary step). Both can edit the mitochondrial mRNA after transcription to change the protein desired. Both have unique splice leaders to the mRNA, conserved at the group level in dinoflagellates and at the species level in euglenids. Both have mRNAs transcribed with multiple genes: in the euglenophytes these are different genes but in the dinoflagellates they are tandem repeats of the same gene. More likely each of these strange features conveys some evolutionary advantage and are not just quirks of nature because we all know that God does not shoot craps to paraphrase an observation made by Albert Einstein on the organisation of the universe.

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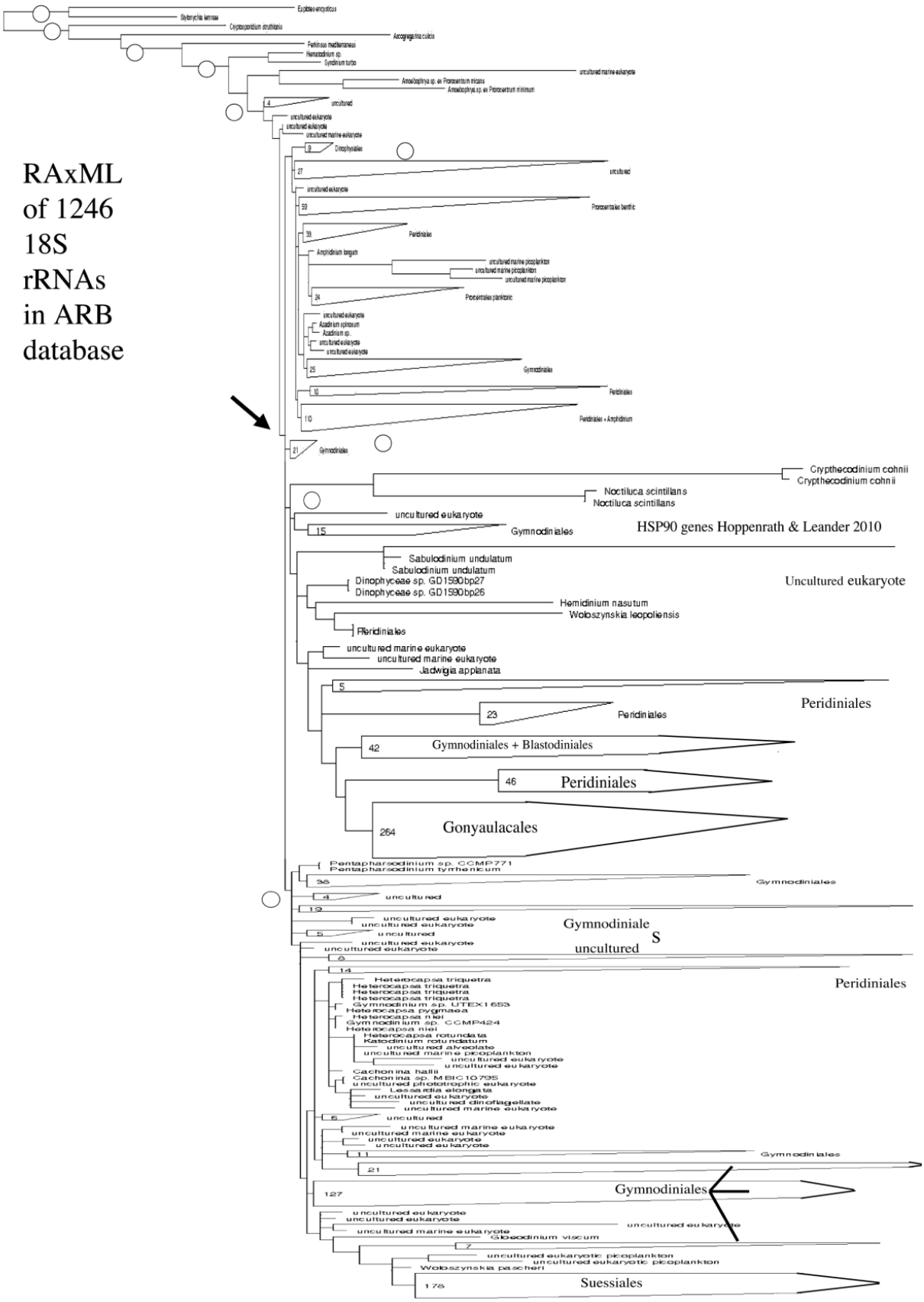
Fig 2. Maximum likelihood analysis of 1296 SSU rRNA seauences currently held in the ARB database and alignment by secondary structure. A = clade 1 and 2, B = clade 3, C = clade 4.



Intragenomic rDNA polymorphism (IRP) can lead to overestimation of species/population diversity in *Alexandrium tamarense/fundyense/catenella* complex

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Abstract

We analyzed 18S rRNA gene (rDNA) in monoclonal strains of *Alexandrium fundyense* and *A. catenella* by both direct and clone-based sequencing, and found 42-50 polymorphic sites in all *A. fundyense* strains and some *A. catenella* strains. The intragenomic 18S rDNA variants within each strain differed by 0-2.5%, some of which exceeded commonly used species boundary (2%). In the phylogenetic trees the polymorphic sequences dispersed across strains forming "polyphyletic" clusters, indicating the possibility of incorrectly grouping the intragenomic 18S rDNA variants as distinct geographic populations or species when analysis is based on a single 18S rDNA clone sequence. Further phylogenetic analyses including all reported and new *tamarense*/*fundyense*/*catenella* data indicated that sequences from this complex formed two major clades. Clade I comprised *A. tamarense*/*fundyense* Northeast America as well as the *A. catenella* Pacific America, all with intragenomic polymorphism (IRP), mixed with reported *A. tamarense*/*fundyense* from Asia. Clade II contained a subclade of *A. catenella* with IRP and *A. tamarense* from various geographic locations without IRP. No relationship was found between IRP and current morphospecies designations, geographic locations, or toxicity. Our result suggests that IRP should be examined when studying species/population diversity in this complex and perhaps other dinoflagellates as well.

Introduction

It is essential to accurately identify the HAB species/strains in order to monitor the dynamics of the causative population and understand the environmental factors regulating the blooms. Unfortunately, *Alexandrium tamarense*, *A. fundyense*, and *A. catenella*, which produce saxitoxin, morphologically differ only by the presence or absence of a ventral pore on the apical plate, making it difficult to discriminate them microscopically. Hence, the three species are usually referred to as *A. tamarense* species complex. Molecular techniques are powerful in resolving closely related species/strains. Ribosomal RNA genes (rDNAs) have been commonly used for species identification and phylogeny. rDNA has been used to determine the phylogenetic relationship among the morphotypes of the *A. tamarense* species complex, particularly the small subunit rDNA (18S rDNA) (e.g. Scholin and Anderson 1993; John et al. 2003). However, phylogenetic trees often are incongruent to morphospecies designations, and grouping based on geographic origin or ability to produce toxins is often inconsistent (Lilly et al. 2007 and ref therein). A potential problem to use rDNAs in dinoflagellate phylogenetic study is the existence of polymorphic copies within the genome (intragenomic rDNA polymorphism or IRP), which although documented sporadically (Scholin and Anderson 1993; Kim *et al*. 2004; Ki and Han 2005) has not been systematically investigated. Whether IRP affects rDNA-based assessment of species or genetic diversity has not been examined. We analyzed IRP in 22 strains of *A. tamarense* species complex: 6 for

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A. fundyense (Afund), 5 for *A. tamarense* (Atama), and 11 for *A. catenella* (Acate). Each of these strains was isolated from a single cell and hence was a monoclonal culture. Cultures were grown in 28 ppt seawater with f/2 medium (Guillard and Ryther 1962) and was kept at 15°C on a 14:10 L:D cycle. Cells were harvested for extraction of DNA and RNA and preparation of cDNA (Lin et al. 2002). 18S rDNA and cDNA were amplified via PCR using 18ScomF1 and 18ScomR1 primer (Zhang and Lin 2005). Amplicons were purified for sequencing directly or after cloning. The result revealed 42 polymorphic (IRP) sites (Fig. 1) in all but one *A. fundyense* strain (CCMP1980). Five of the *A. catenella* strains showed 50 IRP sites, while the other six showed none. IRP sites have been sporadically reported (Scholin and Anderson 1993, Kim et al. 2004), but we found many more per strain. The majority of polymorphic sites was A/C, A/G, A/T, T/C and G/T. cDNA analysis shows that only one of the 18S rDNA copies is expressed. For all the five *A. tamarase* strains we examined, no IRP was observed.

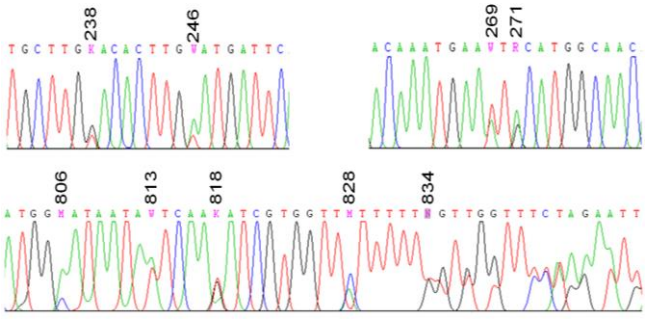

Fig. 1. Some more examples of polymorphic sites in 18S rDNA of *Alexandrium fundyense*. K=T or G; W=A or T; R=A or G; M=A or C; N=T insertion or deletion. Number on top indicates nucleotide positions.

Phylogenetic analyses showed two major clades (Fig. 2). Clade I consisted of strains with IRP, including Gulf of Maine *A. fundyense* strains, some *A. tamarense* strains previously reported from Asia, and the five strains of *A. catenella* from Pacific America. Clade II contained strains from all three morphospecies mostly without IRP except some Asian strains of *A. catenella*. The dispersed distribution of polymorphic 18S rDNA within Clade I and the *A. catenella* subclade of Clade II indicated that strains that would usually be identified as distinct species (2% difference in 18S rDNA sequence) or geographic populations (<2% difference in 18S rDNA) belonged to a same strain.

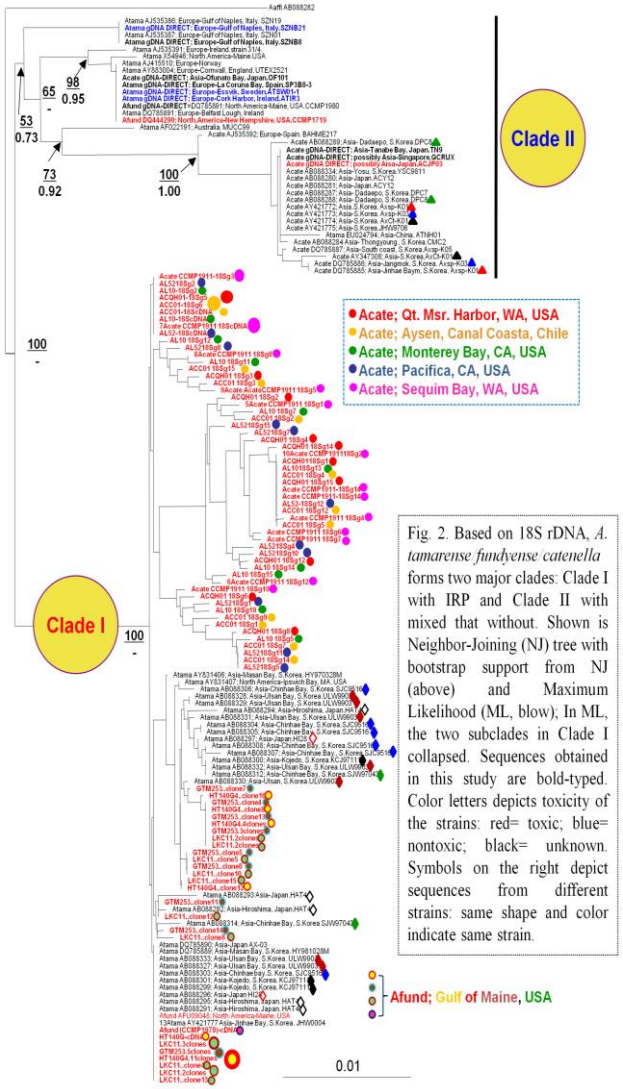

Fig. 2. Based on 18S rDNA, *A. tamarense fundyense catenella* forms two major clades: Clade I with IRP and Clade II with mixed that without. Shown is Neighbor-Joining (NJ) tree with bootstrap support from NJ (above) and Maximum Likelihood (ML, blow); In ML, the two subclades in Clade I collapsed. Sequences obtained in this study are bold-typed. Color letters depicts toxicity of the strains: red= toxic; blue= nontoxic; black= unknown. Symbols on the right depict sequences from different strains: same shape and color indicate same strain.

This suggests that IRP may lead to overestimation of species or population diversity. Overall, no relationship could be found between IRP and morphospecies designation and geographic locality (Fig. 2). Strains with IRP tend to group separately from those without, but in *A. catenella* (in Clade II) the two types clustered together. From our dataset, more IRP strains are toxic than non-IRP strains, but this may be due to sampling bias. This requires further study.

Acknowledgements

We thank Don Anderson for providing many of the *Alexandrium* strains used in this study. The study was supported by the U.S. National Science Foundation (grant EF-0629624) and the CAS International Partnership Project for the Innovational Group on Tropical Marine Ecological Process Studies (grant KZCX2-YW-T001),the NSFC-Overseas Collaboration Fund (grant 40828006).

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The genus *Gambierdiscus* in Ryukyu Islands, Japan

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Abstract

The genetic diversity and morphology of the ciguatera fish poisoning (CFP) related dinoflagellate, *Gambierdiscus* spp., isolated from the Ryukyu Islands were investigated. We established 95 strains from 20 localities. *Gambierdiscus* species were ubiquitous, but the numbers of cells per sample were smaller than reports from the central Pacific. Morphologically, various types were recognized, however, several strains deformed under culture conditions. 11 strains were successfully obtained and 18S rRNA sequences and phylogenetic analyses revealed that the strains could be divided genetically into four different types. One type was closely related to *G. australis* but the other three types were not closely related to known species. In addition to the 11 strains, sequences from a total of 26 strains of 28S rRNA D1D3 regions were obtained. Phylogenetic analyses revealed that the same four types were recognized. One type was genetically and morphologically identified as *G. australes* . The other three types were not closely related to known species. They were distantly related with *G. toxicus* and *G. pacificus,* with *G. caribaeus* and *G. carpenteri,* and with *G. yasumotoi* and *G. ruetzleri,* respectively. These three different types are potentially undescribed species. These results suggest that *Gambierdiscus* species are genetically more diverse than expected.

Introduction

Ciguatera Fish Poisoning (CFP) is the most frequently occurring non-bacterial seafood poisoning syndrome in the world, especially in tropical coral reef areas (Glaziou and Legrand, 1994; Lewis, 2001). 50 to 500 thousand people yearly fall ill (Bomber and Aikman, 1989; CDC, 2007). Benthic dinoflagellates, *Gambierdiscus* spp., are thought to be the origin of CFP (Yasumoto et al., 1977). In 1979, the real producer of CFP was identified as *Gambierdiscus toxicus* by Adachi et Fukuyo (Adachi and Fukuyo, 1979). Since then, 10 species have been described morphologically and genetically (Litaker et al., 2009). In Japan, the Ryukyu Islands have sporadic but regularly occurring CFP outbreaks (Oshiro et al., 2009). In the Ryukyu Islands, however, accurate classification and toxin production studies have not been conducted. The aim of this study is to conduct accurate taxonomic and diversity investigations of *Gambierdiscus* spp. in the Ryukyu islands based on molecular phylogeny and morphological observations.

Materials and Methods

Isolation sources were macroalgae, seagrass, coral rubble, bottom sand, etc. Sampling localities were Okinawajima Island and surrounding islands (Iheya, Tokashiki, Aka Islands), and the Southern Ryukyus (Miyako, Ishigaki, Iriomote Islands). Cells of *Gambierdiscus* were isolated by micro-pipettes to make unialgal clonal cultures. IMK/4 (four times diluted IMK medium, Nippon Pharmaceuticals, Co, Tokyo, Japan) and IMK/4+Soil ex. (5 ml of soil extract was added to IMK/4 medium) were used. Culture conditions were 24 ± 2° C, 14:10 light:dark cycle，and light intensity was approximately 40 µmol photon m^{-2} s^{-1}.

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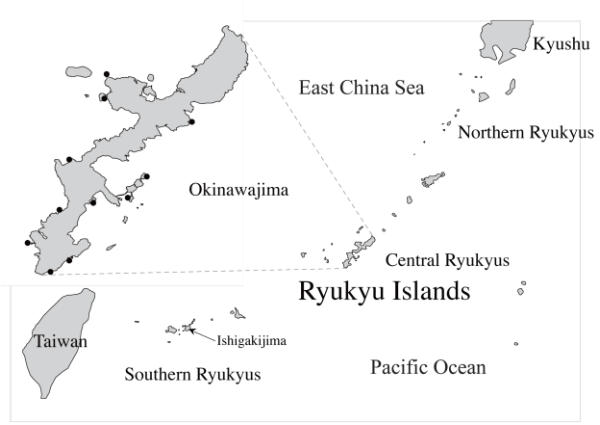

Fig. 1. Sampling sites of Okinawajima (black dots) and Ishigakijima Islands in the Ryukyu Islands, Japan.

Observations were made by Nikon Eclipse 80i light microscope and JEOL-JEM 6060 scanning electron microscope. DNA extraction and purification were done by Quiagen DNeasy Plant MiniKit (Quiagen, MA, USA.) and Gene Clean Kit (BIO 101, OH, USA), respectively. SSU rDNA and LSU rDNA D1-D3 regions were amplified by PCR methods. Primers were according to Takano and Horiguchi (2006). PCR products were further cloned by TOPO TA cloning Kit (Invitorogen Co., CA, USA) and at least three clones were picked up from each strain. Sequencing was performed by Macrogen Japan, Co. Sequences databases were aligned by Clustal X and phylogenetic analyses (MP and NJ methods) were done with PAUP*4.0 b10 (Swofford 2002).

RESULTS AND DISCUSSION

Gambierdiscus cells were found at almost all localities but the numbers of cells were rather small. Usually, each sample had less than 50 cells. Cells were found on geniculate coralline red algae, such as *Actinotrichia fragilis* and *Tricleocarpa cylindrica*, and on brown alga, *Turbinaria ornata*, but in many samples *Gambierdiscus* cells were not found. Other isolation sources were seagrass, floating scum, and sand samples.

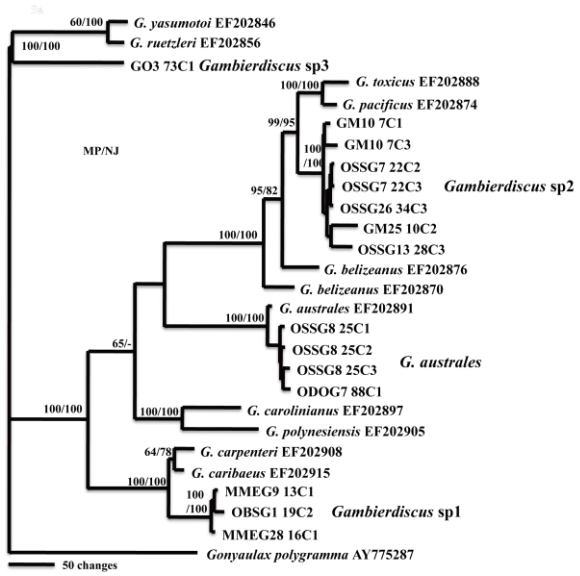

Fig. 2. Phylogenetic tree of *Gambierdiscus* spp. inferred from 18S rRNA sequences (1756 bp) of MP tree. Bootstrap values (1000 replicates) are indicated in nodes (MP:NJ). MP tree was generated by heuristic search with TBR of branch-swapping option and random stepwise-addition option. NJ tree was generated by Kimura's 2-parameter method.

For morphotaxonomy, each strain underwent detailed observations. Cell dimensions (length, width, thickness) and detailed morphology and size of thecal plates were recorded to compare with known species. Under culture conditions, however, several strains became deformed and we could not perform detailed examinations of thecal plate morphologies. Hence molecular phylogenetic characterizations were utilized instead. In addition to the known species, 11 strains were analyzed by SSU rDNA sequences. Litaker et al. (2008) pointed out that *Gambierdiscus* has multiple copies of rRNA genes from each isolate. This is crucial for establishing amounts of species-level divergence and identifying aberrant pseudogene sequences (Litaker, et al. 2009). Even if such risks are present, certain sequences could be obtained to identify species (Litaker et al. 2009). We followed their methodology in identifying the 11 strains in this study.

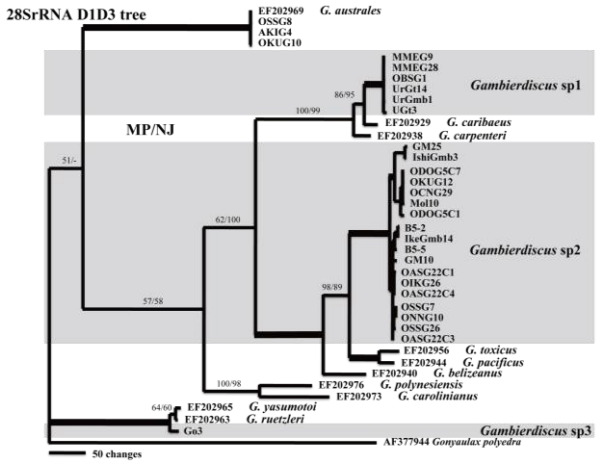

Fig. 3. Phylogenetic tree of *Gambierdiscus* spp. inferred from 28S rRNA D1D3 region sequences (1088 bp) of MP tree. Bootstrap values (1000 replicates) are indicated in nodes (MP:NJ). MP tree was generated by heuristic search with TBR of branch-swapping option and random stepwise-addition option. NJ tree was generated by Kimura's 2-parameter method.

Phylogenetic analyses of SSU rDNA showed that 4 different types of *Gambierdiscus* species were present from the Ryukyu Islands (Fig. 1). Except for *G. australes*, 3 types were not closely related to known species. Subseqeuntly, as an increase in numbers of strains were examined could result in more undescribed species being identified, strain numbers were increased to 26 and we analyzed D1D3 regions of LSU rDNA. Phylogenetic analyses of LSU rDNA revealed that the same 4 types were present (Fig. 2). Strains genetically identified as *G. australes* agreed with the morphological characters of *G. australes* (Chinain et al., 1999; Litaker et al., 2009). Hence, these strains were identified as *G. australes.* The other three types were distantly related with *G. toxicus* and *G. pacificus* (sp2 in Figs. 2 and 3), with *G. caribaeus* and *G. carpenteri* (sp3 in Figs. 2 and 3), and with *G. yasumotoi* and *G. ruetzleri* (sp2, sp3 and sp4 in Figs. 2 and 3), respectively. These three different types are potentially undescribed species. These results suggest that *Gambierdiscus* species are genetically more diverse than expected. Strains of these three potentially undescribed species should be characterized by detailed morphology.

Acknowledgements

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NOVEL SENSORS

Application of fluorescence *in situ* hybridization (FISH) method to detect *Heterosigma akashiwo*

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Abstract

Species-specific probes against cytoplasmic rRNA and nuclear rDNA of *Heterosigma akashiwo* were designed on the basis of LSU and ITS sequences. FISH protocols for whole cell and nuclear were established to detect this alga. The results showed that the rRNA-targeted probe hybridized with cytoplasmic rRNA, showing strong green fluorescence throughout the whole cell, while cells labeled by rDNA-targeted probe exhibited exclusively a fluorescent nucleus. The probes were specific, without cross-reacting to other test algae. The method was also applied successfully to distinguish *H. akashiwo* from mixed field samples. Consequently, the FISH method was proved promising for specific, rapid, precise and semi-quantitative detection for cultured algae as well as field samples.

Introduction

The golden-brown marine alga *Heterosigma akashiwo* (Hada) Hada (Chromophyta: Raphidophyceae) is well known as a eurythermal and euryhaline flagellate in temperate seas. This alga has been found to be responsible for mass mortality of cultured fish in Japan, China, Korea, Norway, Canada, Chile, New Zealand and USA, resulting in great loss to aquaculture (Yamochi 1987; Chang *et al*. 1990; Hard *et al*. 2000). It is crucial for the management of cultured fish in order to avoid stock loss to rapidly identify and quantify *H. akashiwo*, as well as other harmful algal species (Hallegraeff and Hara 1995). Detection of *H. akashiwo* prior to the onset of a bloom event is viewed as important for improving early warning capabilities. However, correct identification and enumeration of *H. akashiwo* is rather difficult and time-consuming. The cells are small, fragile and cell morphology often changes with different water conditions (Guo 2004). On the other hand, traditional monitoring methods for *H. akashiwo* rely on light microscopical examination of live or Lugol's iodine-stained cells. A long time is spent on shipping and counting samples, which greatly delays the warning of potential blooms. To solve these problems, much attention has focused on developing a simple, rapid, reliable, and effective identification and quantification method for this species. In the last decade, fluorescence *in situ* hybridization (FISH), as a promising cell detection technique, has been widely applied. Tyrrell *et al*. (2001) firstly explored the utility of rRNA-targeted oligonucleotide probes to detect *H. akashiwo* cells using FISH. Unfortunately, the authors argued that the application of FISH is problematic, and in their study, this detection method was overlooked and served as an intermediate step for the sandwich hybridization assay. Chen *et al*. (2008) established protocols of FISH for cultured *H. akashiwo*. However, applicability of this method to field samples has not been examined. Therefore, in this study, two specific probes (HA-lsu and HA-its) were designed based on the large-subunit (LSU) ribosomal RNA gene (28S rDNA regions D1-D2) and the internal transcribed spacers (ITS) containing 5.8S rDNA, respectively. These two probes were tested for cross-reactivity to other typical harmful microalgae and a FISH protocol was established for species-specific, quantitative, and rapid detection of *H. akashiwo*.

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Materials and methods

Cultures. *H. akashiwo*, isolated from Jiaozhou Bay, Yellow Sea, was used as targeted species. *Prorocentrum micans*, *Scrippsiella trochoidea*, *Amphidinium carterae*, *Prorocentrum lima* and *Amphidinium operculatum* were used to confirm the species specificity of the designed probes and test for cross-reactivity. The cultures were kept in 250mL flasks containing 100mL f/2 medium at a salinity of 36 psu, at 20–22 °C and a 12:12-h light:dark cycle at an irradiance of 100 μmol photons·m^{-2}·s^{-1} provided by cool white fluorescent tubes. The cultures were stirred daily, nutrient medium replaced weekly.

Probe design. The selected specific probes against cytoplasmic LSU RNA and nuclear ITS DNA were HA-lsu (5′-CGTTCCTCTCTCG-GGTATGCTG-3′) and HA-its (5′-AGTTTTGAC-CCAAACCAA CCTC-3′) . UniC (5′-GGGCATC-ACACGACCT-3′) (Biegala *et al*. 2002) and UniR (5′-GCTGCCTCCCGTAGGAGT-3′) (Amann *et al*. 1990) against cytoplasmic rRNA were used as positive and negative control, respectively. Probes were synthesized commercially with fluorescein 5-isothionate (FITC) attached to the 5′ end (Amersham Pharmacia Biotech). The probes, received in a lyophilized form, were dissolved in 0.1 M Tris-HCl (pH 7.5) to final concentration of 100 μM, and aliquots stored at −20 °C in the dark.

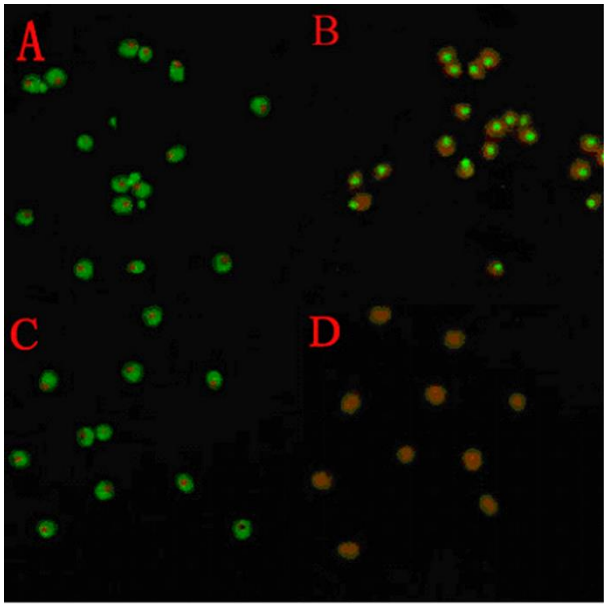

Fig. 1. Photomicrographs of the FISH analyses showing specificity of probes to *H. akashiwo* HA-lsu (A); HA-its (B); UniC (C) and UniR (D).

Fixation protocol. Fixation protocol was determined according to the method of Miller and Scholin (2000) with slight modification. Each culture (0.5 mL) was pipetted gently into a 1.50 mL Eppendorf tube containing 1.0 mL of modified saline ethanol fixative [0.16 mL ddH_2O, 0.10 ml 25× SET buffer (3.75M NaCl, 25 mM EDTA, 0.5 M Tris-HCl, pH 7.80) and 0.74 mL of 95% ethanol]. The mixture was left room temperature for 40min and then centrifuged at 1000 g for 3 min at 4 °C. Approximately 500 μL of lower layer of supernatant was left to resuspend cells. LM observation was performed on preserved cells with a Nikon microscope (E800, Tokyo, Japan).

Fluorescence in situ hybridization. Following fixation with modified saline alcohol fixative as mentioned above, 40 μL of pelleted cells were evenly spotted onto a microscope coverslip, pre-coated by 0.01% polylysine (Sigma, Cat. P8920) and laid in a watch glass. The coverslip was dried using a hair drier. After being rinsed twice with 5× SET hybridization buffer, cells were covered by 100 μL of 5× SET hybridization buffer containing either the positive control, negative control or specific probes. The watch glass was placed in a dark water bath for incubation. After hybridization, the coverslip was washed twice with pre-warmed 1× SET to remove excess probe. The coverslip was stored at 4 °C or −20 °C in the dark before analysis. Specially, for hybridization with HA-its, cells were incubated at 100 °C for 5 min to denature genomic DNA and incubated on ice for 2 min before spotted onto the coverslip. Microscopic observations of cells were performed by epifluorescence microscopy equipped with fluorescein band pass filter set for FITC and counterstain (TRITC. PI) (excitation, 450–490 nm; DM, 505 nm; BA, 520 nm).

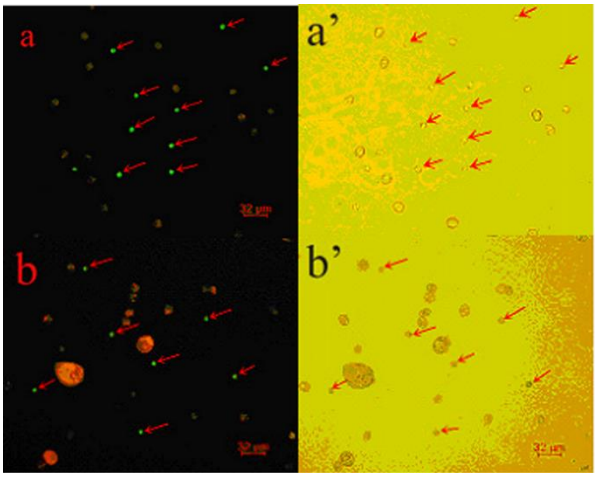

Fig.2. Photomicrographs of *H. akashiwo* hybridized with HA-lsu (a, a') and HA-its (b, b') to identify targeted species using the mixed algal sample. Red arrows showed the cells of *H. akashiwo*.

Results and discussion

Detection of H. akashiwo cells with the optimized hybridization protocol.

When the two specific probes (HA-lsu and HA-its) were applied to hybridize with *H. akashiwo* according to the established protocol, intact cells could be well labeled, with whole cell or exclusive nuclear displaying strong green fluorescence (Fig. 1). This whole cell fluorescence labeled by HS-lsu clearly proved that the rRNA-targeted probe was properly hybridized to abundant rRNA in ribosomes distributed throughout the whole cell. Similarly, use of the positive probe UniC yielded intact cells with green fluorescence over the cells, whereas the negative probe UniR did not stain any cell of this species (Fig.1), indicating that the hybridization conditions were adequate to allow entry of the probes into the cells. The HS-its labeled cells displayed fluorescent nucleus, without fluorescence signal being observed in cytoplasm. This strong fluorescence derived from the nucleus proved that the rDNA-targeted probe was properly hybridized to rDNA distributed throughout the whole nucleus (Fig. 1). Based on these results, we conclude that *H. akashiwo* cells could be detected easily using epifluorescence microscopy, and the sensitivity of the designed rDNA-targeted probe was possibly superior to that of rDNA-targeted probes that hybridize to *Alexandrium catenella* and *A. tamarense* (Adachi et al. 1996).

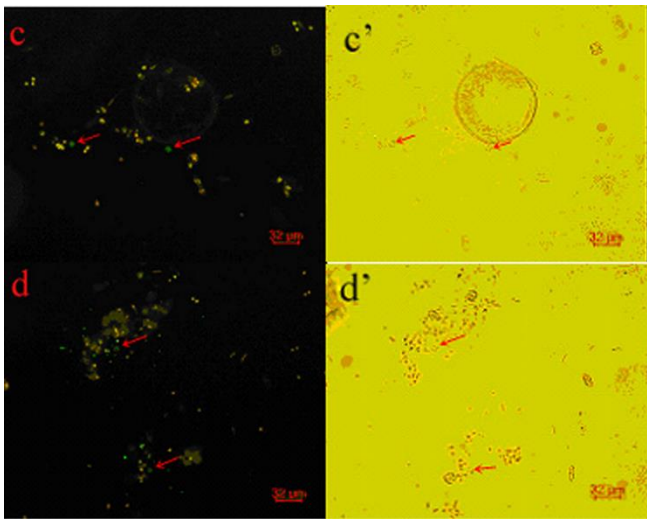

Fig. 3. Photomicrographs of *H. akashiwo* labeled with HA-lsu (c, c') and HA-its (d, d') to identify targeted species using a field sample spiked with targeted species. Red arrows showed the cells of *H. akashiwo*.

To confirm the specificity of HS-lsu and HS-its for the target species, cross-reactivity was examined with many HAB species, including *P. micans*, *S. trochoidea*, *A. carterae*, *P. lima*, and *A. operculatum*. As shown in Fig. 2, HA-lsu and HA-its reacted only with *H. akashiwo* and did not react with all other species tested. This result proved that both specific probes may be useful for molecular identification of targeted species in mixed samples containing different microalgae. To confirm the specificity of HS-lsu and HS-its for the target species in natural seawater, the FISH method was also applied to identify *H. akashiwo* from spiked field samples. The results indicated that both probes showed high specificity and no cross-reactivity to field samples of diatoms, microorganisms and detritus-like amorphous clusters (Fig. 3). Use of these probes in the field appears promising, as *H. akashiwo* cells were successfully detected and identified from the spiked field samples.

Acknowledgements

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New Approach For Direct Detection Of Domoic Acid

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Abstract

Domoic acid (DA) is a dangerous neurotoxin produced by some species of the genus *Pseudo-nitzschia*. An underwater system for assaying domoic acid in seawater was developed. It consists of a fluidic injection system coupled to a Surface Plasmon Resonance (SPR) sensor. The system demonstrated a very high sensitivity in the range 0.1-1 ng/mL during laboratory and shipboard experiments.

Introduction

Toxic species in the genus *Pseudo-nitzschia* are particularly dangerous because they produce domoic acid (DA), a neurotoxin that can bioaccumulate in phytoplankton feeding organisms and which rapidly transvectors throughout the food chain. The production of toxins within and among toxigenic phyto-plankton species varies depending on environ-mental parameters such as temperature, salinity, nutrients and trace elements concen-trations (Granéli *et al.* 2006). Consequently, ambient domoic acid concentrations are difficult to forecast based on cell number alone. Systems capable of detecting DA during bloom formation would therefore be of great interest. In this study, we designed and tested an underwater system for assaying ambient DA concentrations using Surface Plasmon Resonance (SPR) spectroscopy. This optical technique is highly sensitive and measures refractive index changes as biomolecules bind to the surface of the sensor. Refractive index (RI) changes as small as 10^{-6} can be readily measured and only require binding of biomolecules in the tens of picograms range to provide reliable measurements. The SPR surface also can be easily regenerated multiple times which reduces the per sample cost (Homola *et al*, 2006). Because DA is such a small molecule (310 Da), it is difficult to detect in a direct SPR assay. Yu *et al* (2005) were able to overcome this limitation by developing a competition assay capable of detecting DA at concentrations as low as 0.1 ppb in clam extracts. This competitive assay works as follows. First, DA is immobilized on the gold surface during the functionalization step. Next, a sample potentially containing DA is mixed with a solution of anti-DA antibodies. The antibody concentration which is added is carefully calibrated so that there is sufficient antibody to bind all the DA molecules immobilized on the SPR gold surface, but not so much that a great excess of unbound antibody remains. The mixture is then injected in the flow cell of the sensor after a 15 min incubation. The more free DA in the sample, the less unbound antibody available to bind the DA on the SPR surface, and the smaller the refractive index change. Thus, smaller refractive index changes correspond to a greater amount of free DA in the sample. By using standard DA additions, it is possible to determine a consistent relationship between DA concentration and the amount of change in the refractive index relative to a control sample containing no DA. The same approach was taken to develop the underwater SPR sensor reported in this paper. The initial laboratory and shipboard experiments tested the system using PBS solutions spiked with various DA concentrations. Subsequent trials were then carried out using seawater spiked with DA.

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Materials and methods

SPR biosensor. An SPR sensor based on SPR spectroscopy was manufactured in our laboratory and used a commercially prepared chip (Laurent *et al.* 2009). The attachment of DA to the SPR chip (functionalization) was carried out according to the well described protocol by Yu *et al.* (2005). For this assay, the domoic acid antibodies were produced by Mercury Science Inc. They were monoclonal antibodies that had been successfully used in an ELISA assay (Litaker *et al.* 2008). The seawater was taken in Argenton (France). An underwater fluidic system developed and described by Vuillemin *et al* (2009) was used for mixing the chemicals and injecting them into the flow cell of the sensor.

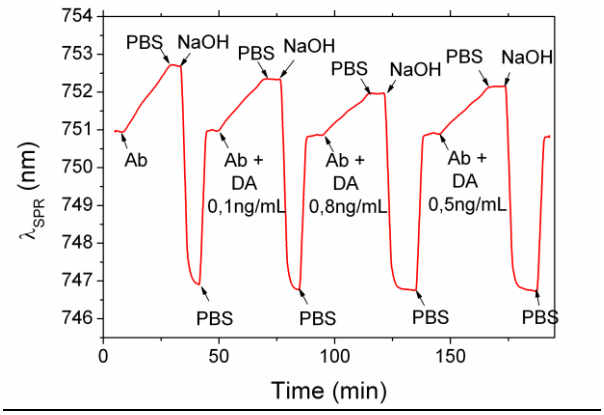

Fig. 1: Sensorgram of the injections of 0, 0.1, 0.8 and 0.5 ng/mL of DA

Experiments

Biosensor characterization. Two sets of experiments were run initially. The first assessed the performance of the SPR system in the laboratory. The second examined how the assay performed in an underwater injection system deployed from a ship under realistic field conditions. In the laboratory experiments, PBS solutions containing anti-DA antibody (1.6 µg/mL) were spiked with DA ranging from 0.1 ng/mL to 10 ng/mL and incubated for 15 min. The mixtures were then sequentially injected into the flow cell for 15 min. After each injection, the flow cell was rinsed with a PBS solution and regenerated using NaOH (100 mM). The field experiments were carried out from an oceanographic boat (Côte de la Manche, INSU). The SPR biosensor and the fluidic system were mounted on a metallic frame along with a CTD (SBE19, Seabird) to measure depth and temperature. The whole system was deployed at 8 m depth.

Seawater analysis. A final set of experiments was carried out using seawater samples containing anti-DA antibody which were spiked with DA at concentrations ranging from 0.1 ng/mL to 5 ng/mL. Samples were incubated for 15 min and run as described above. To compensate for an observed loss of activity in seawater, the antibodies concentration was increased. Further testing showed that the most consistent results were obtained when the higher levels of antibody were used in conjunction with seawater samples that had been diluted 1:1 with PBS.

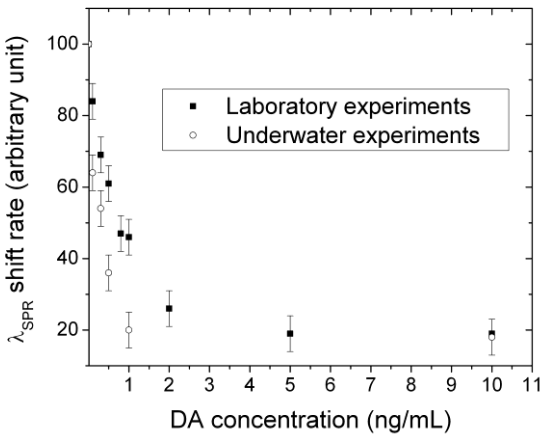

Fig. 2: Sensitivity in PBS during laboratory and underwater experiments.

Results and discussion

Biosensor characterization. Figure 1 presents a sensorgram of four injections of the mixture followed each time by injections of PBS and NaOH. During the sample injection, the signal increased linearly with time as the free antibodies bound the DA molecules affixed to the SPR chip surface. When the PBS was injected, the signal stayed constant indicating that all the antibodies bound strongly to the DA which had been immobilized on the surface. After the regeneration of the SPR chip with NaOH, the signal went back to its value before the injection indicating that antibodies had been completely removed from the surface without a

loss of DA. At higher DA concentrations, the rate of shift in the refractive index with time was also observed to slow due to lower free antibody concentrations.

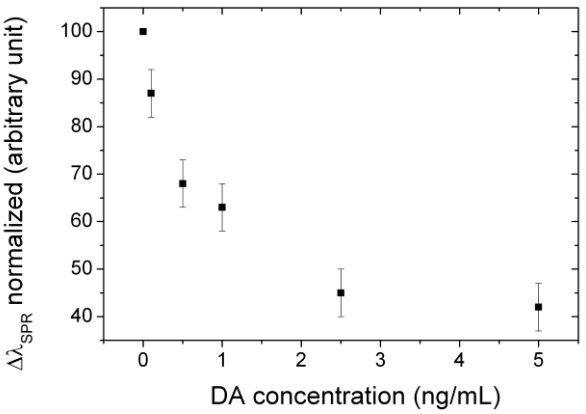

Fig. 3. Assay sensitivity in seawater diluted 1:1 with PBS.

Figure 2 shows the change in SPR shift rates at various DA concentrations under laboratory (squares) and field conditions (open circles). All rates were normalized to solution containing no DA. These plots showed again that the rate of SPR shift rate decreased as the DA concentration increased. This decrease was very sharp between 0.1 to 1 ng/mL. At greater concentrations, the signal reached a plateau. Optimal sensor sensitivity was therefore between 0.1 to 1 ng DA /mL. If greater DA concentrations have to be measured, the antibodies concentration can be increased, but at the cost of the sensitivity. The SPR shift rate for the field samples had a similar shape as the one obtained in the laboratory with identical maximal sensitive range even though the ambient temperature was 8°C.

Seawater analysis. Full strength seawater adversely affected the stability of the antibody and lowered assay sensitivity. This was overcome by increasing the antibody concentration and diluting the seawater samples 1:1 with PBS. Figure 3 shows that this approach produced a shift curve similar to that shown in Figs 1 and 2 and a maximal sensitivity range between 0.1 to 2 ng DA/ml.

Conclusions

A competition assay based on an underwater SPR sensor was developed and assessed in the laboratory and in shipboard experiments. The sensor showed high sensitivity within a lower limit of detection of ~0.1 ng.mL^{-1} and a dynamic range of 0.1 to 1.0 ng/ml in PBS. The assay performed equally well at room temperature and at 8°C in the field. Natural seawater samples had to be diluted 1:1 with PBS for the assay to work reliably over the range of 0.1 to 2.0 ng/ml. As currently configured the system would be sensitive enough to detect dissolved DA in the water column at the end of a bloom. For the assay to be effective in a broad range of monitoring applications it will need to be coupled with a system capable of releasing the toxin from a relatively small number of cells. The next phase of this research will concentrate on developing the extraction systems needed to optimize the SPR DA detection system.

Acknowledgements.

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Field testing for toxic algae with a microarray: initial results from the MIDTAL project

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Abstract

One of the key tasks in MIDTAL (MIcroarrays for the Detection of Toxic ALgae) is to demonstrate the applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events. Water samples are collected from a series of sites used in national phytoplankton and biotoxin monitoring across Europe. The samples are filtered; rRNA is extracted, labelled with a fluorescent dye and applied to a microarray chip. The signal intensity from >120 probes previously spotted on the chip is measured and analysed. Preliminary results comparing microarray signal intensities with actual field counts are presented.

Introduction

Blooms of toxic or harmful microalgae (HABs), represent a significant threat to fisheries resources and human health throughout the world. Since many HABs have significant economic impacts, monitoring programmes which measure toxins that have accumulated in shellfish flesh have become a necessity. In Europe, this requirement for monitoring is established in a series of directives in which monitoring of coastal waters for potentially harmful phytoplankton is mandatory. Traditionally phytoplankton identification and enumeration is carried out using LM. This technique requires a high degree of skill of operator, and is time-consuming. Furthermore, the morphological similarity between different species within or even across phytoplankton genera has meant that light microscopy alone is at times insufficient to assess the potential toxicity of a water sample. A variety of methods based on the sequencing of nucleic acids have been developed which have considerably improved our ability to accurately identify organisms to the species level. These have been outlined recently in a manual for phytoplankton analysis (Karlson *et al*. 2010). Microarrays are state of the art molecular biology for the processing of bulk samples for detection of target RNA/DNA sequences. In MIDTAL, existing rRNA (18S, 28S) probes and antibodies for toxic algal species and their toxins have been adapted for use in a microarray format. This paper presents the first field trial results .

Materials and Methods

Water samples are taken and a measured volume is filtered through nitrocellulose filters (pore size 1-3 µm). The volume of sample filtered depends on turbidity of the water: 0.5-2 l is usually filtered up to when the filter starts to clog. The filter is then immediately submersed in 1ml of Tri-Reagent (Ambion, UK) and an aliquot of *Dunaliella tertiolecta* ($5 .10^6$ cells) added as an internal control for the RNA extraction process. The material is then stored at -80 °C. RNA extraction is carried out through cell lysis, sequential extraction with 1-

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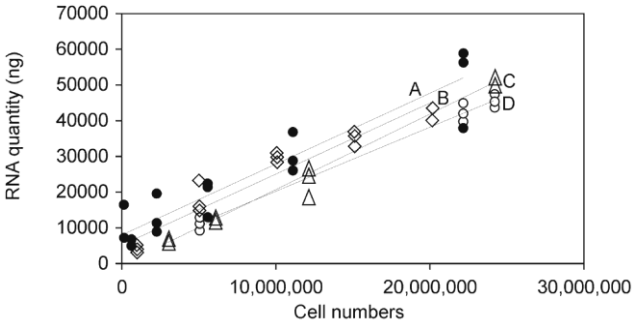

Fig. 1. Comparison of RNA extraction efficiencies on cultures of *Dunaliella tertiolecta* between four MIDTAL partners A (R^2 = 0.8836), B (R^2 = 0.9243), C (R^2 = 0.9848) and D (R^2 = 0.9912).

Bromo-3-chloro-propane (BCP) and isopropanol, followed by ethanol wash. After the final centrifugation step, the pellet is suspended in RNase free water and stored at -80 °C. The RNA is labelled using Platinum Bright 647 Infrared Nucleic Acid kit, fragmented and hybridised to pre-activated epoxysilane-coated microarray chip at 65°C. Unlabelled RNA is removed from the chip surface using 3 washing steps, with different stringency involving EDTA, minimising background noise. The chip, pre-spotted with over 120 oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus and species) is scanned (Genepix 4000B Axon Inc.) and fluorescence intensity from each probe measured. Results are compared with LM of original water sample. This ongoing process will be carried out over 2 years. Preliminary results comparing microarray signal intensities with actual field counts are presented.

Results and Discussion

RNA extraction efficiency. Good yields of high quality RNA were extracted from *D. tertiolecta* cells when a preliminary standard curve was made (Fig. 1). The relationship between cell numbers and RNA content was linear with satisfactory coefficient of determination from four randomly selected project partners.

*Sensitivity of the hybridisations.*The sensitivity of hybridisations onto the microarray were investigated by testing a range of probes which should be highlighted by a particular organism growing under different environmental conditions. Fig. 2 shows results from probes for prymnesiophytes tested on a culture of *Prymnesium parvum.* These probes were adapted for the microarray from those by Lange *et al*. 1996; Simon *et al*. 1997; 2000; Töbe *et al*. 2006; Eller *et al*. 2007. A NanoDrop Spectrophotometer was used to quantify the RNA after the labelling and RNA clean-up steps to determine the exact labelled RNA amount when approximately 1 , 5 , 25 and 100 ng were hybridised to the chip. A pre-selected signal: noise ratio threshold level was applied so that the limit of quantification was represented by a signal of 2. Thus if the optimum probe for prymnesiophytes (PrymS02_25; Lange *et al*. 1996) is applied, then the microarray can not accurately detect RNA amounts below 5 ng (Fig. 2a). Example of image intensities is also shown in Fig. 2b.

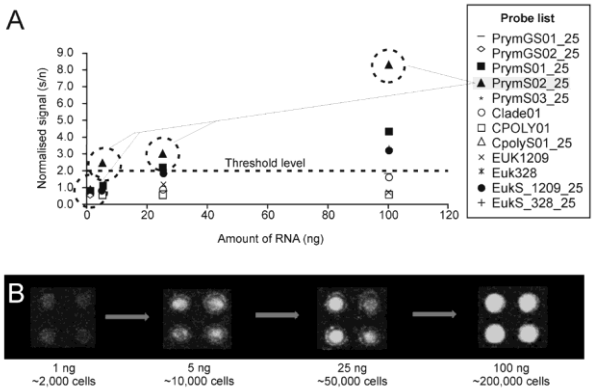

Fig. 2. Standardisation of the *Prymnesium parvum* signal. (A) Calibration curve of RNA (1, 5, 25 and 100ng) against signal intensity for a range of probes. (B) Images of the optimum probe PrymS02_25 when increasing amounts of RNA are hybridised to the microarray. Increasing signal intensity represents increasing cell numbers.

Development of microarray chip. A 1st chip designed for a specified range of HAB species produced weak signals for several species-probe combinations. A 2nd generation chip was designed in which the probes were increased in length to 25 base pairs. This meant a higher melting point temperature was required and thus hybridisation temperature was increased from 58 to 65 °C. This temperature was adopted as standard between all project partners and will be further optimised for the next generation of chip.

Light microscopy and microarray field results. Examples of microarray results are shown in

Figs 3 and 4. Fig 3 compares data obtained from the 1st and 2nd generation chips.

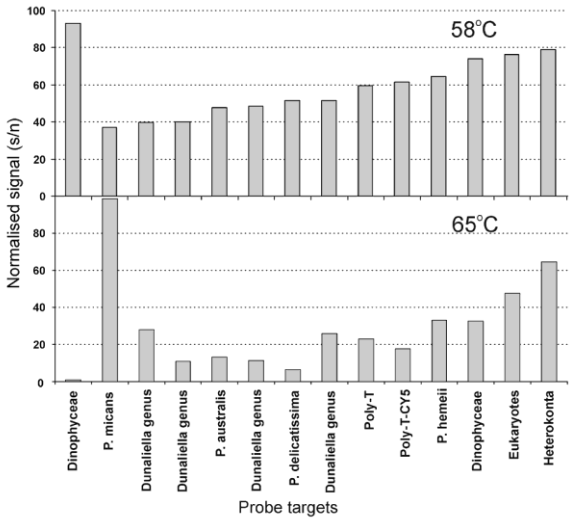

Fig. 3. Microarray results of 1st and 2nd generation chips both hybridised with the same Bell Harbour field extract at temperatures of 58 and 65 °C. Note the difference between the Pmica02 and PmicaD02_25 probe species specific for *P. micans*.

The sample was taken in Bell Harbour, Ireland 2009, during a bloom of *Prorocentrum micans*. LM showed a cell density of 360,000 cells l^{-1}. The *P. micans* probe used on the 2nd generation chip (PmicaD02_25 (98.53 s/n ratio); L.K. Medlin unpubl.) gave a vastly stronger signal to its complement (Pmica02 (37.15 s/n ratio)) on the 1st generation microarray, which was 7 base pairs shorter. A general agreement between microarray signal results and cell counts was obtained. There is also an elevated signal from the class level probe for Dinoflagellates. The strongest signals in Fig.3 signify eukaryotes, heterokonts, dinoflagellates, as well as chlorophyte *Dunaliella* and Poly-T-CY5 used as controls. Cross-reactivity with *P, heimii* will need to be addressed on the 3rd generation chip because it reacts with many target species. A second comparison between LM counts and a selection of 2nd generation microarray results from a sample from Killala Bay, August 2009, is shown in Fig. 4. An assemblage of *P. seriata* group numerically dominated the sample (112,000 cells l^{-1}) (Fig. 4a). The microarray data could identify these as *P. fraudulenta, P. seriata, P. australis,* and *P. multiseries.* A variety of *Alexandrium* probe signals were also evident, which could not be resolved by LM (Fig. 4b) and require EM to confirm the species.

Conclusions

The aim of MIDTAL is to provide a new method to support toxic algal monitoring and reduce the need for mouse bioassay. Demonstration of its capabilities is the first step towards this goal. These field results indicate that there remains further development work to be done but point towards the potential of a 'universal' HAB microarray.

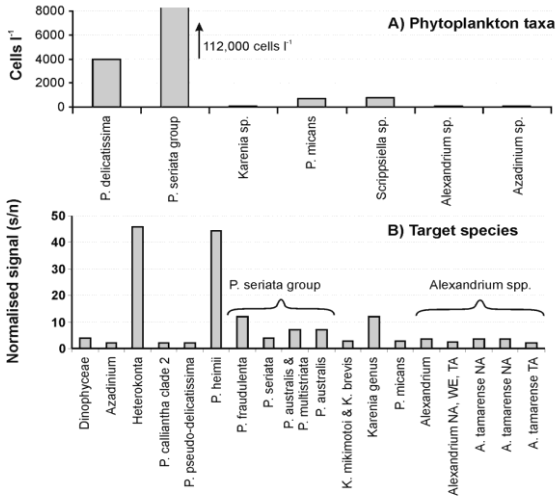

Fig. 4. (A) Cell counts and (B) 2nd generation microarray chip hybridised with RNA at temperature of 65°C from Killala Bay field extract on 15 Aug 2009.

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The Use of Immunoassay Technology in the Monitoring of Algal Biotoxins in Farmed Shellfish

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Abstract

The use of immunoassay technology as an adjunct method for monitoring biotoxins in shellfish was investigated at aquaculture sites in Killary Harbour, Ireland, during summer 2009. Sub-samples of mussels (*Mytilus edulis*) were taken from batches collected as part of the Irish National Phytoplankton and Biotoxin Monitoring Programme (NMP). Samples were analysed for Diarrhetic Shellfish Poisoning (DSP) toxins using a commercially available ELISA immunoassay kit. The results were compared with those obtained by chemical (liquid chromatography with mass spectrometry, LC-MS) and biological (mouse bioassay, MBA) methods from the monitoring programme. DSP levels increased in late June 2009 over the European Union maximum permitted level of 0.16 µg g^{-1} and positive MBA results led to harvest closures. This event was reflected in both the chemical and immunoassay results, where a positive relationship between them was found.

Introduction

Along most of the Atlantic seaboard of Europe, including Ireland, contamination of shellfish with Diarrhetic Shellfish Poisoning (DSP) toxins derived from *Dinophysis* spp. is the biggest problem for shellfish producers (Raine *et al*. 2010). The current standard method within Europe for the analysis of DSP toxins in shellfish is the mouse bioassay (Yasumoto *et al*. 1978). This technique, accepted by EC regulation (Regulation 2074/2005) for monitoring programmes, is now often used in tandem with chemical methods such as high performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC-MS). The ethical issues and limitations of these methods, such as expense, lag time and use in a restricted number of laboratories, have prompted a requirement for new analytical technologies, particularly in peripheral regions. Immunoassay technology is now available for the analysis of amnesic (ASP), paralytic (PSP) and diarrhetic (DSP) toxins in shellfish (Hallegraeff *et al*. 2004). The present study is part of an investigation into the accuracy, reliablility and ease of use of the currently available DSP immunoassay a direct comparison to bioassay and chemical techniques.

Methods

Farmed blue mussels, *Mytilus edulis*, were collected from Killary Harbour (53° 37' N, 9° 48' W) between May and Sept 2009 (Fig 1). Sub-samples were taken fortnightly collected under the Irish National Phytoplankton and Biotoxin Monitoring Programme (NMP). On each occasion, 3 samples were obtained from inner, middle and outer Killary Harbour (Fig 1).

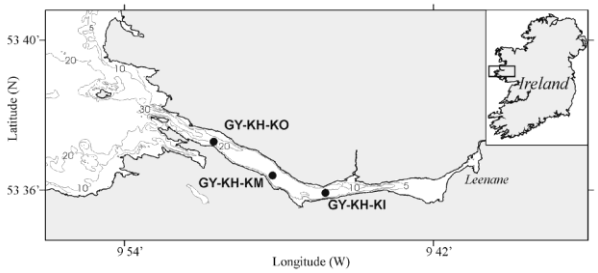

Fig. 1.Map of Killary Harbour showing sampling locations; inner: GY-KH-KI, middle: GY-KH-KM and outer: GY-KH-KO.

Environmental parameters were recorded on each occasion, including water temperature, which was also continuously monitored at the middle sample site using three data loggers (TidbiT, Onset Computer Corporation) suspended at spaced intervals (2, 5 and 13 m depth) on a moored line, and recording data at hourly intervals. Samples for phytoplankton analysis were collected using a 12 mm i.d. tube to achieve an integrated water sample

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over the depth range 0-10 m (Lindahl 1986) and samples were preserved with Lugols Iodine before analysis using an inverted microscope (McDermott and Raine 2010). Mussels were stored at -20 C until analysis. When thawed, mussel tissue was removed from the shell and homogenized. Toxins were extracted with methanol by vortex mixing and centrifuging 1 g mussel tissue with 9 ml 80% (v/v) methanol. The toxin extracts were serially diluted using buffer solution supplied in the analysis kit (DSP, Abraxis) and the analysis proceeded according to the manufacturer's instructions. DSP toxins were quantified both before and after hydrolysis (1.25N NaOH, 100°C, 20 min and then neutralised with HCl), which converts dinophysistoxin (DTX) esters into dinophysistoxins which can be detected by the kit.

The Abraxis DSP ELISA Kit is a rapid assay; it is a direct competitive ELISA, based on recognition of okadaic acid (OA) and DTX-1, DTX-2 by specific anti-bodies. The assay works on a colour reaction. Toxins in positive samples compete against a conjugate enzyme (to which a colour solution binds) for binding sites on antibodies which have been loaded onto a microtitre (96-well) plate. The intensity of the colour produced is inversely proportional to the concentration of toxin present and was read using a micro-plate reader (Biotek). Results were expressed as okadaic acid equivalents i.e. OA and its derivative dinophysistoxins DTX-1, DTX-2 and esters (DTX-3). A standard curve is prepared for each analysis and ttoxin content in each sample is determined by interpolation.

Results

Fig.2 shows levels of DSP toxin in mussel flesh from the three sites in Killary Harbour during tsummer 2009. Chemical (LC-MS) data show that toxin levels exceeded the EU maximum permitted level (MPL) of 0.16 µg g^{-1} on 22 June at outer and middle sites and 29 June at the inner site, suggesting that contamination was transported into the harbour from outside. These dates co-incided with the onset of positive MBA results and enforced the closure of the three areas for harvest (Fig.2).

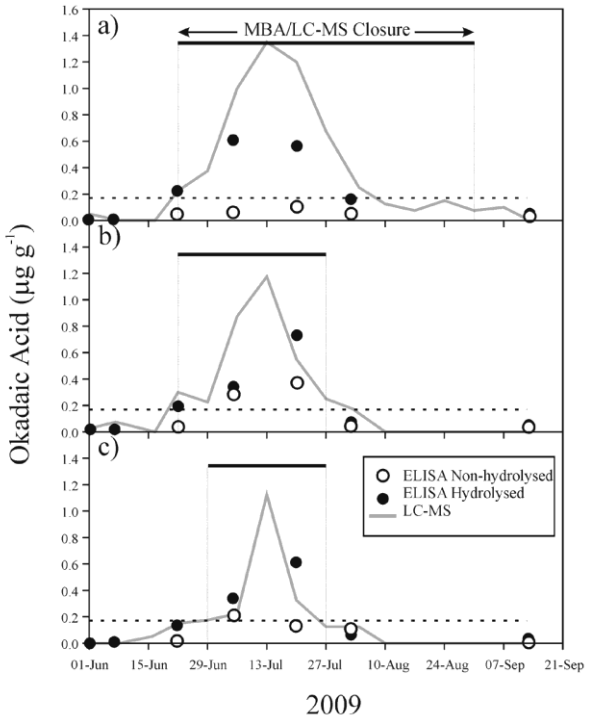

Fig 2. A comparison of DSP toxin levels in shellfish analysed using DSP ELISA immunoassay before and after hydrolysis, LC-MS and mouse bioassay in Killary Harbour 2009 for (a) outer, (b) middle, (c) inner sampling sites.

Subsequently DSP toxin levels rapidly increased at all three sampling sites to ca. 1.2 µg g^{-1}, with toxicity increasing faster at the outer and middle sites than inner site. Toxin levels decreased after mid-July and, with the exception of the outer site, fell to and remained below MPL from 10 Aug. The contamination of mussel tissue with DSP biotoxins coincided with an increase in *Dinophysis acuminata* and *D. acuta* spp. cell densities (Fig. 3a). *Dinophysis* spp. cell densities in integrated samples increased to 2100 l^{-1} on 5 July corresponding to the inital sharp increase in DSP toxin levels at this time.

Water temperatures near the seabed at the middle site increased from 10.8 on 7 June to 15°C on 24 Aug (Fig 3b). This increase was more or less gradual but was punctuated with two sudden peaks. On 16 June temperatures increased by almost 2°C in less than 24 hours.

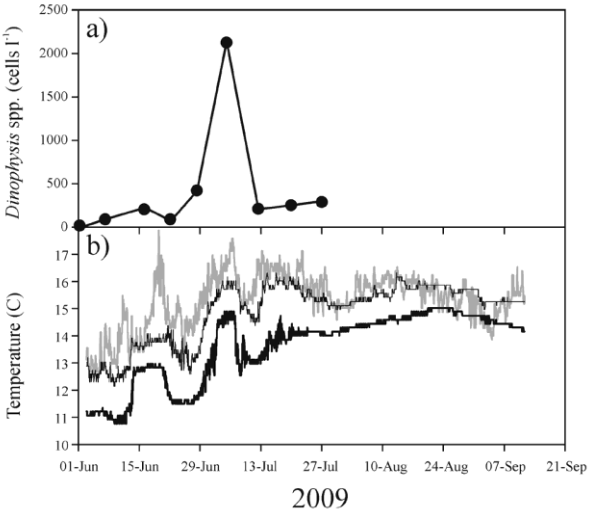

Fig. 3.a) *Dinophysis* cell densities in integrated water samples and b) water temperature data in Killary Harbour, middle site, 2009.

A similar event was observed on 5 July. Both occasions related to increases in *Dinophysis* cell densities, with the second event linked to the sharp increase to >2000 cells l^{-1}, and both followed by a drop in temperature of similar dimension 2-3 days afterwards. It is possible that the increase in *Dinophysis* cell numbers and increase in bottom water temperature were caused by exchange of water between Killary Harbour and near coastal shelf, which brought in the *Dinophysis* population. DSP toxin levels from Killary Harbour during summer 2009 by LCMS were compared with immunoassay (DSP ELISA). Both ELISA and LC-MS showed the same general trend (Fig. 2). Both data sets show an initial non-toxic phase followed by steady increase exceeding MPL, progressing to a steady decline. All hydrolysed samples analysed during the closure period produced positive results by ELISA (Fig.2). Outside this period, no 'false positives' were found in either hydrolysed or non-hydrolysed samples determined by ELISA. On the other hand, most non-hydrolysed samples gave results <MPL during the closure period. All positive (i.e.>EU MPL of 0.16 µg g^{-1}) results determined by LC-MS (and MBA) were also positive by ELISA when hydrolysis was used.

Discussion and Conclusion

The DSP ELISA kit readily detected and quantified presence of DSP toxins in farmed mussels during a toxic event in Killary Harbour in summer 2009. A positive relationship was observed between the MBA, toxin concentrations dby LC-MS and ELISA when samples were hydrolysed. The hydrolysis step thus appeared to be an essential part of the methodology of the immunoassay. The technique has clear potential as an alternative method for toxin analysis, and may prove useful if, for example, screening of shellfish for toxins is required in remote areas where delays in receiving analytical toxin testing results might occur. The immunoassay method proved rapid and easy to use and thus had a number of advantages over chemical methods.

Acknowledgments

The authors acknowledge Simon Kennedy (Killary Fjord Shellfish), H. Kleivdal (Biosense Ltd.) and J.P. Lacaze (Marine Scotland, Aberdeen), Sarah Cosgrove, Gary McCoy and Nicolas Touzet. This work is a contribution to project WATER and part-funded through Northern Periphery Programme.

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TOXINS

The analysis of lipophilic marine toxins

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Abstract

Consumption of lipophilic marine toxin contaminated shellfish can lead to severe intoxications. Methods described in European Union (EU) legislation to test for the presence of these toxins are based on a mouse or rat bioassay. These assays are unethical and have a poor sensitivity and selectivity. For this reason there is an urgent need for alternative methods. Most promising alternatives are the methods based on liquid chromatography - tandem mass spectrometry (LC-MS/MS). A LC-MS/MS method with alkaline chromatographic conditions in which we were able to separate and analyze the most important toxins in a single analysis was developed. Furthermore, a clean up procedure based on solid phase extraction (SPE) was developed. A combination of SPE clean up and alkaline chromatographic conditions resulted in reduced matrix effects for all matrices tested (mussel, scallop and oyster). The developed SPE & LC-MS/MS method was in-house validated using EU Commission Decision 2002/657/EC. With respect to accuracy, repeatability, reproducibility and decision limit the method performed well. The method also performed excellently in view of possible new limits that are 4- to 5-fold lower than current limits for some toxins. A collaborative study was also performed for the most important toxins of the lipophilic marine toxin group.

Introduction

Marine toxins (phycotoxins) are natural toxins produced by at least 40 species of algae belonging mainly to the dinoflagellates and diatoms (Gerssen et al., 2010a). Phycotoxins can accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated shellfish are consumed by humans, this may cause severe intoxication of the consumer (Aune and Yndestad, 1993; Botana et al., 1996; Jeffery et al., 2004). Based on their chemical properties marine toxins can be divided in two different classes: hydrophilic and lipophilic toxins. Toxins associated with the syndromes Amnesic Shellfish Poisoning (ASP) and Paralytic Shellfish Poisoning (PSP) are hydrophilic by nature and have a molecular weight (MW) below 500 Da. Toxins responsible for Neurologic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Shellfish Poisoning (AZP) and other toxins such as pectenotoxins (PTXs), yessotoxins (YTXs) and cyclic imines [spirolides (SPX) and gymnodimine] all have as common denominator a MW above 600 Da (up to 2 000 Da). These toxins have strong lipophilic properties and are generally called lipophilic marine toxins. European Union (EU) legislation prescribes animal tests (mouse or rat) as the official method for control of lipophilic marine toxins in shellfish (Anon, 2005). More than 300000 test animals (mostly mice) are used annually for routine monitoring of lipophilic marine toxins in shellfish within the EU. Besides the ethical aspects of this cruel animal test, it also contradicts with other EU legislation which states the reduction, refinement and replacement of animal tests (Anon, 1986). Furthermore, these animal tests can produce false positive results and have a poor sensitivity and selectivity. In this paper the development of an alternative method for the determination of lipophilic marine toxins is described, based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

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Liquid chromatography mass spectrometry. Traditionally, LC-MS/MS methods used acidic chromatographic conditions for the determination of lipophilic marine toxins (Fux et al., 2007; Quilliam et al., 2001). However, under acidic conditions peak shapes as well as separation of some toxins was poor. With alkaline chromatographic conditions, an acetonitrile/water gradient containing ammonium hydroxide (pH 11), the limit of detection (LOD) for OA, yessotoxin (YTX), gymnodimine (GYM) and 13-desmethyl spirolide C (SPX1) was improved two- to three-fold (Gerssen et al., 2009b). This improvement is mainly due to improved peak shapes. A major advantage of the developed alkaline method is that toxins can be clustered in retention time windows separated for positively and negatively ionized molecules. Therefore, there is no need for rapid polarity switching or for two separate runs to analyze a sample. With this method at least 28 different lipophilic marine toxins can be analyzed in a single run. Separation of the most prominent lipophilic marine toxin groups comprising okadaic acid (OA), dinophysistoxins (DTXs), YTXs, azaspiracids (AZAs) and SPXs was achieved (Figure 1).

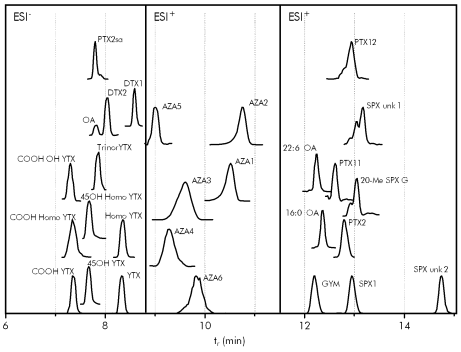

Fig.1. LC-MS/MS separation of 28 lipophilic toxins in a single run.

Matrix effects. It is well known that LC-MS/MS analysis is sensitive to matrix effects (signal suppression or enhancement). This is also the case for lipophilic marine toxins. Therefore, the potential of solid phase extraction (SPE) clean up has been assessed to reduce matrix effects in the analysis of lipophilic marine toxins. A large array of ion-exchange, silica-based and mixed function SPE sorbents was tested. The toxins were best retained on polymeric sorbents. Optimization experiments were carried out to maximize recoveries and the effectiveness of the clean up. This was done by optimization of the wash and elution conditions. Matrix effects were assessed using either an acidic or an alkaline chromatographic system as described in earlier publications (Gerssen, et al., 2009b). In combination with the alkaline LC method this resulted in a substantial reduction of matrix effects to less than 15%, while in combination with the acidic LC method approximately 30% of the matrix effects remained (Figure 2). A combination of the SPE method with the chromatography under alkaline conditions was the most effective (Gerssen et al., 2009a).

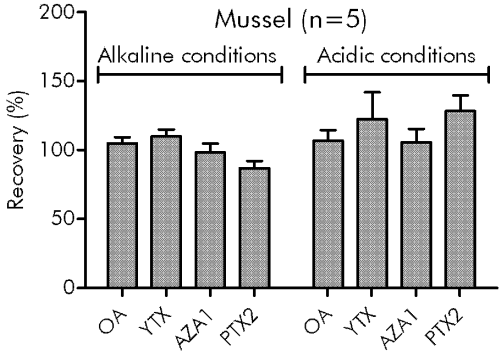

Fig.2. Lipophilic toxin recovery under alkaline and acidic conditions.

In-house validation

Before a method can be officially used in the EU for routine analysis, the method needs to be validated. The in-house validation was performed for the quantitative analysis of OA, YTX, AZA1, PTX2 and SPX1 in shellfish extracts [mussel (*Mytilus edulis*), oyster (*Cassostrea Gigas*), cockle (*Cerastoderma edule*) and ensis (*Ensis directus*)]. Dinophysistoxin-1 (DTX1), -2 (DTX2) and azaspiracid-2 (AZA2) and -3 (AZA3) were not included in the study because the certified standards were not available at that time. The validation was performed using the EU Commission Decision 2002/657/EC as guideline (Anon, 2002). Validation was performed at 0.5, 1 and 1.5 times the current EU permitted levels, which are 160 µg/kg for OA, AZA1 and PTX2 and 1000 µg/kg for YTX. For SPX1 400 µg/kg was chosen as target level as

no legislation has been established yet for this compound. The method was validated for determination in crude methanolic shellfish extracts and for extracts purified with solid phase extraction (SPE). The toxins were quantified against a set of matrix matched standards instead of standard solutions in methanol. In order to save valuable standard the toxin standards were spiked to the methanolic extract instead of the shellfish homogenate. This was justified by the fact that the extraction efficiency is high for all relevant toxins (>90%). The method performed very well with respect to accuracy, intra-day precision (repeatability), inter-day precision (within- lab reproducibility), linearity, decision limit (CCα), specificity and ruggedness. For crude extracts the method performed less satisfactory with respect to the linearity (<0.990) and the change in LC-MS/MS sensitivity during the series (>25%). This decrease in sensitivity could be attributed to contamination of the LC-MS/MS system. SPE purification resulted in a greatly improved linearity and signal stability during long series (more than 20 samples). Recently the European Food Safety Authority (EFSA) has published a number of opinions on the various toxin groups. The EFSA has suggested that in order not to exceed the acute reference dose the levels should be below 45 µg/kg OA-equivalents and 30 µg/kg AZA1-equivalents. If these levels are adapted in legislation this means a 4-5 fold lower permitted limit than the current one. For these toxins a single day validation was successfully conducted at these levels (Gerssen et al., 2010b).

Outlook . Now the developed method has been in-house validated, the next step is a full collaborative study. This study was performed in 2010. In total 13 laboratories participated.Statistical evaluation was performed according to AOAC guidelines for collaborative study procedures (appendix D). HorRat values were good, ranging from 0.71 for AZA total group toxicity till 1.60 for YTX. The final report of this study will be published in the beginning of 2011. Furthermore, recently it is decided to change EU legislation. The new legislation will prescribe the use of LC-MS/MS as the reference method for the analysis of lipophilic marine toxins instead of the animal assay. The method described in this paper can than be adopted as an official method for routine analysis and the mouse and rat bioassay can be abolished. Furthermore, in our opinion more research is needed for the production and isolation of lipophilic marine toxins and method development on functional assays and other new emerging toxins such as palytoxins, cyclic imines and ciguatera toxins.

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First report of spirolides in Greek shellfish associated with causative *Alexandrium* species

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Abstract

During the spring of 2008 and 2009, acute toxicity reactions upon intraperitoneal injections of mussel lipophilic extracts derived from Thermaikos Gulf, Greece, were observed during mouse bioassay (MBA). Symptomatology consisted of neurological signs, convulsions, cramps within a few minutes after injection and death within 30 min. The presence of spirolides was suspected due to simultaneous detection of *Alexandrium ostenfeldii*/*peruvianum*, though in low abundances (<160 cells L^{-1}). Samples of mussels (*Mytilus galloprovincialis*) which provided a positive MBA test for lipophilic toxins with the above acute symptomatology were extracted with methanol and subjected UPLC-MS/MS. The method applied was a multi-toxin method able to quantify okadaic acid, dinophysistoxins, azaspiracids, yessotoxins, pectenotoxins, spirolides, gymnodimine and palytoxin and ostreocins. Results of the analyses revealed the co-occurrence of okadaic acid and of 13-desmethyl spirolide C (SPX1) in all analyzed mussel samples at concentrations up to 10 μg kg^{-1} mussel tissue, confirming the result of the MBA, for which the limit of detection is estimated to be 5.6 μg kg^{-1} shellfish. These findings constitute the first report on the presence of both toxins of the cyclic imines group in Greek shellfish associated with *A. ostenfeldii*/*peruvianum* in Greek coastal waters.

Introduction

The presence of spirolides, toxins of the cyclic imines group, was initially reported in the 1990s in bivalve mollusks from Nova Scotia, Canada and was discovered due to their high acute toxicity in mice upon i.p. injections of lipophilic extracts (Hu *et al.* 1995; Cembella *et al*. 1998). Although spirolides have been previously detected in North America and Europe (Cembella *et al.* 2001; MacKinnon *et al*. 2004; Villar Gonzalez *et al.* 2006), there have been no reports of adverse effects in humans. Spirolides are fast acting toxins, causing rapid death with neurotoxic symptoms in laboratory mice when injected intraperitoneally or administered orally (Hu *et al.* 1996). The marine dinoflagellate *Alexandrium ostenfeldii* has been identified as the causative organism of spirolide toxins (Cembella *et al.* 2001), while recently both *A. ostenfeldii* and the morphologically very similar *A. peruvianum* were associated with spirolides' production (Franco *et al*. 2006; Touzet *et al.* 2008). *A. ostenfeldii* has been found in Denmark (Moestrup and Hansen 1988), Scotland (John *et al.* 2003), Norway (Balech and Tangen 1985) and the Atlantic coast of Spain (Fraga and Sanchez 1985), and can produce either saxitoxin derivates, linked to PSP, or spirolide toxins. Data on the presence of either *A. ostenfeldii* or *A. peruvianum* are few for the Mediterranean Sea; however, strains of both species have been shown to produce spirolides in Spain and Italy (Ciminiello *et al*. 2006; Franco *et al*. 2006; Penna *et al*. 2008; Pigozzi *et al*. 2008). The present study constitutes the first report on the detection of spirolides in Greek shellfish associated with the presence of the potential causative *Alexandrium* species (*A. ostenfeldii*/*peruvianum*) in Greek coastal waters.

Materials and methods

In the framework of the Greek monitoring program for marine biotoxins, shellfish and seawater samples are collected on a weekly basis to detect marine biotoxins and toxic and/or potentially toxic microalgal species, respectively, from all culture and harvesting areas (Fig. 1). *Alexandrium* cells in seawater samples were identified under epifluorescence microscopy and their abundance estimated under an inverted microscope (Utermöhl 1958). Shellfish were first tested for lipophilic toxins' presence by the Mouse Bioassay protocol of Yasumoto *et al.* (1978). Samples which provided a positive test were then extracted with methanol, according to the method of Fux *et al.* (2007) and subjected to chemical analysis by ultra performance liquid chromatography coupled to tandem mass

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spectrometry detection (UPLC-MS/MS) using a Waters Acquity UPLC® TQD system. The MS method was able to determine and/or quantify the following lipophilic toxin groups: okadaic acid (OA) and dinophysistoxins (DTXs), azaspiracids (AZA), yessotoxins (YTX), pectenotoxins (PTX), spirolides (SPX), gymnodimine (GYM) and palytoxin (PlTX) and ostreocins (OST). Certified standards (OA, AZA1, from the National Research Council of Canada were used, where available, to obtain calibration curves, while palytoxin standard was from Wako Chemicals.

Fig. 1. Map of Greece showing cultivation and harvest areas (indicated by *) sampled in the framework of the National Monitoring program for Marine Biotoxins.

Results and Discussion

Alexandrium ostenfeldii and *A. peruvianum* (average length: 33µm, width: 35µm) were identified by the large ventral pore (Vp) in the 1' plate (Fig. 2A-D) characteristic of these two species, distinguished mainly by the shape of their sulcal anterior (sa) plate (narrow triangular in *A. peruvianum;* wide rectangular in *A. ostenfeldii*) The fact that both species exhibit the same toxin profile and the slight morphological differences among them has been suggested to imply synonymy (Franco *et al*. 2006). The environmental temperature differences among the two species type localities call for precaution before reaching a conclusion (Penna *et al.* 2008). Specimens were identified as *A. ostenfeldii/ peruvianum*, due to taxonomical ambiguities and that in this sparse *Alexandrium* population in Thermaikos Gulf, cells with both narrow and wide sa plates (Fig. 2A, 2B) were found. Cells were detected in seawater collected from Thermaikos Gulf during the winter-spring of 2008 and 2009, coinciding with detection of spirolides in mussels (*Mytilus galloprovincialis*) sampled from the same area. In 2008, their presence was scarce and abundances did not exceed 80 cells L^{-1}. In 2009, however, they were found in more stations, still in relatively low cell densities (<160 cells L^{-1}) but higher t

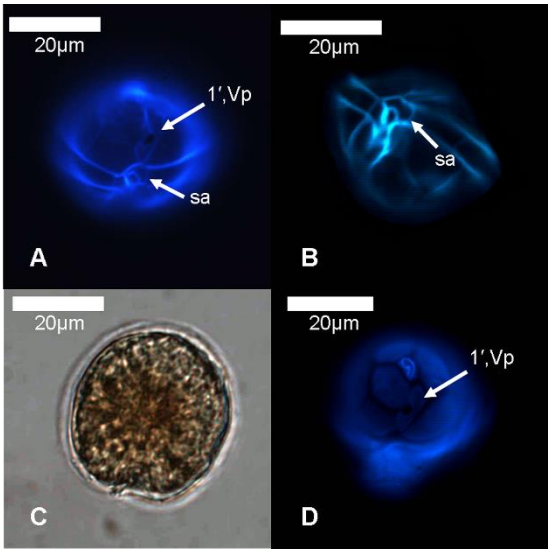

Fig. 2. *Alexandrium ostenfeldii/peruvianum* cells from Greek waters: (a, b, c) Cells under epifluorescence microscopy showing the large Vp in 1' plate and the different shapes of s.a. plate, (c) cell in bright field (apical view).

In April 2008, the presence of spirolides in mussels from Thermaikos Gulf was a coincidental finding, during UPLC-MS/MS confirmatory analyses of samples found positive in MBA tests for lipophilic toxins. The positive tests resulted from the presence of OA in high levels: symptomatology of most mice before death was typical of the OA toxins group, while an intense *Dinophysis* spp. bloom had been recorded. In one of the samples, however, one of the mice died within 15 min of injection with neurological symptoms atypical of OA. The maximum concentrations of 13-desmethyl spirolide C (SPX-1) in 2008 were at 5 µg kg^{-1} mussel tissue (Figure 3a). Detection of spirolides fitted with the identification of the few *A. ostenfeldii/peruvianum* cells found. In late January–early February 2009, however, all positive MBA tests for lipophilic toxins in mussels from Thermaikos Gulf were characterized by acute toxicity reactions and with symptomatology atypical of the common OA group: neurological signs, convulsions, cramps within a few min after the injection and death within 15-30 min. When the animals survived they fully recovered and their subsequent appearance and behaviour were normal. At the same time, no

Dinophysis spp. were present in seawater; instead, cells of *A. ostenfeldii/peruvianum* were found in abundances not exceeding 160 cells L^{-1}. The presence of spirolides was suspected and maximum concentrations were 26 μg kg^{-1} mussel tissue (Fig. 3b).

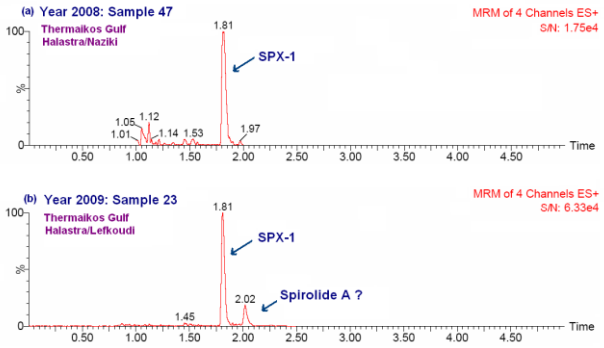

Fig. 3. Chromatograms of mussel samples from Halastra in Thermaikos Gulf analyzed by UPLC-MS/MS (GYM & SPX channel): (a) Sample 47, April 2008 (4 μg kg^{-1}), (b) Sample 23, January 2009 (26 μg kg^{-1}). Method LOQ: 2 μg kg-1.

The intraperitoneal LD_{50} of SPX-1 is reported to be 6.9 μg kg^{-1} mouse body weight, indicating a limit of detection (LOD) for this compound in the MBA of about 5.6 μg kg^{-1} shellfish (Munday 2008). This explains the positive MBA results obtained in the present study in comparison to the concentrations determined by UPLC-MS/MS. The maximum SPX-1 concentration of 26 μg kg^{-1} is within the typical concentrations of 20-50 μg kg^{-1} reported in shellfish for 13-desmethyl SPX C (EFSA, 2010). In the chromatogram of sample 23/2009 (Fig. 3b) another peak eluting at 2.02 min was evident, which could be an isomeric compound of SPX-1, the spirolide A, which had the same m/z ratio and therefore, similar fragmentation pathway. However, quantification of this compound was not possible because there exists no commercial standard available for this toxin. The possibility of the presence of spirolide A in Greek shellfish should be further investigated since this toxin can be produced by hydrolysis of a shellfish metabolite, spirolide E, which is inactive in the mouse bioassay (Sleno *et al.* 2004).

Conclusions

This study reports for the first time 13-desmethyl spirolide C (SPX-1) and possibly spirolide-A in Greek shellfish and its association with the potentially toxic dinoflagellates *A. ostenfeldii /peruvianum*. Generally, symptomatology in the mouse bioassay test for lipophilic toxins was a useful indicator of spirolides' presence in shellfish samples. On the other hand, the UPLC-MS/MS method used was very sensitive (LOQ: 2 μg kg^{-1} shellfish tissue**)** and could be employed for the confirmation of SPX-1 presence in shellfish.With regard to microalgal species identification, the establishment of cultures is considered necessary to further determine the identity and toxicological properties of the spirolide-producing dinoflagellates in Greek waters; furthermore, the combination of both morphological and molecular analyses of isolates from the type localities, is necessary to better assess the validity of *A. ostenfeldii* and *A. peruvianum* as separate taxa.

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An investigation into solid phase adsorption toxin tracking (SPATT) for the detection of domoic acid, the amnesic shellfish poison, in seawater

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Abstract

Previously proposed as a tool to facilitate monitoring of lipophilic shellfish toxins (LSTs), solid-phase adsorption toxin tracking (SPATT) was assessed for its capability to adsorb and recover domoic acid (DA), an amnesic shellfish toxin (AST) responsible for site closures in Scottish waters. SPATT bags containing SP-700 resin beads were deployed in Loch Ewe (Scotland) on a weekly basis in conjunction with sampling of phytoplankton and local bivalve shellfish. During four sampling periods throughout summer 2009, an automated water sampler was deployed at Loch Ewe, flushing daily seawater through SPATT cartridges containing SP-700 resin and collecting water samples for phytoplankton identification. SPATT resin samples were extracted and analysed by LC-MS/MS and ELISA. Although no direct correlation could be drawn between the concentration of DA in SPATT extracts and the phytoplankton community present in water samples, significant variations of toxin concentration could be observed between sampling periods. Importantly, the detection of DA in all SPATT cartridges proved that although a limited amount of DA could only be desorbed from the resin after initial adsorption, the quantity recovered was enough to show daily variations of DA concentration in seawater. Results suggest that SPATT cartridges may possibly be used as an early warning tool to inform shellfish farmers of an incoming or declining toxic event and could potentially help better understand AST events.

Introduction

Domoic acid (DA) is a naturally occurring toxin produced by pennate diatoms, mainly from several species of the *Pseudo-nitzschia* genus. Accumulation of DA in shellfish through filter feeding on toxic producing *Pseudo-nitzschia* can lead to Amnesic Shellfish Poisoning (ASP) intoxication in humans, which needs to be closely monitored to protect consumers health. Despite the current monitoring methods involving weekly phytoplankton monitoring and analysis of shellfish samples for biotoxins, accurate prediction of the evolution of a toxic event is still not possible. This leaves shellfish farmers in great uncertainty when harvesting time starts. With the aim of increasing our understanding of harmful algae dynamics, we used solid-phase adsorption toxin tracking (SPATT) which was developed in New Zealand in 2004 (MacKenzie et al. 2004). This technique is based on the theory that significant amounts of toxin are dissolved in seawater when toxic algae are present in the water column. *In vivo* adsorption of the toxin onto synthetic resin and subsequent extraction and analysis constitutes a powerful tool to assess variation of toxin concentrations in the water column. Previous studies have shown the resin's capability to track rise and fall of lipophylic toxins concentrations in the water column (Turrell et al. 2007, Fux et al. 2009), but to our understanding, no reports of such findings with DA have yet been published. After testing several adsorbent candidates (results not shown in this report), a synthetic resin (Sepabeads® SP-700, Mitsubishi

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Chemical Corporation) was selected for its ability to adsorb DA dissolved in seawater and was then used in SPATT field deployments.

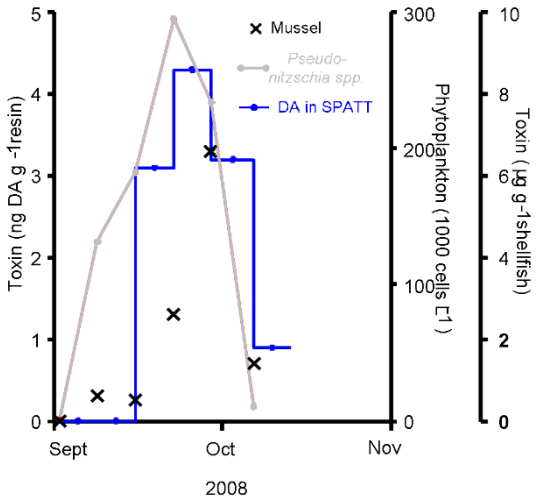

Fig.1. Domoic acid in mussels and SPATT bags compared with Pseudo-nitzschia, Sep-Oct 2008.

Material and Methods

Nylon mesh bags (95 µm) filled with SP-700 resin (12 g) were suspended to a weighed line at a depth of 7 m on the West coast of Scotland at Loch Ewe (57°49.38'N - 5°37.03') and were deployed and retrieved on a weekly basis from 2008. At the same time, water and shellfish samples were taken for further phytoplankton and biotoxins analysis. Phytoplankton taxa were counted and identified using the Utermöhl method (Kelly and Fraser, 1998). SPATT bags and shellfish samples were stored frozen (-20 °C) prior to toxin extraction and analysis. SP-700 resin (0.8 g) was used to fill 1 mL cartridges (RezorianTM Tube Kit, Supelco) which were installed onto an automated water sampler (Aqua Monitor WM-2, EnviroTech LLC) programmed using a sampling script. The water sampler, deployed at Loch Ewe at a depth of 7 m, was programmed to flush 3.6 L of seawater through a cartridge every day at noon. At the same time, a discrete water sampler (180 mL) was collected using the auto-sampler and stored in EVA/EVOH bags containing 1 mL of Lugols' iodine for preservation. From 07/05/09 to 14/05/09, mussels samples were collected daily from a nearby location and stored in a freezer prior to further analysis. Resin contained in the SPATT bags was first removed and washed with 300 mL of ultrapure water, before an aliquot (5 g) was vortex mixed with 10 mL of 50 % v/v MeOH/water. The solvent was left to soak the resin for 30 min before being eluted with an extra 90 mL of 50 % v/v MeOH:water. A 10 mL aliquot was evaporated to dryness and re-suspended in 50 % v/v MeOH:water.

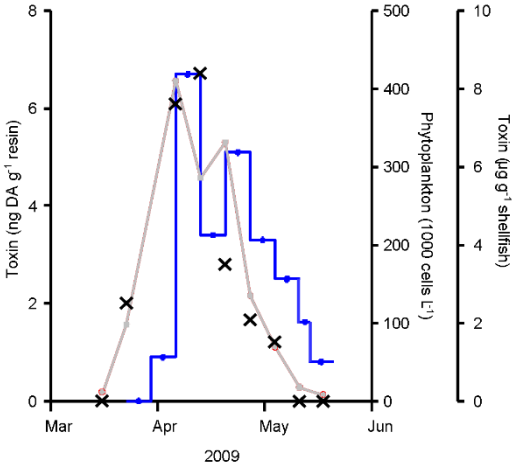

Fig.2. Domoic acid in mussels and SPATT bags compared with Pseudo-nitzschia, Mar-May 2009.

Resin from SPATT cartridges was extracted following a similar protocol with different volumes: washed with 30 mL of DI water (Mili-Q), vortex mixed with 2.5 mL of 50 % MeOH and eluted with 17.5 mL of 50% MeOH. The whole portion (20 mL) was evaporated to dryness and re-suspended in aqueous methanol. Shellfish aliquots (~2 g) were mixed in a polypropylene centrifuge tube (50 mL) with 50 % v/v MeOH:water for 1 min using an Ultra-Turrax (13,000 rpm). Extracts were then centrifuged (3,000 rpm for 10 min) and the supernatants were filtered (0.2 µm PVDF syringe filter). Finally, extracts were analysed using LC-MS/MS (Hess et al. 2005) and ELISA immunoassays (Kleivdal et al. 2007).

Results and discussions

DA was successfully detected in SP-700 SPATT bags, even when levels in corresponding mussels were lower than regulation (~6.5 mg/kg, compared to 20 mg/kg limit set by EC). The peak of DA concentration was reached simultaneously in mussel and the SP-700 resin (see Figs. 1, 2), a week after the maximum abundance of *Pseudo-nitzschia spp*. was detected in the water column.

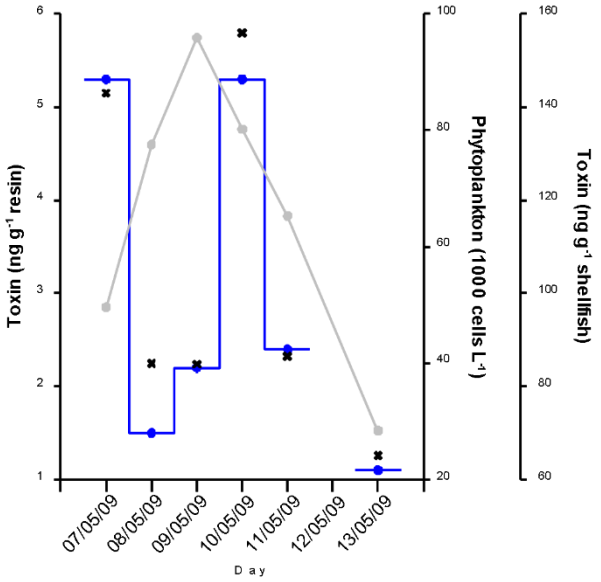

Fig.3. Domoic acid in mussels and SPATT bags compared with Pseudo-nitzschia, in a daily sample series 7 to 14 May 2009.

The volume of seawater (3.6 L) flushed through the SPATT cartridges was sufficient to allow the adsorption of quantifiable amounts of DA onto the resin and variations of dissolved DA present in the water column could be observed between two consecutive days. The highest amount of DA recovered from a SPATT cartridge coincided with highest concentration of DA in a mussel sample collected on the same day. This happened to be the day after the peak of *Pseudo-nitzschia spp.* (Fig.3). Correspondence between DA concentration extracted from the resin and the *Pseudo-nitzschia spp.* counts did not occur every time. Indeed, (shown in Fig. 4), the peak of DA in the cartridge (21 ng/g) was detected when counts of *Pseudo-nitzschia spp.* were low in water samples (18,000 cells/L).

Conclusion

SPATT devices containing SP-700 resin have the ability to trace the variations of concentration of DA dissolved in the water column, even when levels of toxic phytoplankton being present in the water column are low. In combination with DA quantification using ELISA testing, it has the potential to be a relatively quick and simple method which could help shellfish farmers in their harvesting management. Indeed, the use of SPATT cartridges flushed with seawater is able to identify daily variations of DA in the water column.

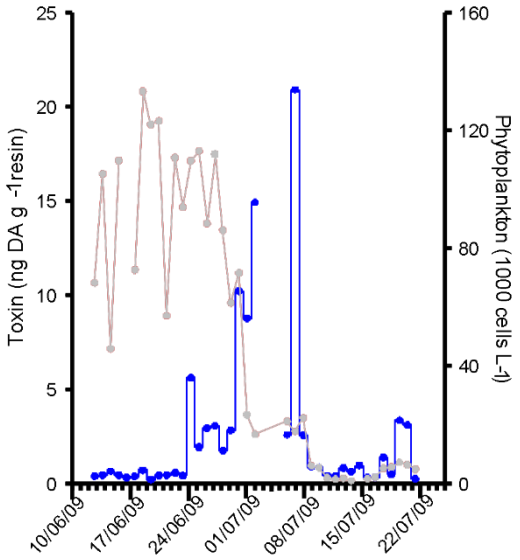

Fig.4. Domoic acid in SPATT bags compared with Pseudo-nitzschia, June-July 2009.

Acknowledgements

We thank P. MacLachlan and J. Grant for assistance with deployment and retrieval of SPATT. This work was financed by the Scottish Government Marine Directorate, the 6th Framework Programme of the European Community (SPIES-DETOX 0302790-2) and the NPP-WATER project.

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Update on the Application of Solid Phase Adsorption Toxin Tracking (SPATT) for Field Detection of Domoic Acid

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Abstract

Recent publications have identified the analysis of phycotoxins in sentinel shellfish as a problematic tool for environmental monitoring purposes. Domoic acid (DA), produced by some species of *Pseudo-nitzschia*, can remain undetected in sentinel shellfish stocks during toxic blooms and subsequent marine bird and mammal mass mortality. Solid Phase Adsorption Toxin Tracking (SPATT) has previously been described as a new tool useful for monitoring of lipophilic toxins; a methodology extending its applicability towards the hydrophilic phycotoxins [DA and paralytic shellfish toxin (PST)] has been recently described. The original method development included 18 months of weekly SPATT deployments in Monterey Bay, California (U.S.A.). Here, we update the original results with new field data from 2010 (Jan – Nov), presenting DA-signaling from SPATT deployments in conjunction with concurrent, traditional monitoring practices. The sensitivity of SPATT to the onset of a toxic *Pseudo-nitzschia* bloom (spring 2010) is presented for comparison to the sensitivity in the original method. Unlike in years for which SPATT-DA signaling has been described, toxigenic *Pseudo-nitzschia* abundance remained elevated throughout 2010, allowing for a unique evaluation of SPATT-DA performance during an exceptionally sustained toxic event.

Introduction

Solid Phase Adsorption Toxin Tracking (SPATT) is a passive sampling technology to detect lipophilic phycotoxins as a 'man-made' sentinel mussel, enabling monitoring of lipophilic toxins in coastal waters without the cost and complications of shellfish analysis (MacKenzie *et al.* 2004, Fux *et al.* 2009, Rundberget *et al.* 2009, MacKenzie 2010). The SPATT technology has most recently been extended for the detection of the hydrophilic phycotoxin domoic acid (DA produced by toxigenic species of *Pseudo-nitzschia* (Lane *et al.* 2010). The method development of SPATT for its application with DA included 18 mo (July 2007-Dec 2009) of weekly-rotation field deployments at Santa Cruz Municipal Wharf (SCMW) in northern Monterey Bay. As described, 3 adsorptive synthetic resins identified as SPATT-DA candidate resins (HP20, SP700, SP207) were sealed separately into Nitex mesh using a plastic bag sealer. The SPATT 'bags' were secured to a weighted rope and suspended in the water column alongside sentinel mussels sampled weekly as part of the California Department of Public Health (CDPH) Marine Biotoxin Monitoring Program. After one week the bags were recovered (and replaced with new bags), transported to UCSC on blue ice, and extracted; the extract was then analyzed for DA (Lane *et al.* 2010). Concurrent traditional sampling techniques included (a) collection of integrated-depth whole water (0, 1 and 3 m) for cell counts of toxigenic *Pseudo-nitzschia* (*P. australis* and *P. multiseries*) and analysis of DA (particulate and dissolved), and (b) collection of a net [5 x 10 ft vertical tow for assessment of *Pseudo-nitzschia* (genus) relative abundance within the phytoplankton by light microscopy. Two significant toxic events occurred during the 18-month period; in both, DA detection by SPATT preceded shellfish toxicity by 7-8 weeks and afforded unique advance warning of the toxic bloom events. Here we update the initial performance report described as part of the development of SPATT

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for application with DA. The times series described in Lane *et al.* 2010 is updated for Jan -Nov 2010 with data from field deployments of SPATT at SCMW and sampling of *Pseudo-nitzschia* (genus) relative abundance, toxigenic *Pseudo-nitzschia* cell counts, particulate DA, and sentinel mussels. We describe two refinements since description of the original extraction protocol: (1) extraction of SPATT as free resin, (2) same-day preparation of ammonium acetate reagent for use in SPATT extractions.

Methods

Continued weekly field deployments of SPATT and concurrent sampling of *Pseudo-nitzschia* relative abundance, toxigenic *Pseudo-nitzschia* cell counts, particulate DA and sentinel mussels were conducted as described in Lane *et al.* 2010. High resolution (daily) sampling of particulate DA was carried out 6-21 Oct across 2 wk-long SPATT rotations, allowing for comparison between the two approaches (discrete daily grab sampling of particulate toxin vs week-integrative SPATT deployment). The treatment of SPATT resins recovered from the field was consistent with Lane *et al.* 2010 until 8 Feb 2010; from that rotation all SPATT resins were extracted in-column as free resins. The extraction of SPATT as free resin is possible with adjustment of the original extraction protocol, as follows: the SPATT bags are recovered from the seawater, immediately rinsed with Milli-Q (2 x ~200 mL) and transported to the lab on blue ice. One side of the bag is cut open and the resin is Milli-Q-rinsed out of the bag into an extraction column. During this process the extraction column spigot is in the 'closed' position, so that the volume of Milli-Q used in resin transfer can be held constant (~10 mL). The resin is then extracted as in Lane *et al.* 2010, with the specification that the 1M ammonium acetate in 50% MeOH reagent is prepared the same day it is used for extraction.

Results and Discussion

DA detection data from week-integrative SPATT deployments at SCMW are shown in Fig 1, with concurrent weekly data for *Pseudo-nitzschia* relative abundance, toxigenic cell abundance, particulate DA, and DA in mussels. *Pseudo-nitzschia* (genus) and toxigenic species were observed every week from 31 Mar onward, with few exceptions. Particulate DA tracked closely with toxigenic cell counts, although detection of particulate DA was sporadic in the weeks prior to (and zero the week of) the initial observation of toxigenic *Pseudo-nitzschia* on 14 Apr. Shellfish data from UCSC indicate low-level DA in sentinel mussels for all samples collected in 2010, and elevated DA (>1 ppm) in sentinel mussels during the height of the bloom (5 May-8 Sept) with only a single exception (28 Jul; 0.679 ppm).

DA was detected in the first SPATT deployments of the year, from 29 Dec 2009 – 12 Jan 2010 (Figure 1). SPATT and sentinel mussel monitoring was

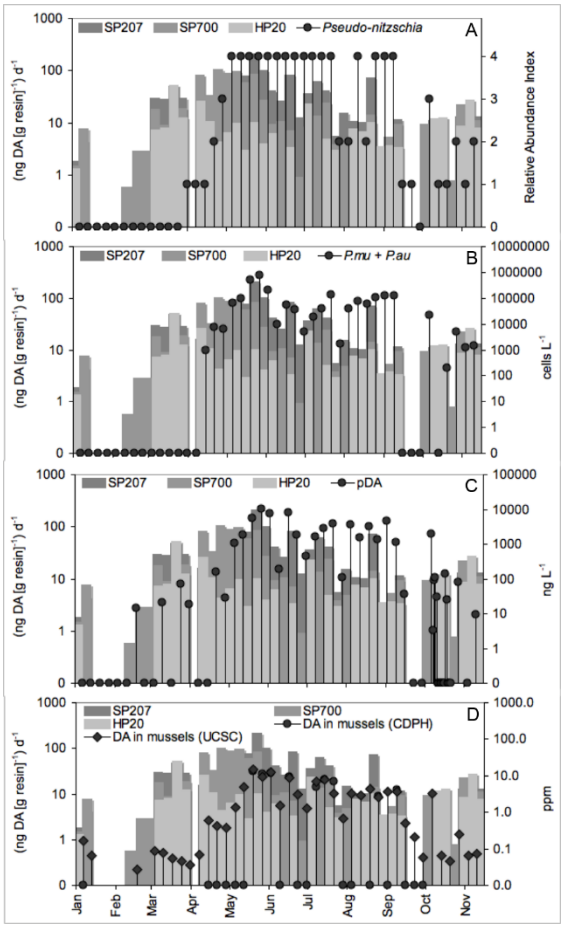

Fig 1. Domoic acid (DA) detection by week-integrative deployments of SPATT (HP20, SP700, SP207) at Santa Cruz Municipal Wharf, with the following weekly data from discrete sample collections: *Pseudo-nitzschia* (genus) relative abundance within the phytoplankton [0, 1=1% or less, 2=1-9%, 3=10-49%, 4=50% or more] (A), toxigenic *Pseudo-nitzschia* cell abundance (*P. australis* + *P. multiseries*) (B), particulate DA (C), and DA in mussels (D).

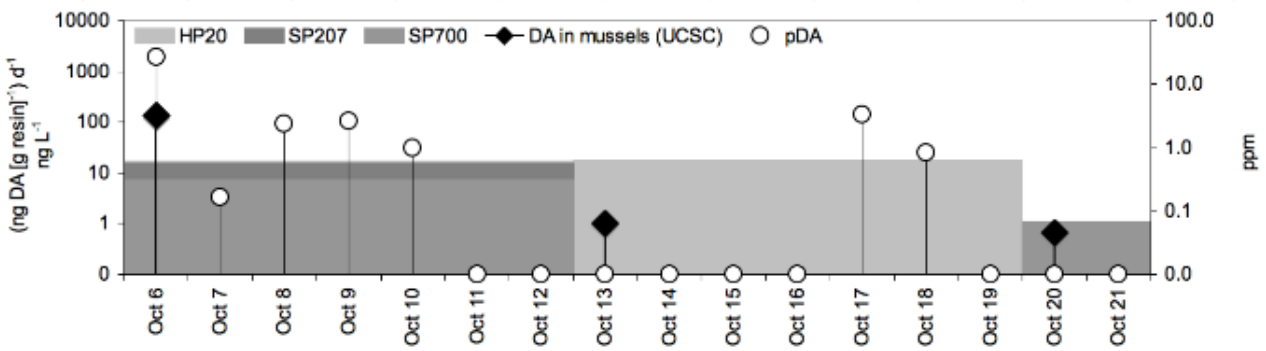

Fig. 2. Domoic acid (DA) detection by week-integrative deployments of SPATT (HP20, SP700, SP207) at the Santa Cruz Municipal Wharf, with DA in mussels (sampled weekly) and particulate DA (sampled daily).

disrupted from 13 Jan – 09 Feb due to loss of the wharf deployment platform in a winter storm. DA was detected by SPATT upon the resumption of weekly deployments on 10 Feb, and was detected in every deployment rotation thereafter until 08 Sept, with a single exception (rotation 31 Mar – 06 Apr) which coincided with two weekly samples in which particulate DA was not detected (0.00 ng L^{-1}; 07 and 14 Apr). The continual detection of DA by SPATT began on 07 Apr; this signal preceded the observation of toxigenic *Pseudo-nitzschia* by 1 week, the detection of particulate DA and *Pseudo-nitzschia* (genus) at relative abundance >1% by 2 weeks, the recognition of bloom onset (toxigenic *Pseudo-nitzschia* abundance >10,000 cells L^{-1}) and impending shellfish toxicity (>1 ppm) by 4 weeks, and the detection of shellfish toxicity by the regulatory agency (CDPH) by 6 weeks. SPATT DA with daily-sampled particulate DA and weekly-sampled DA in sentinel mussels are presented for 06-21 Oct (Figure 2). Particulate DA was variable from day-to-day, ranging from zero to 1935 ng L^{-1}. In the most exceptional example of this variability, particulate DA fell to 3.22 ng L^{-1} on 07 Oct after its highest measurement the day before (1935 ng L^{-1}). DA detection by SPATT was moderate for the first two week-long deployment rotations (06-13 Oct and 13-20 Oct), then became relatively weak [(0.78 ng g^{-1}) d^{-1} by SP700]. Weekly samples of DA in sentinel mussels indicated low and decreasing shellfish toxicity during this period (3 ppm on 06 Oct; <1 ppm on 13 & 20 Oct). These data illustrate the utility of SPATT, which, as an integrative passive sampling technology, can provide a monitoring perspective that is less susceptible to the variability introduced through discrete sample collections (i.e. SPATT data inform the collector of toxin encounters across the deployment period, while discrete-sample data inform the collector of conditions in the water parcel that was collected). However, since SPATT is not approved for regulatory decisions, it remains complementary to traditional sampling.

Acknowledgements

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Production of Stigmasterol by the Harmful Marine Dinoflagellate *Karenia brevis*

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Abstract

Karenia brevis is an important bloom-forming dinoflagellate that has been shown to produce two primary sterols, gymnodinosterol ((24*R*)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol) and brevesterol (its 27-*nor* isomer). We present the identification of a previously undetected sterol as stigmasterol (24*S*-ethylcholest-5,22E-dien-3β-ol), a common plant sterol that is often found in other algal classes but which is, to our knowledge, rarely found in dinoflagellates.

Introduction

In a recent study on sterol biosynthesis in *Karenia brevis*, Leblond *et al*. (in press) found that exposure to the fungicide fenpropidine led to production of two tri-unsaturated intermediates, 4α-methyl-5α-ergosta-8,14,22-trien-3β-ol and 5α-ergosta-8,14,22-trien-3β-ol. Gas chromatography/mass spectrometry (GC/MS) examination of acetate derivatives of untreated control culture sterols revealed the presence of an unidentified $C_{29:2}$ sterol (approx. 39% of total sterol complement) that eluted after gymnodinosterol and brevesterol, two major sterols produced by *K. brevis* (Leblond & Chapman 2003; Giner *et al*. 2003). This $C_{29:2}$ sterol was not present in the chromatogram of trimethylsilyl ether (TMS) derivatives, indicating that it coeluted with gymnodinosterol, and was not present in GC/MS analyses of the acetate derivatives of fenpropidine-treated culture sterols, indicating that fenpropidine somehow inhibited its production. Reexamination of the GC/MS chromatograms of the acetate derivatives from Leblond & Chapman (2002) revealed the presence of the unidentified $C_{29:2}$ sterol at an average relative percentage of approxately 12% for the free sterols of three *K. brevis* isolates.

The objective of this manuscript is to identify this $C_{29:2}$ sterol and discuss its taxonomic relevance.

Materials and Methods

Culture replicates of *K. brevis* (Davis) Hansen et Moestrup strain Piney Island [referred to as FMRI in Leblond & Chapman (2002)] were grown to exponential phase in 2 L of L1 medium treated with 15 µL of dimethylsulfoxide (DMSO) to replicate the carrier solvent conditions used in Leblond *et al*. (in press). Cultures were grown under conditions that replicated as closely as possible those of Leblond & Chapman (2000); however, these replicates were grown in a different incubator with a different seawater supply. Furthermore, *K. brevis* Piney Island has been in continuous culture for approximately ten years in our laboratory, and the effect of absolute culture age on sterol composition is unknown. Cells were harvested and extracted in accordance with Leblond & Chapman (2000).The total lipid extract was saponified, derivatized to form TMS or acetate derivatives of sterols according to Leblond & Chapman (2002), and analyzed via GC/MS on a Finnigan GCQ (Thermo Finnigan, Waltham, MA, USA) according to Leblond *et al*. (in press). Relative retention times (RRT) to cholesterol were calculated according to Jones *et al.* (1994). Authentic stigmasterol (Sigma-Aldrich, St. Louis, MO, USA) was utilized for comparative GC/MS and NMR analyses.

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Isolation via high-performance liquid chromatography (HPLC) and characterization of the unidentified C_{29} sterol via NMR was performed as in Leblond *et al*. (in press). Proton NMR spectra were obtained using an ECA 500-MHz FT NMR spectrometer (JEOL, Peabody, MA, USA). Freshly purchased $CDCl_3$ (D, 99.8%, Cambridge Isotope Laboratories (Andover, MA, USA) was used to prepare all samples in 528-PP NMR tubes (Wilmad, Buena, NJ, USA). Chemical shifts (δ) were referenced to the residual proton resonance of the solvent, which was set to 7.240 ppm.

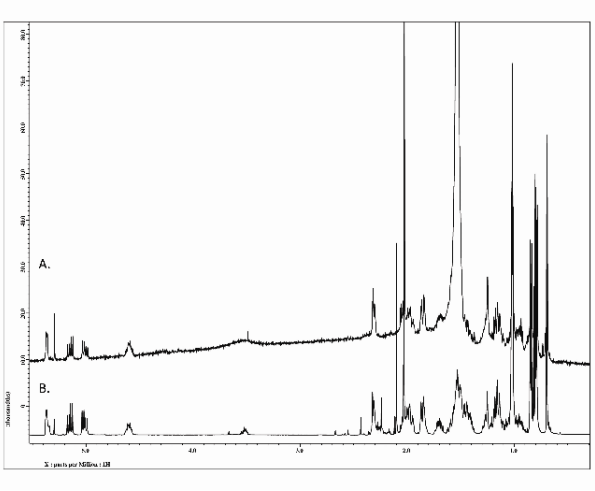

Fig. 1. Proton NMR spectra of A.) unidentified $C_{29:2}$ sterol and B.) authentic stigmasterol (as sterol acetates). The peak at 5.28 ppm is residual methylene chloride from sterol acetate derivative processing.

Results and Discussion

K. brevis produced three major sterols with the following relative percentage values and retention times for acetate derivatives: brevesterol (37.2±1.2%, 44.76 min., RRT of 1.082), gymnodinosterol (23.6±2.3%, 46.96 min., RRT of 1.531), and an unidentified $C_{29:2}$ sterol (39.2±2.1%, 47.77 min., RRT of 1.723). Note that minor sterols, such as those observed by Giner *et al*. (2003), are not reported. The $C_{29:2}$ sterol did not appear as a distinct peak in the chromatograms of TMS derivatives. Stigmasterol-TMS was found to have the same retention time (45.65 min.) as gymnodinosterol-TMS, and a coinjection of the two revealed one uniform peak (data not shown). Stigmasterol-acetate also had the same retention time as the $C_{29:2}$ sterol, and a coinjection of stigmasterol-acetate with the acetate of the $C_{29:2}$ sterol revealed one uniform peak (data not shown). The unidentified $C_{29:2}$ sterol displayed a mass spectrum that was almost identical in fragmentation pattern to that of authentic stigmasterol (data not shown). Comparison of the vinyl and methyl regions to those of authentic stigmasterol (Fig. 1) via proton NMR revealed them to be the same compound; assignment of protons is provided in Leblond *et al*. (2005). The presence of stigmasterol in dinoflagellates is rare. It is found as a minor component of the sterols of *Glenodinium hallii* (Alam *et al.* 1981), cultured endosymbionts from marine invertebrates (Withers *et al*. 1982), and a strain of *Amoebophrya* sp. that infects *Karlodinium veneficum* (Place *et al*. 2009).*K. brevis* possesses a plastid of presumed haptophyte ancestry (Tengs *et al*. 2000). The sterols of haptophytes have been shown to have stigmasterol (or its epimer poriferasterol) as a major component (Conte *et al*. 1994; Véron *et al*. 1996; Ghosh *et al*. 1998). Future studies should examine the underlying genetics of sterol biosynthesis in *K. brevis* and a haptophyte(s) before a conclusion can be made about a potential haptophyte origin for stigmasterol in *K. brevis*.

Acknowledgments

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.

Potency of pinnatoxins produced by dinoflagellates isolated from New Zealand and South Australia

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Abstract

Vulcanodinium rugosum., the scrippsielloid dinoflagellate producer of pinnatoxins, was isolated from surface sediments collected from Northland, New Zealand, in summer 2008/2009. The dinoflagellate was isolated and cultured predominantly in cyst form, with the New Zealand isolates producing pinnatoxins E and F and Australian isolates predominantly G and less E and F. *V. rugosum* killed sea urchin and paua larvae and caused morbitity in sea slug larvae; Pacific oyster and *Artemia salina* larvae became slightly moribund. Greenshell™ mussel larvae were unaffected. Depuration rates of 50% of pinnatoxin E and F from Pacific oysters were 14.5 and 16 d respectively. Crude extracts of mass cultures of the New Zealand and Australian isolates tested for toxicity in mice by intraperitoneal injection, gavage and voluntary consumption resulted in toxicity ratios of 1.0: 1.8: 4.5 and 1.0: 2.9: 7.8 respectively.

Introduction

Pinnatoxins E, F and G were newly characterized from razor clams (*Pinna bicolor*) and Pacific oysters (*Crassostrea gigas*) from South Australia in 2007 (Selwood et al., 2010) and a year later pinnatoxins E and F were detected retrospectively in Pacific oysters collected and archived between 1994 and 2007 from Northland, New Zealand (McNabb et al., 2008; Selwood et al., 2010). The toxins caused the death of mice during regulatory biotoxin testing, which led to precautionary harvesting closures for shellfish in both South Australia and New Zealand. The scrippsielloid producer of pinnatoxins E and F, *Vulcanodinium rugosum*, was first identified from Rangaunu Harbour sediments in 2009 (Rhodes et al., 2010a,b,2011a), and the producer of pinnatoxin G was cultured from South Australian sediments in 2010 and determined by DNA sequence analysis to be a similar species (Rhodes et al. 2011b). The dinoflagellate mainly occurs in cyst form, and cysts have been shown to divide asexually (Rhodes et al. 2011a). Mass cultures of cysts were grown to compare DNA sequences and morphology and for shellfish feeding and depuration studies, invertebrate larval assays, and toxicity testing. The acute intraperitoneal toxicity of some purified pinnatoxins has been established (Munday, 2008; Selwood et al., 2010), but there is no information on their oral toxicity. The extracts of mass cultures of the pinnatoxin E and F producing New Zealand strain, CAWD163, were of interest as toxicity in mice was similar whether by intraperitoneal injection, gavage or voluntary consumption (Rhodes et al. 2010a). The toxicity of extracts from the pinnatoxin G producing strain, CAWD180 isolated from Australia, was also determined to allow comparison with the pinnatoxin E and F producer.

Methods

Vulcanodinium rugosum, cultures are maintained in the Cawthron Institute Collection of Micro-algae (CICCM). Mass cultures were grown in seawater-based K medium (Keller et al., 1987) in Erlenmeyer flasks or horizontal plastic bags, at 25ºC and 100 µmol $m^{-2}s^{-1}$ photon flux (14:10 h L:D). Light microscope images: Inverted epifluorescent microscopes (Olympus). Dinoflagellate cultures were extracted using the UltraClean™ Soil DNA isolation kit (MoBio Laboratories Inc., CA, USA), and LSU ribosomal DNA and ITS regions were amplified and phylogenetic trees created as described previously (Rhodes et al. 2011a).

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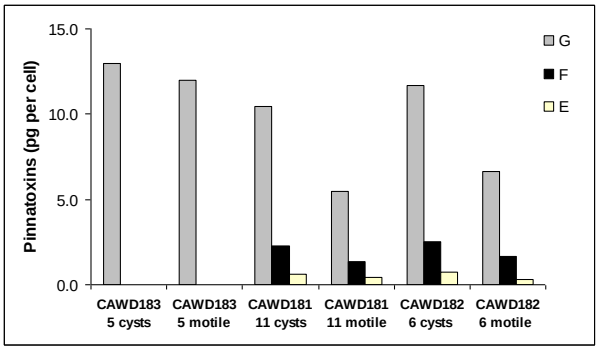

Fig. 1. Pinnatoxin E, F and G production by Australian isolates of *Vulcanodinium rugosum*, comparing cysts and motile cells.

Cultured *V. rugosum*, cysts for LC-MS analysis were centrifuged, pellets extracted with methanol and pinnatoxin analyses completed as described (Rhodes et al. 2010b). Pinnatoxins E, F and G isolated from South Australian shellfish (Selwood et al., 2010) were the reference standards.

Larvae (6-10 of each species in triplicate) were transferred to 24-well tissue culture plates (Becten Dickinson, USA) and *V. rugosum* cysts added at specified concentrations. *Artemia salina* larvae were tested at age 48 h. Greenshell™ mussel, Pacific oyster, sea urchin and paua larvae (raised at the Glenhaven Hatchery, Nelson), were 6 d old. Sea slug larvae were raised at Cawthron Inst. Larvae were observed for 24 h, and scored to indicate if they were healthy, moribund or dead, and toxicity estimated over time. To determine the toxicity of *V. rugosum* from Australia, freeze-dried pellets from cultures of CAWD180 (3.1 $x10^{-6}$ cells) were extracted with ethanol as described previously (Rhodes et al., 2010a). Residue (29.8 mg) was taken up in methanol and aliquots evaporated under N, then extract taken up in 1% Tween 60 in saline and injected intraperitoneally (i.p.) at various dose levels into Swiss albino mice (18-20 g). LD_{50} values were determined by the up-and-down procedure (OECD, 2006). Uptake and depuration of pinnatoxins were assessed for Pacific oysters (from Glenhaven Hatchery, Nelson) by feeding with *P. honu* CAWD167: (i) 10 young oysters (shell length 3-5 cm, av. 0.9 g w.w. minus shell) were fed cysts concentrated by settling and removal of supernatant then agitated to re-suspend. Seawater was exchanged daily. Four oysters were sampled (0 h) as controls. Oysters were fed throughout day for 5 d and sampled for analysis on days 3 and 5. Cell densities and toxin conc. of cultures were measured daily. Controls, and oysters immediately prior to depuration, were fed *Isochrysis* sp. and sampled biweekly. (ii) Uptake and depuration in mature oysters (shell length 6-8cm, av. 11.5 g w.w. minus shell): 12 oysters treated as (i) with one micro-algal addition daily for 3 d. (iii) 10 oysters treated as (ii), but cysts not concentrated and 2 oysters sampled weekly.

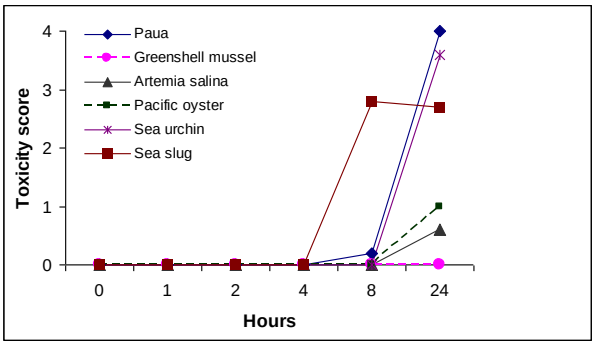

Fig. 2. Time to death of invertebrate larvae in presence of *V. rugosum* cysts containing 120 and 410 ng per ml pinnatoxins E and F (1 ml addition in triplicate). Scale: 0, healthy; 1, settled; 2, immobile; 3, moribund; 4, dead.

Results and Discussion

Pinnatoxins appear widespread globally, with reports in shellfish from Japan (Chou et al. 1996; Takada et al. 2001), Norway (Miles et al. 2009), Australia and New Zealand (Selwood et al. 2010). Pinnatoxins were also detected in sediments from Japan (LCMS of surface sediments from eel grass beds in Okinawa, by Dr Suda, Univ. Ryukyus, Nishihara, Okinawa), from Franklin Harbour, South Australia, and Rangaunu Harbour, New Zealand (Rhodes et al. 2010b, 2011b). *V. rugosum* cysts were cultured from New Zealand and Australia, and were morphologically and genetically similar. All isolates produced pinnatoxins (LCMS), although the 11 clonal New Zealand isolates tested did not produce pinnatoxin G and pinnatoxins E and F concentrations ranged from 0-6.5 and 0.3-39.0 pg/cell respectively. Cysts produced predominantly pinnatoxin F, whereas motile cells produced predominantly E, although as F is readily oxidised to E this may an artifact of extraction. South Australian isolates produced pinnatoxin G and lesser concentrations of E and F, ranging from 28-52, 1-3, and 8-14 pg/cell respectively, and cysts produced more G than motile cells (Fig. 1).

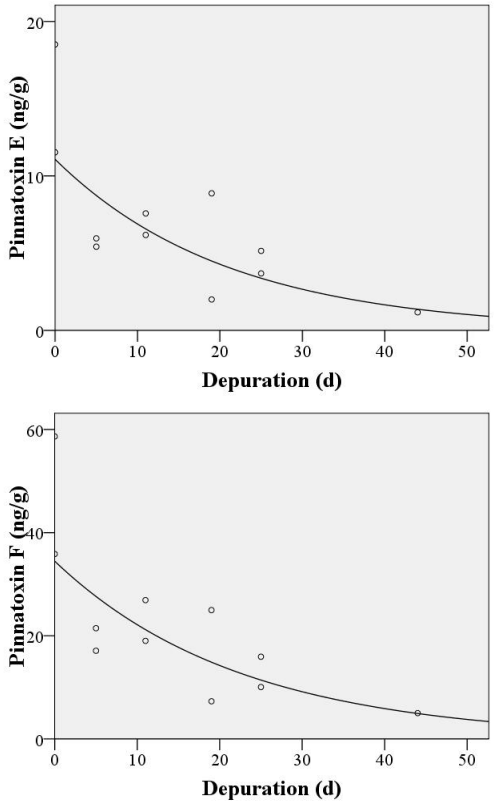

Fig 3. Depuration of pinnatoxins E (L) and F (R) from Pacific oysters following uptake of 14.5 and 47 ng g^{-1} respectively. An exponential model ($y = e^{ax} + b$) was fitted by least squares regression; R = 0.816 and 0.830 ($p<0.01$) and B = -0.048 and -0.044 ($p<0.01$) for E and F.

The effect of pinnatoxins on invertebrate larvae varied (Fig.2): Greenshell™ mussel larvae were unaffected by 1 ml additions of *V. rugosum* cells (120 and 410 ng/ml pinnatoxins E and F respectively). However, 100% of sea urchin and paua and 60% of sea slug larvae, were killed between 8 and 24 h. Pacific oyster and *Artemia salina* larvae became moribund within 24 h; 20% died. Young oysters died when fed high concentrations of cysts over 5 d (4.8 $x10^6$ per d); the conc. of pinnatoxin E and F determined in these oysters was 30.6 and 97.9 ng g^{-1} respectively. 11% of mature oysters died with an uptake of 11.6 and 10.5 ng g^{-1} pinnatoxin E and F, and the balance died within the next 7 d despite feeding having stopped. Deaths were probably due to acute exposure to pinnatoxins released into the seawater when cysts were concentrated and agitated. In later feeding trials, with no concentration/agitation, uptake and depuration was achieved and depuration of 50% of pinnatoxins E and F took 14.5 and 16 d respectively (Fig. 3). Oysters survived a maximum conc. of 14.5 and 47 ng g^{-1} pinnatoxin E and F respectively at the time of feeding cessation. Controls were toxin free. Toxicity of crude culture extracts of *V. rugosum*, tested in mice by i.p. injection, gavage and voluntary consumption, exhibited little difference between i.p. and oral administration (Rhodes et al. 2010, 2011). Ratios between different extract administrations for New Zealand and Australian isolates were 1.0: 1.8: 4.5 and 1.0: 2.9: 7.8 respectively. The toxins caused respiratory paralysis, as do purified pinnatoxins and other cyclic imines (Munday 2008; Selwood et al. 2010).

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Development of a new HPLC-FLD method for the determination of DSP toxins using the fluorescence tagging reagent 3-bromomethyl-7-methoxy-1, 4-benzoxazin-2-one

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Abstract

A new sensitive HPLC method for the determination of the diarrheic shellfish toxins, okadaic acid (OA) and dinophysistoxin-1 (DTX-1) in mussel tissue was developed and validated. The extraction of the analytes from the dried (lyophilized) mussel samples was achieved by using acetonitrile, followed by solid phase extraction of the mussel extract on C18 cartridges. The eluant containing the toxin analytes was then derivatized, using the sensitive reagent 3-bromomethyl-7-methoxy-1, 4-benzoxazin-2-one, with the addition of N-ethyldiisopropylamine as catalyst base. The derivatives were further cleaned up by normal phase SPE using alumina cartridges and the toxins were determined by HPLC on a C18 analytical column using acetonitrile:water gradient elution and fluorescence detection (excitation at 345 nm – emission at 440 nm). Derivatization reaction was optimised before application in the method. Calibration was performed by the standard addition method and was linear in a typical analytical range of 160-400 ng gr^{-1} wet tissue.

Introduction

Shellfish species as mussels are very often contaminated with various types of lipophylic toxins which are produced by phytoplankton species. Consumption of contaminated shellfish can cause severe intoxications. Okadaic acid and dinophysistoxin-1 are included in the okadaic acid group of DSP toxins and are responsible for gastrointestinal disorders in humans. Legislation established for the protection of the consumers is referred to 160 μg Kgr^{-1} of whole edible shellfish tissue which is the sum of all relevant OA and DTXs. Chemical analysis of these toxins used to be performed by the analytical technique of HPLC-FLD, until LC-MS was successfully implemented for this analysis. In the case of HPLC-FLD analysis the free carboxyl group of the toxins are pre-column and off-line derivatizised so as to give fluorescent signals. There are two challenges when working on such reactions, first the application of such a reagent that can increase sensitivity of the detection of the derivatives and second the usage of effective clean-up procedures that will provide increased selectivity. Such methods are sensitive to matrix effects that usually lead to false estimation of the toxin content of the sample analyzed. Various cleaning up methods are usually applied for the reduction of the matrix effects (liquid-liquid extraction, spe pre- or after derivatization with a variety of stationary phases).

In this work the derivatization of the toxins is performed by the introduction of the fluorescent moiety of 3-bromomethyl-7-methoxy-1,4 benzoxazin-2-one (BrMB) (Yamada *et al* 1991; Nakanishi *et al* 1992; Kusaka and Ikeda 1993) in presence of N-ethyl-diisopropylamine (DIPEA), as the base catalyst. A methodology for successful cleaning up of the mussel extracts was developed using dry (lyophilized) mussel tissue spiked with the particular toxins.

Materials and methods

Chemicals and standards

Water used was purified by passing through a Milli-Q water purification system. The solvents used were acetonitrile (ACN), hexane, ethyl acetate (EtOAC), acetone, methanol (MeOH), dimethyloformamide (DMF), hexamethyl-phosphoramide (HMPA), pyrrolidone, di-methylsulfoxide (DMSO), The reagents used for the derivatization of the toxins were N-ethyl-diisopropylamine (DIPEA), 3-bromomethyl-7-methoxy-1,4benzoxazin-2-one (BrMB). The stock solutions were prepared by the standards OA (Calbiochem, 100 μg 99,4%, Germany) and DTX-1 (Wako, 100 μg 98%, Japan). The bulk stationary phase for the spe tubes used were silica C18

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(Merck, Supelclean LC-18), alumina (Merck, 90 active acidic, 0,063-0.200 mm).

Preparation of extracts

Whole tissue of blank mussels was dried by the technique of freeze-drying. The dry tissue was homogenated to fine powder. Portion of the powder, proportional to 1 gr of whole tissue, was spiked with the proper volume of toxin and then it was extracted once with 4 mL ACN, by vortex mixing for 3 min. The extract was then centrifuged for 10 min at 1500 rpm. 0.5 mL of the supernatant was transferred and concentrated, under stream of N_2, up to 0.2 mL for the following silica C18 spe cleaning-up.

SPE clean-up

The portion of 0.2 mL of spiked mussel extract was spe cleaned up with C18 silica glass columns. The ACN eluant which contained the toxin analytes was derivatized, using the sensitive reagent 3-bromomethyl-7-methoxy-1, 4-benzoxazin-2-one, with the addition of N-ethyldiisopropylamine as catalyst base. The derivatives were further cleaned up by normal phase SPE using alumina glass columns. Elution of the toxin esters was achieved with ethyl acetate/MeOH. The eluant was dried and diluted in 100 μL ACN for the HPLC-FLD analysis.

HPLC-FLD analysis

Chromatographic separation was achieved using Shimadzu HPLC system. Separation was achieved on a Supelcosil C18 column (Supelco 4.6 × 250 mm, 5 μm). Mobile phase A was water and mobile phase B was ACN. Flow rate was 1 mL min^{-1}. The gradient started at 60% B, this composition was kept for 10 min and then changed linearly in 10 min to 100% phase B. It was kept at 100% phase B for 10 min and returned to 60% B in 10 min. An equilibration time of 10 min was allowed before the next injection. Injection volume was 20 μL and the column temperature was 20 ^{0}C. Fluorescence detector was tuned at λex: 345 nm, λem: 440 nm.

Results and discussion

Derivatization optimization

The reaction is performed according SN2 mechanism. The reaction was optimised through experiments for the determination of the optimum concentrations of the reagents added, the most proper polar aprotic solvent for the completion of the reaction and the selection of the optimum temperature-time combination. The solvents that were checked are ACN, acetone, DMF, DMSO, pyrrolidone, HMPA. According to the results: the optimum concentrations for the added reagents are 50 μL BrMB (2 mg mL^{-1}) and 50 μL DIPEA (7 $\mu L mL^{-1}$) both solutions in ACN. ACN proved to be, for the specific experimental design, the polar aprotic solvent that favors the most this reaction. Optimum temperature-time combination for the completion of the reaction is 70 ^{0}C for 60 min. BrMB is an excellent fluorescent reagent for the analysis of these toxins.

Sample pretreatment

The proposed Sample pretreatment for sample pre-treatment before HPLC measurement proved to be efficient for the removal of undesirable matrix components, as is seen in the chromatograph [Fig.1], but the matrix effect was not eliminated as concluded by the estimated recovery at Table 1. LOQ of the instrument and LOQ of the method are satisfactory for both toxins.

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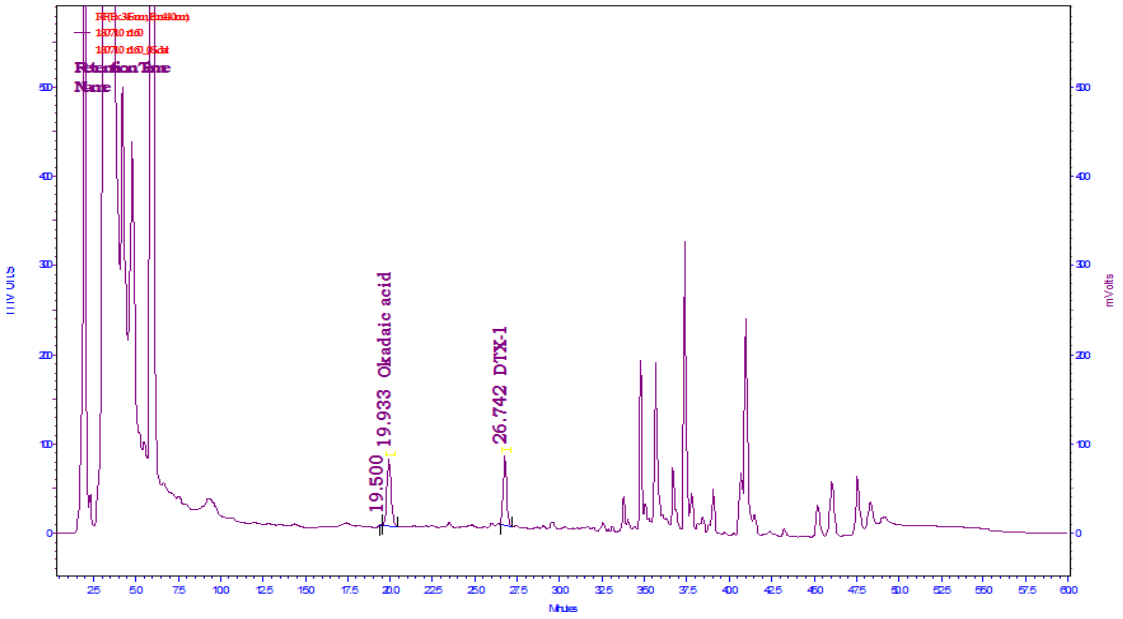

Fig. 1 HPLC-FLD chromatogram of blank mussel spiked with 160ng of OA and 160ng of DTX-1, treated with proposed methodology.

Table 1 Results of the proposed method.

	R^2 [a]	Recovery	Repeatability [b]	LOD*instr.*	LOQ*instr.*	LOD*meth.*	LOQ*meth.*
OA	0.9773	125.35 (±4.13)	3.3	2.2	6.6	21.8	66.1
DTX-1	0.9719	141.5 (±7.56)	5.3	11.1	33.6	39.9	120.9

[a] Correlation obtained from blank mussel spiked, pretreated and analysed as described
[b] %RSD of 6 independent measurements of blank mussel spiked with 160 ng gr^{-1}.

Comparison of the influence of different extraction procedures and analytical methods on the qualitative and quantitative results for PSP toxins determination

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Abstract

Using toxic mussels from outbreaks of *G. catenatum*, the results of PSP intercalibrations and PSP toxin evaluation are presented between laboratories responsible for monitoring shellfish production areas in Cataluña, Galicia and Andalucía. Different extraction and clean up procedures are evaluated. PSP toxicity was estimated from HPLC analyses with pre-column and post-column oxidation and compared with the reference method. HCl 0.1 N can be considered a universal extractant since it can be used in both Mouse Bioassay (MBA) and HPLC with pre-column or post-column oxidation. On the other hand, using pre-column methods it is not possible resolve pairs of toxins with different toxicity and quantitative results are underestimated relative to MBA.

Introduction

Marine toxins limit the development of the bivalve aquaculture industry. To guarantee the maximum safety level for consumers, monitoring programs need to implement robust and reliable methodologies for quantification of toxins in shellfish. Currently, there is controversy concerning the methodology to use in monitoring programs for quantification of PSP toxicity. The MBA method for PSP (AOAC 959.08) is the reference method in the EU, but it the possibility has been opened to use the Lawrence method (AOAC 2005.06). As a consequence, a research project "JACUMAR" started in 2008 with laboratories responsible for monitoring programs in shellfish production areas in Cataluña, Galicia and Andalucía and also laboratories specialized in PSP toxin evaluation. The results obtained with toxic mussel samples from outbreaks of *G. catenatum* are presented. Different extraction and clean up procedures are evaluated. PSP toxicity was estimated from HPLC toxin analyses with pre-column and post-column oxidation and then compared with the reference method.

Methods

Extraction procedures

Sample of mussels (*Mytilus galloprovincialis*) from outbreaks of *Gymnodinium catenatum* in Andalucia coast were used. Four extraction procedures were applied: a) Boiled HCl 0.1 N extracts following MBA method for PSP (AOAC 959.08); b) Boiled Acetic acid 1% extracts following Lawrence method (AOAC 2005.06); c) Unboiled HCl 0.1 N extracts (modification of AOAC 959.08) and d) Unboiled acetic acid 1 % extracts (modification of AOAC 2005.06) (Fig.1).

Sample cleaning.

Since HPLC determination of PSP toxins do not include an organic cleaning phase as in determination of lipophilic toxins, apolar compounds are retained in the column head. The yellow colour may be due to modified carotenoids produced by the mussel. This significantly limits column life and application of HPLC in monitoring programs. To resolve this several cleaning procedures have been tried: 1) 30% trichloracetic acid (TCA). (Rourke et al., 2008). 2) Liquid-liquid partition. Hexane and ethyl acetate. 3) OSTRO Sample Preparation Plate (Waters). 4) Solid phase extraction with Sep pak light C18 Cartridges. 5) Matrix dispersion purification with Lichroprep RP18 (40-63 μm) (Merck). For this last procedure 300 mg of LichroprepRP18 (40-63 μm) were added into centrifuge tubes and conditioned with 2 mL of

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Methanol and 8 mL of Mili-Q water. After removing methanol and water 2 mL of HCl 0.1 N mussel extract were mixed with the resin and centrifuged at 10400g for 15 min in order to separate resin and extract. Finally, supernatant was collected and filtered through 0.2 µ before HPLC.

Results

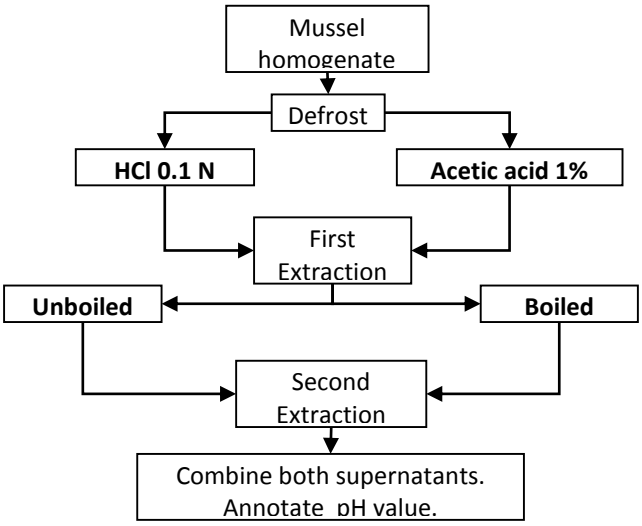

Fig. 1 Scheme of the extraction procedures applied.

Extraction procedures

Results with different extraction procedures are summarized in Table 1. Since acetic acid kills mice, these extracts cannot be used in MBA. HPLC post-column analyses confirmed that unboiled acetic acid 1% does not extract PSP toxins. Comparison of quantitative results of both boiled HCl 0.1 N and acetic acid 1% extracts by HPLC with pre and post column derivatization demonstrated that HCl 0.1 N extraction is more effective than acetic acid 1%. HCl 0.1 N extracts can be used for determination by HPLC with pre-column oxidation and also MBA and hence can be considered a universal extractant. Slight differences in toxin profile were observed depending on extraction procedure and analytical method. It was not possible to separate some pairs of toxins with pre-column methods (Table 2).

Sample cleaning

TCA is toxic for the mouse and does not remove the yellow colour of mussel samples. Partioning with hexane and ethyl acetate did not remove the colour and OSTRO purification plate showed low recovery and diluted the sample. Good results have been obtained with sep pak light C18 cartridges and matrix dispersion purification with Lichroprep RP18 (40-63µm). The main drawback of sep pak is that the toxin is distributed between fractions 1 and 2 (Table 3), and therefore diluted. Best results have been obtained with matrix dispersion addressing the high area % obtained (Table 3). The sample colour reduction was estimated to be <50% by measuring absorbance at 490 nm of the original samples and four replicates after purification with Lichroprep RP18 (Table 4).

Conclusions

HCl 0.1 N can be considered a universal extractant since it can be used in both MBA and HPLC with pre-column or post-column oxidation. Using pre-column methods it is not possible resolve pairs of toxins with different toxicity and quantitative results are underestimated relative to those by MBA. Matrix dispersion is an easy and fast procedure with recovery around 80% based on area percentages for each toxin obtained by HPLC with post-column oxidation.

Acknowledgements

Junta Nacional Asesora de Cultivos Marinos (JACUMAR) by project “Comparison of methodologies for the evaluation of Paralytic Shellfish Poisoning (PSP) toxins in bivalves. Application for aquaculture in Spain”. Thanks due to Institut de Recerca I Tecnologia Agroalimentàries (IRTA) and Instituto Español de Oceanografía (IEO) Spain.

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J.F. Lawrence et al. (2005) J AOAC Int, 88, 1714-1732;

W. A. Rourke et al. (2008) J A
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Table 1. Toxicity values from Chromatographic data are expressed as µg STX 2.HCl equivalents per Kg of whole flesh. Toxin concentrations have been computed using the toxicity data of Oshima (1995). Toxicity values from MBA were calculated from Sommer´s Tables (AOAC 959.08).

	HCl 0.1 N		Acetic acid 1%	
µg STX 2.HCl eq / Kg	**Unboiled**	**Boiled**	**Unboiled**	**Boiled**
MBA		10773 (n=11, SD 10%)		
HPLC Pre-column	6068 (n=9, SD 4%)			4785 (n=4, SD 8%)
HPLC Post-column	9443 (n=3, SD 6%)		No Detect	3130 (n=4, SD 20%)

Table 2. Toxin profiles obtained with the extraction procedures and analytical methods applied.

Toxin profiles	C1	C2	GTX6	GTX4	GTX1	dcGTX2	dcGTX3	GTX5	GTX2	GTX3	neoSTX	dcSTX	STX	dc-NEO	C3	C4
Post column (HCl 0.1N unboiled)																
Pre column (HCl 0.1N) Unboiled	UNRESOLVED					UNRESOLVED			UNRESOLVED						UNRESOLVED	
Pre column (Acetic acid 1%) Boiled	UNRESOLVED					UNRESOLVED			UNRESOLVED						UNRESOLVED	

Table 3. Relative area percentages for each toxin determined by HPLC post-column. Values corresponding to samples after matrix dispersion and Sep pak C18 purification procedures. 100% value is given to the area of each toxin in the original extract.

	GTX6	dcGTX3	dcGTX2	GTX5	GTX2	neoSTX	dcSTX
Matrix dispersion	85	85	79	96	74	88	94
Sep Pak C18 (fraction 1)	62	71	67	64	24	64	59
Sep Pak C18 (fraction 2)	22	28	24	30	27	18	19
Sep Pak C18 (fraction 3)	0	0	2	0	0	0	0

Table 4. Absorbance readings at 490 nm of original sample and four replicates of sample after purification with Lichroprep RP18.

Mussel extract	Absorbance (490 nm)
original	0.711
1	0.306
2	0.318
3	0.327
4	0.313

MECHANISMS OF TOXICITY

Fibrocapsa japonica (Raphidophyceae) shows high extracellular haemolytic activity at low cell densities

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Abstract

Haemolysins are thought to be involved in the ichthyotoxicity of the raphidophyte *Fibrocapsa japonica*. So far, all haemolytic data were based on the analysis of intracellular haemolysins in concentrated extracts of cells at the end of the exponential or in the stationary growth phase. To gain more insight in the mechanisms of haemolysin production we studied differences in intracellular and extracellular haemolysin activity at various cell densities during exponential growth. The haemolytic activity of *F. japonica* changed significantly as a function of cell concentration. At increasing cell concentrations, extracellular haemolytic activity decreased while intracellular activity increased. Possibly, the very high extracellular haemolytic activity at low cell concentrations could be caused by growth inhibiting or allelopathic haemolysins.

Introduction

The harmful algal bloom species *Fibrocapsa japonica* Toriumi and Takano (Raphidophyceae) is potentially ichthyotoxic. The fish kills caused by *F. japonica* are ascribed to toxicity and/or mechanical damage to the fish (Khan *et al.* 1996; de Boer *et al.* 2005; Pezzolesi *et al.* 2010). Haemolysins are one of the proposed toxins that are involved in the ichthyotoxicity of *F. japonica*. Bioassay guided characterization of haemolytic fractions from the *F. japonica* Wadden Sea strain (CCRuG Cl 3) yielded the polyunsaturated fatty acids (PUFAs), C18:4n3 (OTA), 20:5n3 (EPA), and C20:4n6 (AA) (Fu *et al.* 2004). Next to these haemolytic PUFAs, potent light dependent haemolytic compounds were found in the intracellular fraction (Boer et al. 2009). A synergistic effect of the reactive oxygen species (ROS) superoxide and EPA on the ichthyotoxicity of *C. marina* was demonstrated earlier (Marshall *et al.* 2003), but also suggested to occur in *F. japonica* (Pezzolesi *et al.* 2010). Generally the haemolytic activity of *F. japonica* cells is high and comparable to other haemolytic harmful algal bloom species like *Prymnesium parvum* (de Boer *et al.* 2009). So far, all haemolytic data were based on the analysis of intracellular haemolysins in concentrated extracts of cells at the end of exponential or in stationary growth phase. Because fish mortality not only occurs during these growth phases but also at lower cell densities (Khan *et al.* 1996), extracellular haemolysins could also be involved in ichthyotoxicity. In the present study we investigated intra- and extracellular haemolysin activity during various stages of *F. japonica* growth. To distinguish between intra- and extracellular substances we used different procedures. Haemolytic activity was assessed using the earlier described erythrocyte lysis assay (ELA).

Material and Methods

In two independent experiments, growth and haemolytic activity of in total four replicate cultures of the non-axenic Dutch *F. japonica* Wadden Sea strain W420 were followed. The cultures were pre-cultured for at least 2 generations in exponential growth phase. Four 1L Erlenmeyer flasks were incubated for experiments, with an initial cell density of 700 ± 100 cells ml^{-1}. All cultures were grown in autoclaved natural sea water (Doggersbank, North Sea) adjusted to salinity of 25 with Milli-Q and enriched with f/2-Si medium. The cultures were maintained at 18 ± 1 °C in a 16:8h light:dark photoperiod with photon flux density of 45 ± 5 µmol photons m^{-2} s^{-1}. Mucocyst ejection was monitored by daily observation. Samples for cell density were taken daily in the light period, for 19-25 days. After sampling cell numbers and biovolume were immediately determined using a Coulter model ZM

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particle counter equipped with channelyzer 256 and 100 µm aperture tube (de Boer *et al.* 2005).

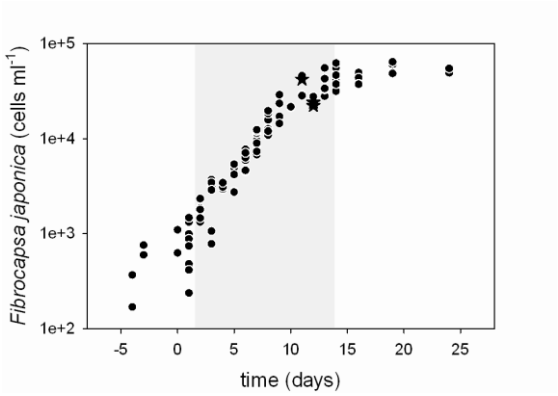

Fig.1. Growth curves of 4 *Fibrocapsa japonica* cultures. One culture was corrected for longer lag phase. Grey = exponential growth, * = samples with mucocyst ejection

Since *F. japonica* cells are easily damaged, several procedures were introduced to separate intracellular (I) and extracellular (O) haemolysin fractions. The following types of samples were used: Type I: whole, non-treated cultures containing living cells (O); Type II: whole cultures, frozen at -20 °C in order to break the cells, giving total haemolysins (I+O); Type III: filtered cells followed by extraction (I); Type IV: filtrate by standard filtration (O); and Type V: filtrate by reverse filtration (O). Samples were processed using all five procedures: for experiment 1 Type I, III and IV, and for experiment 2 Type II, III, IV and V. For Type III 10^5 cells of each culture were collected on a polycarbonate filter (2 µm, Ø 25 mm) using mild vacuum (standard filtration). Filters, cultures and filtrates were stored at -20 °C until further analysis, except Type I samples which were immediately analysed for haemolytic activity. Cell extracts (Type III) were obtained as described in de Boer *et al.* (2004). ELA was performed as described previously (de Boer *et al.* 2004). For analysis of haemolytic activity of living cell samples (Type I) the ELA buffer was adjusted to salinity of 25. The absorptions of the negative and positive control of ELA buffer were both significantly higher than the standard ELA buffer (one way ANOVA p=0.018 and p=0.006). In both ELA methods, f/2-Si medium and ELA buffer served as controls and did not show any haemolytic activity. Saponin (Sigma) was used as a reference, with an EC_{50} value of 4.3 µg ml^{-1}. The cell densities of *F. japonica* from the dilution series were plotted against the % erythrocyte lysis and were fitted by a sigmoidal curve or, alternatively, by a linear regression. EC_{50} was calculated as the concentration of *F japonica* cells (cells ml^{-1}) responsible for 50% erythrocyte lysis. Statistical analysis was performed using SPSS and Statistica.

Results

All cultures showed similar growth curves (p=0.64) with initial lag phase, followed by exponential growth phase between days 1 and 11-14 (Fig. 1). Specific growth rates at the applied conditions were also comparable (p=0.68), and on average 0.34 ± 0.02 d^{-1}. The exponential growth phase of the replicate cultures was defined between 1.3 ± 0.15. 10^3 and 4.2 ± 0.3. 10^4 cells ml^{-1}. Mucocyst ejection started at a cell concentration of 2.2. 10^4 cells ml^{-1}.When cell concentrations were below 0.5. 10^4 cells ml^{-1}, no significant differences in haemolytic activity were found between filtrates Type IV and V (p=0.59) nor between filtrates and whole culture (Type I) (p=0.64, Fig. 2). Above this cell concentration, maximum lysis of erythrocytes was mostly not sufficient to calculate extracellular haemolytic activity. This indicates that below 0.5. 10^4 cells ml^{-1} the calculated extracellular haemolytic activity was not caused by damaged or broken cells during sample processing. Highest extracellular haemolytic activity was 20 x higher (EC_{50} = 600 cells ml^{-1}) than maximum intracellular activity from Type III samples (EC_{50} = 1.2. 10^4 cells ml^{-1}, Fig. 3). The cell concentration was positively correlated with the intracellular haemolytic activity and negatively correlated with extracellular haemolytic activity (Spearman rank correlation; R^2=0.97 and R^2=-0.65). Mucocyst ejection did not affect the haemolytic activity neither inside nor outside the cell. Only the maximum erythrocyte lysis of the frozen samples (Type II) of days 16 and 19, was sufficient to calculate haemolytic activity (data not shown). These EC_{50} values were not different from Type III samples (p=0.09).

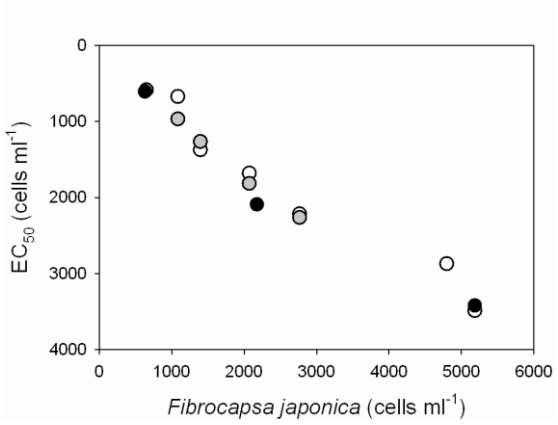

Fig 2. Effect of cell concentration on haemolytic activity (EC_{50}) with different sampling methods. White: filtrate by standard filtration; grey: filtrate by reverse filtration; black: whole, non-treated cultures containing living cells.

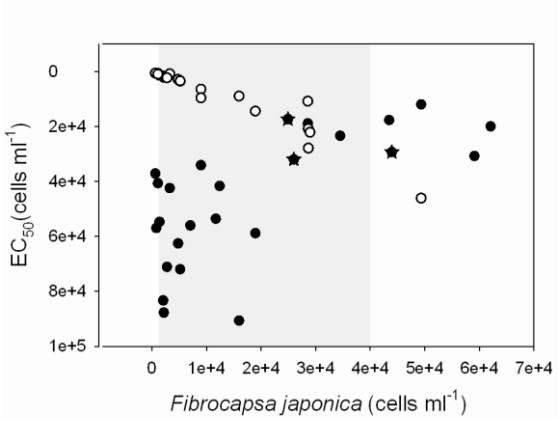

Fig. 3. Effect of *Fibrocapsa japonica* cell concentration on haemolytic activity (EC_{50}) measured intracellular (black) and extracellular (white) Grey = exponential growth phase; * = samples with mucocyst ejection.

Discussion

Different procedures for estimating extracellular haemolytic activity showed similar results. This implies that, although cells of *F. japonica* break easily, standard filtration may be used for distinguishing between extracellular and intracellular haemolysin fractions. When cells were damaged by freezing, results were similar to intracellular activities derived from filtered cells. It is unclear why high extracellular haemolytic activity was not observed in the frozen samples. Haemolytic activity of *F. japonica* significantly changed as a function of cell concentration during exponential growth, i.e. when growth rate was maximal and constant. Very high extracellular haemolytic activity at low cell concentrations could have ecological advantages by improving the competitive abilities. More specifically, the haemolysins might be the same growth inhibiting or allelopathic compounds released by *F. japonica* at low cell concentrations during exposure to bacteria (van Rijssel *et al.* 2008). The intracellular haemolysins showed highest haemolytic activity at high cell concentrations when nutrients were getting scarce and mucocysts were released. The intracellular haemolytic activity could be attributed to the PUFAs and haemolytic compounds other than PUFAs, as described earlier (Fu *et al*. 2004; de Boer *et al.* 2009). So far, the mechanism of the ichthyotoxicity of *F. japonica* was ascribed to the production of these PUFAs (Marshall *et al.* 2005, de Boer *et al.* 2009, Pezzolesi *et al.* 2010), however in the present study we have shown that extracellular haemolysins might have different structural and ichthyotoxic characteristics.

Acknowledgements

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Rapid and drastic decreases of body temperature in mice intraperitoneally injected with lethal dose of okadaic acid

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Abstract

The mouse bioassay for diarrhetic shellfish poisoning toxins has been used as the official method in Japan and also the world. However, its scientific basis is poorly understood. A lethal dose of OA was i.p. injected into mice. The rectal temperature of the mice was measured by an electronic thermometer or the body surface temperature monitored by infrared camera before and every hour after inoculation. One hour after inoculation, the rectal temperature decreased to below 35°C in all mice injected with OA. Two and/or 3 hours after inoculation, rectal temperature decreased to below 30°C in some mice. Such rapid and drastic decreases of the body temperature were also detected by using an infrared camera. Mice death could be obtained at 24 hours after inoculation with a lethal dose of OA, but rapid and drastic decreases of the body temperature were observed within a few hours. These indicate the possibility that decreases of the body temperature might be used as a rapid index especially for negative results.

Introduction

The mouse bioassay (MBA) for diarrhetic shellfish poisoning (DSP) toxins was established by Yasumoto *et al*. (1978). MBA for DSP toxins was determined as the official method in Japan since 1981 (Japanese Ministry of Health and Welfare 1981) and has also been widely used in many countries of the world (FAO 2004). MBA for DSP has been used for monitoring or surveying the food safety for 30 years, but the scientific basis of MBA is poorly understood. I have been studying pathophysiological changes of the mice after intraperitoneal (i.p.) injection of DSP toxins. In this study, I found here, the rapid and drastic decreases of body temperature in the mice i.p. injected with a lethal dose of okadaic acid.

Materials and Methods

Animals: Four-week-old, male, ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were housed in plastic cages with woodchip bedding and fed commercial pellets and tap water *ad libitum*. Mice were adapted for 1 day and used at 18–20g of body weight. All animal experiments were conducted with the approval of the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

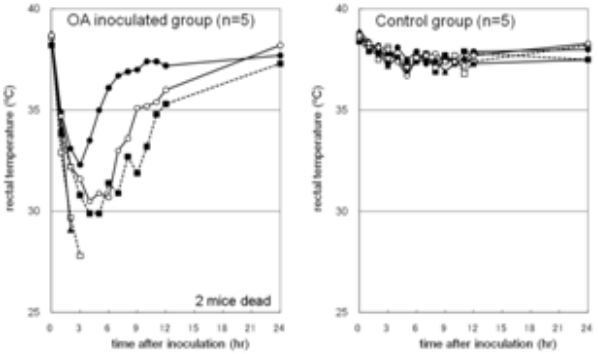

Fig.1. Rectal temperature after OA inoculation

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Toxin: Okadaic acid was purchased from LC Laboratories (Woburn, MA, U.S.A.). Okadaic acid was first dissolved in acetone and mixed with soybean oil. Acetone was removed by evaporation and the residue was suspended in 1% Tween 60 saline and sonicated. The final inocula contained 10% soybean oil.

Experimental design: A lethal dose of okadaic acid (4μg/ml/mouse) was i.p. injected into mice. Only carrier solvent was i.p. injected into the control mice. Both experimental and control groups consisted of 5 mice each. The rectal temperature of the mice was measured before and every hour after inoculation by using an electronic thermometer (CTM-303; Terumo Corporation, Tokyo, Japan) in the first experiment. The body surface temperature of the mice was monitored, instead of measuring the rectal temperature, by using an infrared camera (FLIR i5, FLIR Systems, Inc., Wilsonville, OR, U.S.A.) in the second experiment.

Results and Discussion

One hour after inoculation with a lethal dose of okadaic acid, the rectal temperature decreased to below 35°C in all mice (Table 1). Two and/or 3 hours after inoculation, the rectal temperature decreased to below 30°C in some mice. Out of 5 mice in the experimental group, 2 mice died and 3 mice survived. At 24 hours after inoculation, the rectal temperature of 3 surviving mice was almost recovered to that of before inoculation. In the control group, the rectal temperature of the mice showed variations but not such drastic decreases as seen in the experimental group. Drastic decreases of the body temperature were also detected by using an infrared camera (Table 2). At 2 hours after inoculation, thermograms of the experimental group changed from white to gray, suggesting drastic decreases of body surface temperature. On the other hand, the thermograms of the control group did not show such changes. In this experiment, 4 mice were dead 24 hours after inoculation. The thermograms of 1 survived mouse showed gray even at 24 hours after inoculation, indicating nonrecovery of the body temperature. This mouse survived at 24 hours after inoculation, but died in a few hours after the end of the experiment. (In the official protocol of Japan, this case is considered as negative, because the protocol requires judging the results 24 hours after inoculation.) Mice death at 24 hours after

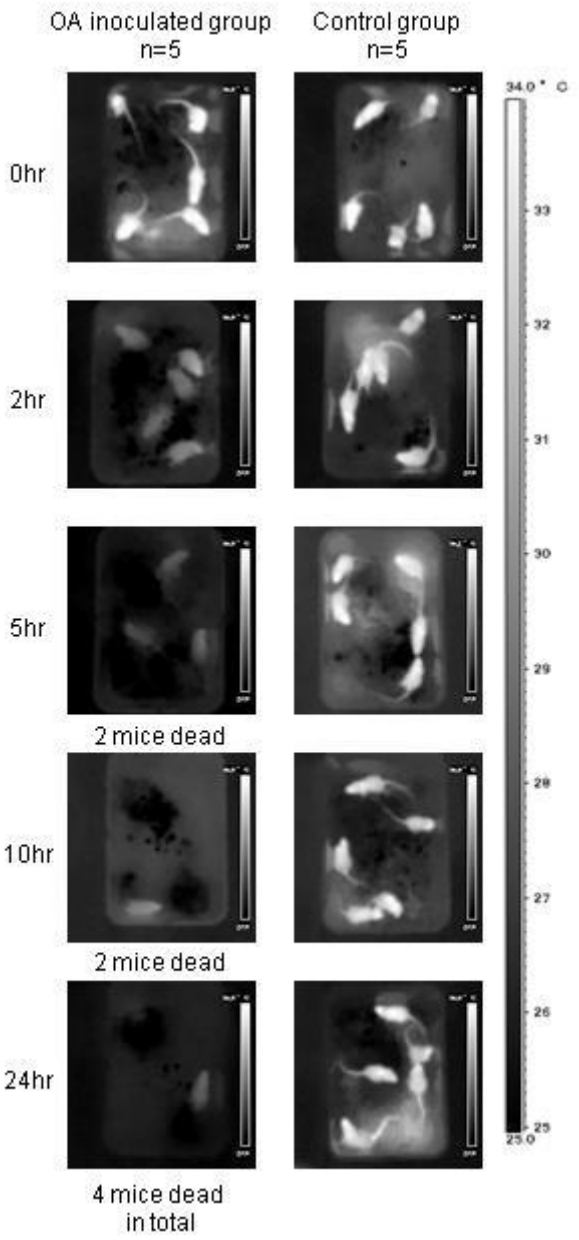

Fig.2. Thermograms after OA inoculation

inoculation was 40% in the first experiment and 80% in the second experiment. On the other hand, the rapid and drastic decreases of the body temperature were observed within a few hours after inoculation in all the mice. These indicate the possibility that the decrease of the body temperature might be

used as a rapid index especially for negative results. Moreover, by using infrared cameras, stressless monitoring of the body temperature is possible.

Acknowledgements

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Possible involvement of hemolytic activity in contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*

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Abstract

We investigated the effects of two strains (SUO-1 and FUK) of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. The SUO-1 strain was highly toxic to rotifers, whereas the FUK strain was less toxic. Both the cell-free culture supernatant and the ruptured cell suspension prepared from these strains were not toxic to rotifers. Furthermore, when direct contact between *K. mikimotoi* and rotifers was interrupted with a cell-impermeable membrane (3 µm pores), the toxicity of both the SUO-1 and FUK strains of *K. mikimotoi* to rotifers were completely inhibited. Cell suspensions of SUO-1 showed hemolytic activity toward mammalian erythrocytes, but the FUK strain did not. The cell-free supernatant and the ruptured cell suspension of SUO-1 showed no significant hemolytic activity. These results suggest that this highly toxic strain of *K. mikimotoi* causes lethality in rotifers by direct contact in which live cell-mediated hemolytic activity might be a contributing factor.

Introduction

Karenia mikimotoi (formerly *Gyrodinium aureolum*, *G.* cf. *aureolum*, *G.* type-'65, *G. nagasakiense*, and *G. mikimotoi*) is a common dinoflagellate that causes red tide in many coastal waters. HABs due to this species have been reported in Western Japanese waters (Honjo, 1994), the North Atlantic (Gentien, 1998), and other coastal areas (Lu and Hodgkiss, 2004; Sun et al., 2007), and are frequently associated with severe damage to wild fish, aquaculture fish, and shellfish. Previous studies demonstrated that *K. mikimotoi* produces various toxic agents, including low molecular weight, hemolytic toxins (Arzul et al., 1994; Mooney et al., 2007), cytotoxic polyethers (Satake et al., 2002), and reactive oxygen species (ROS) (Yamasaki et al., 2004). Matsuyama (1999) showed that *K. mikimotoi* strongly inhibited the filtration rate of bivalves. Sellem et al. (2000) demonstrated that the 18:5n3 fatty acid produced by *K.* cf. *mikimotoi* delayed or inhibited the firstcleavage of sea urchin (*Paracentrotus lividus*) eggs and produced abnormalities in their embryonic development. Mitchell and Rodger (2007) reported that an algal bloom of *K. mikimotoi* during summer 2005 in Ireland was associated with mortalities of both fish and shellfish. They also reported histopathological changes in the gills, gastrointestinal tracts, and livers of fish and shellfish killed by *K. mikimotoi*. Despite the toxicity potential of *K. mikimotoi*, the exact mechanism of toxicological action remains unclear. Herbivorous zooplankton, such as rotifers and copepods, have been previously used to elucidate toxic mechanisms of HAB species (Wang et al., 2005). For instance, previous studies have demonstrated that several dinoflagellates had lethal effects on *Brachionus plicatilis* (Kim et al., 2000). To gain insight into the toxic mechanism of *K. mikimotoi*, we examined the effects of two strains, SUO-1 and FUK, on the rotifer *B. plicatilis* under various experimental conditions. The hemolytic activity of these *K. mikimotoi* strains toward various mammalian erythrocytes was also examined.

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Materials and methods

Two strains of *K. mikimotoi* were isolated from the Fukuoka Bay (FUK), Japan in 2004 and Suo Nada (SUO-1), Japan in 2006. These clonal strains were maintained at 26°C in 200-mL flasks containing 100 mL of modified seawater medium (SWM-3) at a salinity of 25 (Yamasaki et al., 2007). In addition, cultures were kept under a 12:12-h photoperiod using a cool-white fluorescent lamp (200 ± 5 µmol m^{-2} s^{-1}). Culture cell numbers were counted microscopically using a hemocytometer (Erma Inc., Tokyo, Japan). Cell-free culture supernatant and the ruptured cell suspension were prepared as previously described (Zou et al., 2010). The rotifer *B. plicatilis* was provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with *Nannochloropsis oculata* as described previously (Kim et al., 2000).

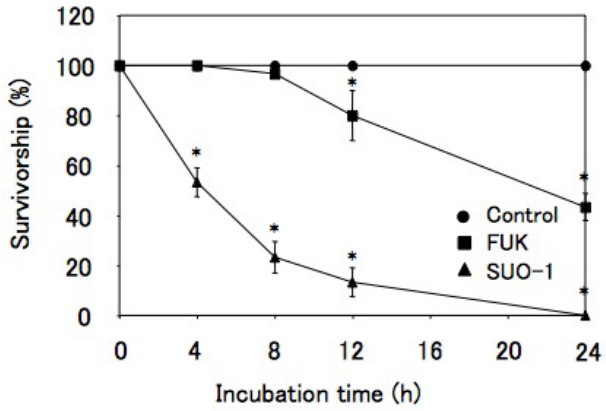

Fig 1. Effects of the SUO-1 and FUK strains of *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*.

Rotifer toxicity test was conducted in 48-well plates. Each well contained 10 individual rotifers in 100 µL of modified SWM-3 added to 900 µL of each *K. mikimotoi* cell suspension (2 x 10^4 cells mL^{-1}). As a negative control, 10 individual rotifers were cultured in 1 mL of the modified SWM-3 alone. The number of dead rotifers was counted every two hours with stereomicroscopic observation over the course of a 24-h incubation. Three wells were used per treatment. Rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan) and was used within seven days of receipt. The erythrocytes were washed three times with phosphate-buffered saline (PBS) and adjusted to a final concentration of 4% (v/v) in PBS. Duplicate of 50-µL aliquots of serial twofold dilutions of intact cell suspension, cell-free culture supernatant, or ruptured cell suspension of each *K. mikimotoi* strain using modified SWM-3 were added to round-bottom 96-well plates. Each well contained the same 2% (v/v) suspension of erythrocytes in each sample solution; the well plates were gently shaken. After incubation for 5 h at 26℃ under illumination from a fluorescent lamp (200 µmol $m^{-2}s^{-1}$), the plates were centrifuged at 900×g for 5 min. Aliquots (70 µL) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates. Released hemoglobin was determined by measuring absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOHCo., Ltd., Tokyo, Japan). Negative controls (zero hemolysis) and positive controls (100% hemolysis) were included using erythrocytes suspended in modified SWM-3 alone and in modified SWM-3 containing 1% (v/v) Triton X-100, respectively.

Results and discussion

Rotifer toxicity testing was conducted with two strains of *K. mikimotoi* (SUO-1 and FUK) at the same cell density (2 ×10^4 cells mL^{-1}). As shown in Fig. 1, both strains of *K. mikimotoi* were significantly ($p < 0.05$) lethal to rotifers, although the potency of the strains were quite different. The toxicity of the SUO-1 strain was greater than that of the FUK strain, and all the rotifers exposed by the SUO-1 strain died after a 16-h exposure. After exposure to SUO-1, rotifer stress was immediately apparent and their movement declined within 1 h of exposure. Dead rotifers with partial morphological changes, especially to the corona located at the anterior end of two ciliated rings, started to appear after exposures of 6 h. Relative to control rotifers, damaged coronas with excessive secretion of mucus and abnormal foam-like structures in the corona were observed in some rotifers exposed to the SUO-1 strain. The FUK strain was only weakly toxic to the rotifers in comparison to SUO-1, and about 40% of the rotifers were viable after the 24-h exposure. The cell-free culture supernatants and ruptured cell suspensions of both *K. mikimotoi* strains were not toxic to *B. plicatilis*. Furthermore, when direct contact of *K. mikimotoi* cells and rotifers was interrupted with a cell-impermeable membrane, the toxic effects of both strains completely disappeared even though the 3.0

μm pore size of the membrane is sufficient to allow large molecular weight compounds to transfer from *K .mikimotoi* to the rotifers. These results suggest that the direct attack of *K. mikimotoi* cells is probably a key mechanism causing the lethality observed in rotifers. It is unlikely that soluble toxins released from *K. mikimotoi* cells are responsible for the observed toxicity. Hemolytic activities of the FUK and SUO-1 strains of *K. mikimotoi* toward rabbit erythrocytes were examined. As shown in Fig. 2, intact cell suspensions of the SUO-1 strain were strongly hemolytic to rabbit erythrocytes in a cell density-dependent manner. No hemolytic activity was observed in the FUK strain.

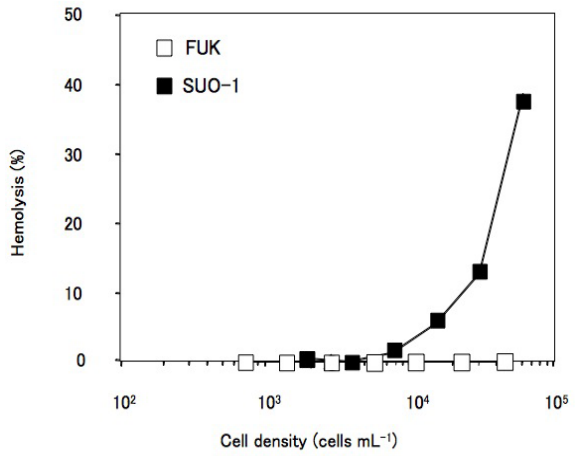

Fig 2. Hemolytic activity of the SUO-1 and FUK strains of *Karenia mikimotoi* toward rabbit erythrocytes.

There seemed to be a positive correlation between the toxicity of *K. mikimotoi* to *B. plicatilis* and its hemolytic activity. The SUO-1 strain was more toxic to rotifers and induced potent hemolytic activity, whereas the less toxic FUK strain showed no hemolytic activity (Figs. 1 and 2). Furthermore, the cell-free supernatant and the ruptured cell suspension of the SUO-1 strain, which were incapable of killing rotifers, did not show any hemolytic activity.These results suggest that the live cell-mediated hemolytic activity might be linked with the toxic effects on rotifers. The rotifer corona may be especially sensitive to the SUO-1 strain due to a toxic agent located on the cell surface that is responsible for hemolytic activity. Thus, the gradual accumulation of damage to rotifers caused by live SUO-1 cells may eventually lead to their mortality. Morphological changes, concomittant with the secretion of mucous-like substances and the formation of foam-like structures, observed around rotifer coronas exposed to SUO-1 live cells suggest that the corona is sensitive to attack by SUO-1 cells. It is possible that certain toxic agents located on the SUO-1 cell surface may cause membrane damage leading to impaired membrane permeability. Such an agent could also induce hemolysis which may attack erythrocyte membranes through direct cell-to-cell contact and damage membrane structures.

Acknowledgements

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MANAGEMENT

Socio-Economics of Mitigating Chesapeake Cyanobacteria Blooms

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Abstract

Blooms of harmful algae are characteristic of the Chesapeake and its tributaries, leading to dissolved oxygen problems, fish and crab mortalities, and in some cases, toxin production and threats to domestic animals and the public. Over the last 3 years, a technique has been developed to efficiently remove cyanobacteria from surface waters using mixtures of clays and acidified chitosan, a by-product of crab shells. The technique appears to be adaptable for field use but public willingness for field application, previously preventing use of a similar technique for 'red tides' off Florida's west coast, as well as the costs for use of the technique could limit adoption as a routine mitigation strategy in regional waters. We conducted a two part study to estimate costs of using the technique in field control of blooms as well as surveying local citizens to assess public support for use of the technique at some expense to each household. Cost per household is estimated at $0.15 USD, suggesting modest impacts on the state's taxpayers. More importantly, citizens were generally supportive of routine use of the technique for field bloom mitigation. Our survey of the University of Maryland community, local farmers, watermen, and municipal officers yielded about 67% respondent support for general use of the technique, with slight variances detected between the groups surveyed.

Introduction

Globally harmful algal blooms (HABs) have been treated with numerous mitigation techniques; however, very few assess accompanying environmental and economic impacts. Mitigating such blooms through clay flocculation of local sediments (Zou *et al*. 2006) is an innovative method that theoretically minimally impacts system ecology at modest costs for materials and to local businesses. In our research, we have expanded this previously documented clay flocculation technique by conducting a cost assessment and surveying regional citizens on willingness for mitigating local blooms. This paper focuses on the socio-economics of bloom mitigation specific to the state of Maryland, USA. Our clay mixture has been proven to be effective in removing tidal-fresh harmful algal blooms present in the Chesapeake Bay (Savranskaya-Gallo *et al*. in prep.). For the current research to be implemented as a routine mitigation technique in bloom control by the state, cost consideration is critical because the funds for bloom mitigation are ultimately derived from taxpayers. In addition, public support must be neutral to positive: Maryland citizens must be willing to allow bloom mitigation however successful and harmless the technique appears, or state officials will not adopt the technique as a standard mitigation protocol. The basis for our research stems from experiences with an attempt to mitigate Florida red tides. The Florida coastline has long been affected by red tides of the dinoflagellate, *Karenia brevis*. Like the HABs present in the Chesapeake Bay, *K. brevis* brevetoxins have harmed flora, fauna, and humans who have been exposed to its toxins (Sengco *et al*. 2002). Specifically, red tides

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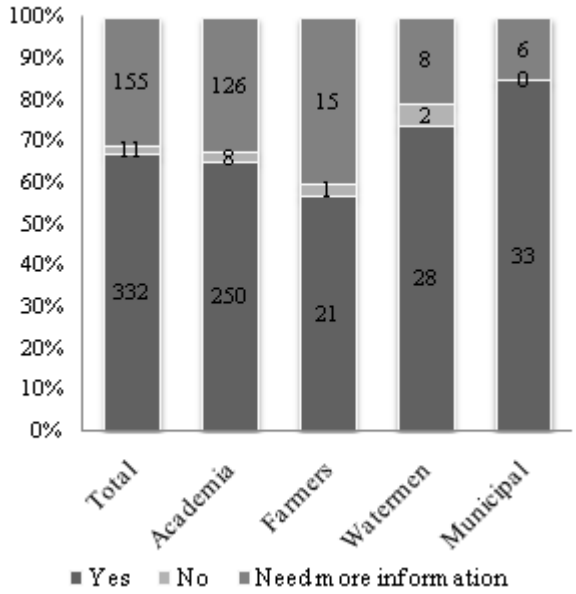
have led to fish kills and for humans, gastrointestinal symptoms such as nausea, diarrhea, abdominal pain, and respiratory distress as well as neurological symptoms such as headache, vertigo, and poor coordination (Alcock 2007). Laboratory and field microcosm research results indicated that an effective clay mitigation technique could likely be applied to field blooms to remove bloom biomass; however, their technique could not be implemented due to public dissent (Kuhar *et al.* 2009; M. Sengco, pers. Comm..). We hoped to assess initial reactions to the technique by surveying Maryland residents from various regions and professions following delivery of a short summary on bloom impacts and possible costs of the strategy to the average household.

Methods

Two methods were used, the first determining the overall cost of the production and application of the clay mixture and the second the development and analysis of a public survey of willingness to support the method's routine use. In calculating the cost of producing and applying the mixture on the Chesapeake Bay, we considered the costs of the clay and relevant components including the flocculant chitosan, transporting and storing clay, and costs of manpower for application. Transportation costs are a major factor with necessary rental costs of barge, boat, and truck. Monitoring is a future cost that is not associated with the clay mixture, but is already part of the state's existing field program. We calculated the final individual tax by measuring the surface area of the affected parts of the Bay and dividing it by the number of tax-paying households in the state of Maryland. The purpose of the survey was to gauge public opinion for the application of the clay+chitosan mixture on the Bay as well as its willingness to pay for the intervention. We have analyzed our results through cross-tabulation of demographic groupings versus willingness to support and pay. We also used Microsoft Excel to perform chi-square tests to analyze for significance of public approval.

Figure 1. Willingness to Support



Results

Following collection of costs of the mitigation elements below (Table 1), we obtained an annual cost per taxpaying household of $0.15. The estimate was derived from the small region of Maryland's bay that has supported previous cyanobacteria blooms.

For the survey, we received 498 complete surveys. Of those, 384 responses were garnered through electronic forms distributed via social media and during Maryland Day at the University of Maryland College Park. This survey group ("academia") consisted mainly of undergraduate and graduate students attending the university as well as family members and members of the community of the city of College Park. The second survey was taken at the Farm Bureau's Annual Picnic in Caroline County (37 responses, "farmers"), while the third and fourth surveys were distributed and retrieved at the Calvert County Watermen's Day Festival (38 responses, "watermen") and the Maryland Municipal League 2010 Fall Conference (39 responses, "municipal"), respectively. Support for use of the technique for bloom mitigation was seen across all four groups. Overall 66.7% of the surveyed population indicated that they would support the method's routine use. Results for the four

groups were University of Maryland (65.1%), farmers (55.6%), watermen (73.7%), and municipal officials (84.6%) (Fig. 1).In addition, 330 people (66.2%) indicated that they were willing to pay at least $1 USD for technique implementation, an overall favorable willingness to pay. Demographic characteristics of the surveyed populations such as gender, age, household income, education, and geographical location did not prove to be significant factors ($p>0.05$) in the way the different groups responded.

Table 1. Cost Breakdowns

Item	Cost (in USD)
Labor	175,000
Barge Rental	3,000
Tugboat Rental	480,000
Chitosan	168,000
Sediment*	Free
Total	826,000
Number households in state of Maryland	5,500,000
Cost per household	0.15

*Local sediment at flocculation site is at no cost

Discussion

The minimal costs calculated for routine mitigation of cyanobacteria blooms recurring in Maryland's tidal-fresh areas suggest that funding should not be a major problem for adoption of the technique as a 'standard field procedure' in state waters. Costs could be even be lower, considering results using sediments from the shore surrounding blooms in China, eliminating transportation costs (Zou *et al*. 2006, Pan *et al*. 2010). The general support shown from the small surveyed population of Maryland citizens also suggests that public sentiment might not be as restrictive as noted in Florida for routine treatment of its recurring red tides. However, critical to the positive response identified in the survey is the importance of effective communication with citizens prior to distribution of the questionnaire. At the Watermen's Festival and Maryland Municipal League Conference, members of our research team were able to answer questions prompted by participants and provide more detailed information. A portion of the academic population obtained information from our team at Maryland Day; participants at the Farm Bureau's Picnic were informed through a 5 minute oral summary. The effectiveness of outreach and education could be further tested in the future by carrying out within-group studies, giving varying levels of information to people within the same group and analyzing for differences in willingness to support. Efforts can be made to familiarize the public at, for example, town hall meetings, radio spots, and televised video clips. Another way to further understand factors behind support for or rejection of the proposed method is to ask questions of the groups uncertain on technique application, thereby further indicating the importance of effective education in public willingness and method adoption.

Acknowledgements

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The removal of microcystins in drinking water by clay minerals

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Abstract

Korean Yellow Clay (Whangto) can be used to efficiently remove the cyanobacterial toxin microcystin-LR when present in water. This removal technique can be employed by rural and remote communities when surface water is contaminated with a toxic *Microcystis* sp. bloom. In most cases two sequential applications of clay can bring the dissolved microcystin toxin content below WHO guidelines for drinking water (1 ug/L) (WHO, 1998). The removal process is characterised by adsorption to the clay particle, with the Langmuir isotherm fitting the results marginally better than the Freundlich isotherm.

Introduction

Quality drinking water is a fundamental necessity for the wellbeing of all humans. Cyanobacteria may pose a serious threat to the quality of drinking water, especially to rural and remote communities who are primarily dependent upon surface waters. In the developed world, most of the major cities have water purification systems which can in most cases effectively remove the toxins produced by these organisms. However, in rural and remote communities, where complex water purification systems are unavailable, the populations are at risk of being exposed to cyanobacterial toxins. This risk is heightened when the organisms are in bloom. Microcystins (MCs) are one group of cyanobacterial toxins with worldwide distribution. They are produced by a number of cyanobacterial genera including *Microcystis* sp.. MCs are cyclic heptapeptides and are among the most potent natural toxins known. To date, over 80 different congeners have been characterised. The structure of one of the most common congeners, microcystin-LR (MC-LR), is shown in Figure 1. In continuing studies regarding removal of cyanobacterial toxins from drinking water, we have investigated a simple removal technique that can be easily adapted by rural and remote communities when a *Microcystis* sp.

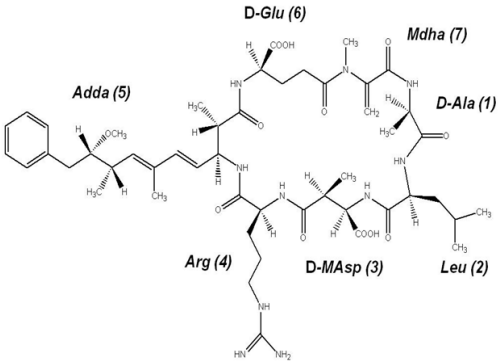

Fig. 1. Structure of microcystin LR

bloom is present. *Microcystis* sp. blooms are the most common cyanobacterial blooms to be found and have a worldwide distribution. Korean Yellow Clay (Whangto) has historically been used to mitigate harmful algal bloom (HAB) events in Korean waters. It is a mineralogically complex alumino-silicate mineral, with a typical composition as summarized in Table 1. Preliminary investigation with this material showed promising results, with rapid equilibration of

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MC-LR achieved in approximately 7 minutes (Fig. 2).

Table 1. Composition of Whangto by X-ray fluorescence

Al_2O_3	CaO	$Fe_2O_3^*$	K_2O	MgO	MnO	Na_2O	P_2O_5	SiO_2	TiO_2	L.O.I.	Total
21.07	0.1	5.1	3.06	0.53	0.03	0.17	0.05	60.23	0.7	8.16	99.2

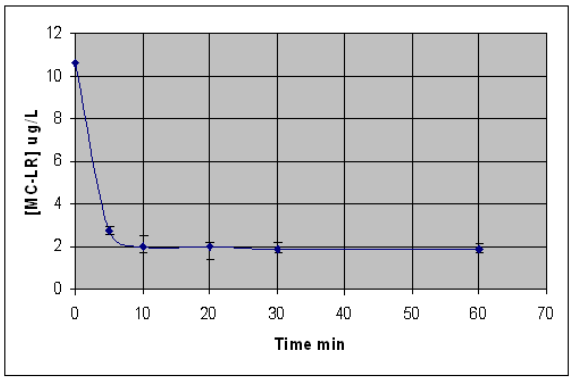

Fig. 2. Removal of MC-LR by Whangto over time

Materials & Methods

Batch sorption experiments were conducted at ambient temperature (23 ^{O}C +/- 2 ^{O}C) in duplicate. The clay material in powder form was added to the test solution (500 mL) in a 1 L Schott bottle. The test solutions consisted of MC-LR dissolved in Milli-Q water. Prior to the addition of clay material, a sub-sample was taken to establish the initial concentration of MC-LR. The bottles were capped and mixed manually for 1 minute every 5 minutes for 1 hour. Two sets of batch sorption experiments involving the Korean Yellow Clay and one set involving bentonite as a sorbent were undertaken. In the first set, using the Korean Yellow Clay, the amount of clay used was kept constant (10 g) while the MC-LR concentration was varied (1.5, 7.3, 13, 17.8, and 22 µg/L). In the second set, the toxin concentration was kept relatively constant (5.9 - 7.3 µg/L), while the amount of clay added was varied (2.5, 5, 10, and 25 g). The bentonite sorption experiments were conducted with a constant MC-LR concentration (5.5 µg/L) and variable amounts of clay (5, 10, and 15 g) only. After one hour, a sample was taken from each of the test solutions and centrifuged at 13,200 rpm to remove any suspended clay. The samples were then analysed in triplicate using an ELISA method. The ELISA kit used (Abraxis, Warminster, PA, USA) was found to give reliable results at these concentration levels.

Results & Discussion

A typical set of results obtained with Korean Yellow Clay are summarised in Table 2. This clay material removed almost 90% of the MC-LR content in a single run.

Table 2. Typical removal data for Whangto

Korean Yellow Clay (Whangto) 10g (20g/L)		
Initial MC-LR Conc (µg/L)	**Final MC-LR Conc (µg/L)**	**% Removed**
22.02	1.93	91.2
17.76	1.17	93.4
13.03	1.72	86.8
7.27	0.96	86.8
1.47	0.17	88.4

Bentonite showed only about 60% removal (data not shown), indicating Whangto type clays are better suited for removal of MCs from drinking water. Coulombic interactions may potentially play an important role in the sorption of MC-LR onto clays. MC-LR possesses two carboxylic acid groups and an amino group capable of ionisation. The pKa values of MC-LR were determined by de Maagd and coworkers (1999) to be 2.09, 2.19 and 12.48. The pH of clay suspension (10 g/L) was found to be 5.6, suggesting that MC-LR in the test solution was largely ionised (anionic). However, since clay colloids are also known to carry negative charges (Tan 1998), the mechanism of sorption of MC-LR on the surface of the clay is unclear. The importance of clay as a sorbent was also found by Chen and co-workers (2006) who observed that the clay content of a studied soil played a more important role in sorption of microcystins than the organic content. Based on their findings, however, the authors proposed an additional sorption mechanism, namely a chemical bonding with metal ions on the surfaces of soil/clay particles (Chen et al. 2006). According to Chen and co-workers (2006), nitrogen and oxygen atoms in the structure of microcystins may enable them to act as chelating ligands with metal ions on the clay surface. The authors further found that from the studied microcystin congeners, MC-RR was more strongly sorbed on the soils than

MC-Dha7LR or MC-LR. Chen et al (2006) suggest that the chemical structures of these microcystins most likely explains the variability, noting that MC-RR contains three extra nitrogen atoms compared to MC-LR, which may provide additional binding positions compared to the other studied MC congeners. However, it has also been found that MC-RR is slightly more hydrophobic than MC-LR with log *Kow* values of 4.4 and 4.2 respectively (Rivasseau et al. 1998). The utility of the various clay materials for drinking water treatment may be determined by the extent to which they are capable of removing the toxin from water. Morris and coworkers (2000) report a removal of more than 81% by marine sediment consisting of naturally occurring clay minerals, predominantly montmorillonite (bentonite) and kaolinite. Their findings are consistent with our results of overall removal of more than 83% of MC-LR from aqueous solution observed for the Korean Yellow Clay. Commercial grade bentonite (montmorillonite) by itself, however, showed somewhat lower removal of MC-LR from water, averaging at about 55%. It was also observed that most of the Korean Yellow Clay settled out of the solution in approximately 5 to 10 minutes after mixing, while bentonite remained suspended in the solution for several hours. This may have important practical implications for drinking water treatment favouring the Korean Yellow Clay over bentonite, with less load on the filters for eventual removal of the sorbent.

Conclusion

Laboratory scale experiments have shown that Korean Yellow Clay (Whangto) can efficiently remove a significant amount of dissolved microcystin-LR. In most cases, two sequential applications of this clay can bring the dissolved microcystin toxin content below WHO guidelines for drinking water (1 ug/L) (WHO, 1998). The removal process is characterised by adsorption to the clay particle, with the Langmuir isotherm (Figure 3) fitting the results marginally better than the Freundlich isotherm (goodness of fit 94 and 87% respectively).

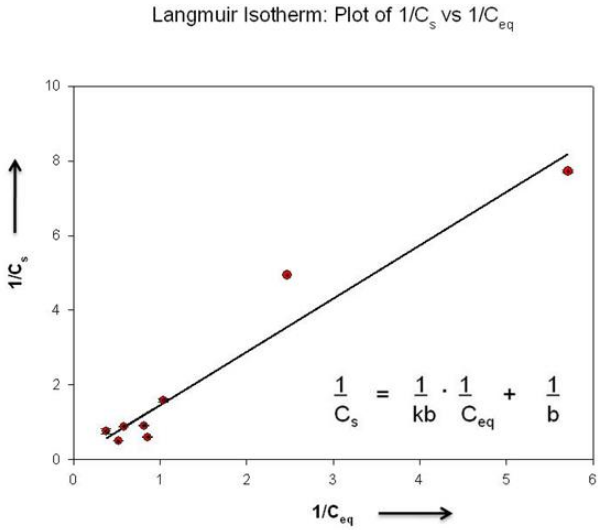

Figure 3. Langmuir adsorption isotherm for MC-LR/Whangto system

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Suppression of the dinoflagellate *Noctiluca scintillans* by algicidal bacteria for improving shrimp aquaculture

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Abstract

For suppressing growth of the dinoflagellate *Noctiluca scintillans*, which often causes diseases in shrimp culture and economic losses due to increased shrimp mortality, we tried to isolate and use algicidal bacteria to assess the possibility for improving shrimp aquaculture. In 260 isolates, 10 strains of algicidal bacteria were isolated from shrimp culture ponds. Among these, strain BS2 showed the strongest killing activity on *N. scintillans*.-When BS2 was not added to the culture of two species of shrimp (*Penaeus monodon* and *Litopenaeus vannamei*) with *N. scintillans*, nearly 80-90 % of shrimp died within 7 days. However, when BS2 was added to the culture of shrimp with *N. scintillans,* this strain could inhibit *N. scintillan*s growth in shrimp culture within 48 hours of incubation and shrimp survival rates increased from 23 to 87 % and 13 to 87% of *P. monodon* and *L. vannamei*, respectively. BS2 did not impact on shrimp condition at all. The results suggest that applying algicidal bacteria for suppressing *N. scintillans* in the field would be promising for stable shrimp aquaculture.

Introduction

Blooming of dinoflagellate *Noctiluca scintillans* in shrimp culture stresses the shrimps and results in lower or loss of shrimp production by the occurring of diseases in shrimp culture (Songsangjinda et al., 2006). Algicidal bacteria could behave in suppressing or killing HABs in coastal areas (Fukami et al., 1992; Yoshinaga et al., 1997; Doucette et al., 1998). However, no studies refer to the role of algicidal bacteria in shrimp culture ponds. Thus, it is essential to study the effect of isolated bacterial strains which inhibit the growth of HABs, in particular *N. scintillans* in order to evaluate the possibility of using algicidal bacteria in shrimp culture ponds. This is expected to be an alternative method to maintain and improve shrimp growth and production.

The aim of this study was to isolate algicidal bacteria from shrimp culture pond water and to evaluate the killing activities on *N. scintillans* under laboratory condition. The suppressing effect of algicidal bacteria on the growth of *N. scintillans* for improving shrimp survival was investigated.

Materials and methods

N. scintillans was isolated from Angsila, Mueang, Chonburi province, Thailand. The culture was maintained by incubating at 28±1 ºC in SWM III media. under 12:12 h light:dark cycle with *Dunaliella* sp. as prey. Algicidal bacteria were isolated from the shrimp culture pond during the phytoplankton bloom in June-July 2008 at Dumrong shrimp farming, Tepha, Songkhla province, Thailand. The procedure of bacterial isolation is shown in Fig. 1. All isolates were screened for killing effect on *N. scintillans* and cell change was observed by light microscopy. A bacterium BS2, showing the greatest killing effect on *N. scintillans*, was used for estimating its effect on other phytoplankton species (*Heterosigma akashiwo, Chattonella antiqua, Chaetoceros ceratosporum, Prorocentrum lima* and *Dunaliella sp*.), to ensure its killing specificity. This strain was also used for a trial on suppressing growth of

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N. scintillans under shrimp culture conditions. *Litopenaeus vannamei* (White shrimp) and *Penaeus monodon* (Black tiger shrimp) with a body length about 1.5-1.8 cm (small size) and about 3.5-4.0 cm (big size) were used. Initial algal density was 10 cells/ml and algicidal bacterial density was ~10^5 cells/ml. The algal abundance and the shrimp survival were daily investigated up to 7 days of the trial.

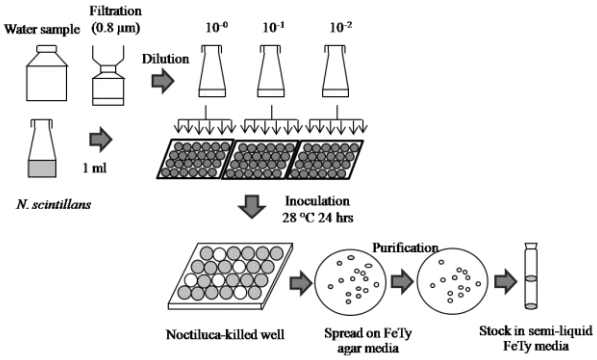

Fig 1 The procedure of algicidal bacteria isolation using a 24-wells tissue microplates.

Results and Discussions

In 260 bacterial isolates, 10 strains showed some killing effect on *N. scintillans*. Among these, strain BS2 showed the greatest killing effect (data not shown). After inoculation of BS2 to *N. scintillans*, the normal vegetative cell became rounded, expanded and finally bursted. In the present study, we emphasized the bacterial effects on the growth of another phytoplankton species. As shown in Fig. 2, the strongest *Noctiluca*-killing strain BS2 showed killing effect only on *N. scintillans* but no influence on other species of phytoplankton. So far, most isolates killed many plankton species and very few were species-specific (Fukami et al, 1991). Some bacteria in natural environments were controlling the growth of HABs (Fukami et al., 1991; Clinton et al., 2005) and inhibiting the development and/or terminating of HABs populations (Fukami et al., 1995; Imai et al., 1998).

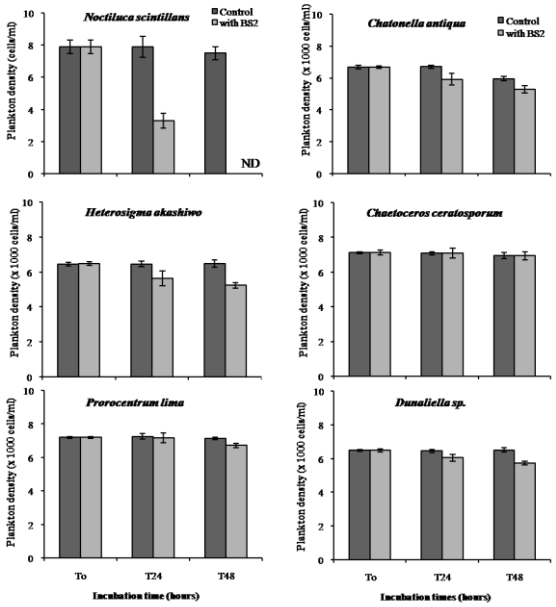

Fig 2. Effects of algicidal bacteria (BS2) on growth of other phytoplankton (*H. akashiwo, P. lima, C. antique, C. ceratosporum and Dunaliella sp.)* at 0, 24 and 48 hours of incubation time. Control without BS2 cell. ND is not detected plankton cells. Data are means of 5 replicates with standard deviations.

Our results indicate that *Noctiluca*-killing bacteria are distributed widely and may control *Noctiluca* in the shrimp pond. Bacterial isolate BS2 was tested in the treatment against *N. scintillans* in shrimp rearing conditions (SNB), along with a control (SN) (Fig. 3). The result showed that the *N. scintillan*s cell abundances in treatment SN, with no adding bacterial BS2, increased after incubation 48 hours and were about 20 cells/ml at 120 hours of the incubation time. In contrast, in the treatment to which bacteria BS2 (SNB) were added, *N. scintillan*s cells decreased and disappeared after 48 hours of incubation (data not shown). In treatment without BS2, nearly 80-90 % of shrimp died within 7 days (Fig. 3), probably due to suppression by *N. scintillans.* The effect of *N. scintillans* was stronger on smaller size than bigger size of shrimp. On the contrary, strain

BS2 could inhibit the *N. scintillan*s growth in shrimp culture within 48 hours of inoculation time and small shrimp survival rates (small size) were improved from 13 to 87% and 23 to 87 % in *L. vannamei* and *P. monodon*, respectively (Fig. 3). In addition, the algicidal bacteria strain BS2 did not show any impact on growth and mortality of shrimp. In conclusion, algicidal bacteria could inhibit harmful phytoplankton growth and improve shrimp survival and the algicidal activity showed no impact on shrimp. Therefore, to use algicidal bacteria in shrimp culture conditions is promising and the possibility for applying algicidal bacteria must be further studied.

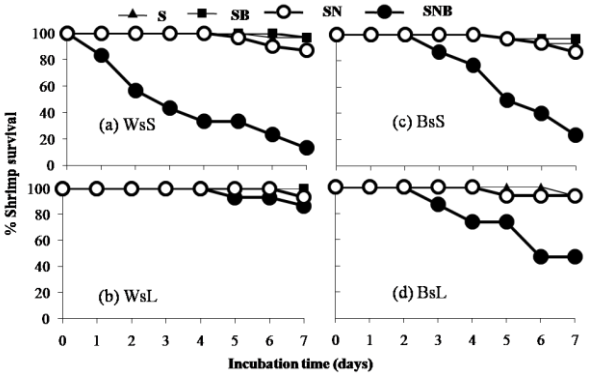

Fig. 3. Percentage of shrimp survival after inoculation of *N. scintillans* and algicidal bacteria strain BS2. (S: shrimp, SB: shrimp+BS2, SN: shrimp+ *N. scintillans*, SNB: Shrimp + *N. scintillans+BS2*). WsS: *L. vannamei* (small size) WsB: *L. vannamei* (big size), BsS: *P. monodon* (small size) and BsB: *P .monodon* (big size).

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Levels of PSP toxins in bivalves exposed to natural blooms of *Alexandrium minutum* in Catalan harbours

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Abstract

The development, validation, comparison and evaluation of analytical methods for marine toxins rely on the availability of toxic material. Within the project JACUMAR PSP, our interest is mainly focused on autochthonous bivalve species with the toxic profile of *Alexandrium minutum*, since this is the principal species involved regionally in PSP outbreaks. Mussels and oysters were exposed during few days in the harbor of Vilanova i la Geltrú, to blooms reaching a maximum *A. minutum* concentration of 200,000 cells L^{-1} in 2008, and 40,000 and 800,000 cells L^{-1}, in 2009. Mussels, oysters and clams were exposed to one bloom of 22,000 cells L^{-1} in the harbor of Cambrils in 2009. In all situations higher toxic levels analyzed by HPLC-FD with postcolumn oxidation were observed in mussels (i.e. 1,200-2,500 µg eq. STX kg^{-1}) than in oysters (i.e. 60-800 µg eq. STX kg^{-1}) exposed to the same bloom. Blooms with higher concentrations of *A. minutum* did not correspond to higher levels of PSP toxins in bivalves. These differences may be explained by differences in *A. minutum* population dynamics, toxin production or in the physiological state or behaviour of shellfish. These results confirm that mussels concentrate more PSP toxins from *A. minutum* than oysters and clams.

Introduction

Marine toxins limit the development of the bivalve aquaculture industry. In order to guarantee the optimal development of the aquaculture industry and to assure the maximum safety level for consumers, monitoring programs for marine toxins need to implement robust and reliable methodologies for the quantification of toxins present in shellfish. Within the Spanish aquaculture framework, paralytic shellfish poisoning toxins, PSP (saxitoxins, STX, and derivatives) that are potentially responsible for serious food-borne intoxications are of particular relevance and are present all along the Spanish coastline. The project "Comparison of methodologies for the evaluation of Paralytic Shellfish Poisoning (PSP) toxins in bivalves. Application for aquaculture in Spain", funded by Junta Nacional Asesora de Cultivos Marinos (JACUMAR) aims to compare five methods for detecting PSP toxins, applied to samples from Spanish coasts: mouse bioassay (MBA), chromatographic methods (HPLC-FD with pre- or post-column derivatization), Jellett™ Rapid Test kits and cell-based assays (CBA). One of the first difficulties was the obtaining of large amounts of shellfish samples naturally contaminated with PSP toxins produced by any of the two microalgal species mainly involved in toxic outbreaks in Spain, *Gymnodinium catenatum* and *Alexandrium minutum*. The acquisition of shellfish samples contaminated with PSP toxins produced by *A. minutum* was by exposing mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and, in one occasion, clams (*Ruditapes* sp.) to blooms which occurred in two Catalan harbours in 2008 and 2009. This activity produced the amounts of samples required for the right performance of the comparison between techniques and participants, as well as data about the contamination of different shellfish species exposed to the same bloom in natural conditions.'

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Materials and methods

The exposure of shellfish to blooms of *A. minutum* in a harbour was made four times; three in the harbour of Vilanova i la Geltrú (41º12'N 1º44'E), and one in the harbour of Cambrils (41º03'N 1º03'E). In each exposure, shellfish were collected from one of the two Ebro Delta bays, Alfacs Bay or Fangar Bay. The different species of shellfish were suspended at the same point, the same depth and for the same time (Table 1). At the end of the determined time of exposure, all the shellfish were opened by cutting the adductor muscle, edible tissues were completely removed from the shell, and drained in a sieve to remove salt water before homogenization of the pooled individuals of the same species. Samples were then kept frozen (less than -15ºC) until their analysis. Aliquots of 5 ± 0.1 g of the homogenate were accurately weighed into a 50-mL falcon tube. Each aliquot was extracted with 10 mL of HCl 0.1N (double extraction, without heating); 5 mL of the extract were purified with 250 μL trichloroacetic acid 30% (TCA 30%) and the final pH was corrected to 3 with NaOH 1M. The purified extracts were filtered through 0.2 μm before their analysis by HPLC with fluorescent detection (HPLC-FD) with post-column derivatization (Franco and Fernandez Vila, 1993). A sample of seawater was taken at the same point where shellfish were suspended, at the beginning of each exposure, and fixed with buffered formaldehyde for the determination of the density of *A. minutum* by the method of Utermöhl (1958).

Results and discussion

The strategy of exposing shellfish to blooms of *Alexandrium minutum* (densities over 1,000 cells L^{-1}) occurring in small harbours succeeded in the acquisition of large amounts of contaminated shellfish matrixes (Table 2), except in the case of clams. The main PSP toxins were gonyautoxins 1, 2, 3 and 4 (GTX1, GTX2, GTX3 and GTX4); the typical toxic profile produced by *A. minutum*. The ratio of each PSP toxin varied depending on the shellfish species and the level of contamination of the sample. Mussels presented higher levels of PSP toxins than oysters and clams. Nevertheless, the content of PSP toxins did not correlate to the densities of *A. minutum*. It can be observed that the highest concentration of PSP toxins (exposure in Vilanova i la Geltrú on February 2009; the density of *A. minutum* was 39,840 cells L^{-1}) did not relate to the highest concentration of *A. minutum* (exposure in Vilanova i la Geltrú on March 2009; the toxin content in mussels was 1,256.9 μg eq. STX kg^{-1} flesh). This can be explained by the aim of this strategy. The main objective was the acquisition of large amounts of contaminated shellfish, thus, we lack of additional data for explaining the differences found between experiences. We can hypothesize explanations such as: differences in *A. minutum* population dynamics, differences in the toxin production of populations of *A. minutum* geographically separated or blooming at different seasons of the year, differences in the physiological state or filtering behavior of shellfish, differences in the hydrodynamics of the harbour, etc. It can be even a sampling bias, because we have a single estimate of *A. minutum* concentration for each exposure so we do not know to what concentration of *A. minutum* were exposed .

Conclusions

The exposure of shellfish to natural blooms of *A. minutum* in harbours is a good strategy for the production of naturally contaminated samples, useful for the development and validation of analytical methods or to be used as reference material. Mussels accumulate more PSP toxins produced by *A. minutum* than oysters and clams under the same exposure conditions.

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Table 1. Exposures of shellfish to natural blooms of *A. minutum* in two Catalan harbours during 2008 and 2009.

Date	Shellfish origin	Shellfish species	Harbour	Days of exposure
April 2008	Alfacs bay	Mussels Oysters	Vilanova i la Geltrú	4
February 2009	Alfacs bay	Mussels Oysters	Vilanova i la Geltrú	6
March 2009	Fangar bay	Mussels Oysters	Vilanova i la Geltrú	3
May 2009	Fangar bay	Mussels Oysters Clams	Cambrils	8

Table 2. Results obtained in each exposure. Note that the percentages of PSP toxins (GTX 1, 2, 3 & 4) are the proportion of each compound in the total concentration of the extract, not their contribution to the total toxicity (μg eq. STX kg^{-1} flesh).

Date	Harbour	*A. minutum* density (cells L^{-1})	Shellfish species	Toxic flesh (g)	μg eq. STX kg^{-1} flesh	%GTX1	%GTX4	%GTX2	%GTX3
April 2008	Vilanova i la Geltrú	200,000	Mussels Oysters	1,000 100	1,277.5 1,043.0	26 44	67 42	3 5	4 5
February 2009	Vilanova i la Geltrú	39, 840	Mussels Oysters	1,000 100	2,702.7 849.1	34 29	37 44	27 25	2 2
March 2009	Vilanova i la Geltrú	817, 550	Mussels Oysters	1,000 250	1,256.9 30.5	42 0	27 50	27 50	1 0
May 2009	Cambrils	21,995	Mussels Oysters Clams	1,200 600 250	45.9 1.5 n.d.	0 0 0	100 100 0	0 0 0	0 0 0

Risk Management of Farmed Mussel Harvest Bans due to HAB Incidents in Greece

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Abstract

The severity and the consequences of site closures to shellfish commercial harvesting, a protection measure for public health against toxicity inflicted by harmful algal blooms, has been estimated for the Mediterranean mussel (*Mytilus galloprovincialis*) farming in Greece. Estimations were carried out in a semi-quantitative manner at the farm level. Results showed that the financial losses depended on the season and duration of the harvest ban. Since the product becomes marketable from late spring to early autumn site-closures longer than 6 weeks within that period could be catastrophic for a farm. Consequences include yield losses due to extended stocking of ready to harvest mussels in the farm and ex-farm price reduction due to oversupply after the harvest ban. Moreover, mussel seed collection and placement within the farm is delayed due to lack of space as the bulk of mussels remain un-harvested putting in danger next season's production. Proposed strategies to minimise losses consisting of differential handling of the marketable mussels and of extraordinary spatial extension of farm facilities due to harvest bans caused by HABs were discussed.

INTRODUCTION

The shellfish harvest ban imposed by the authorities due to toxic phytoplankton blooms (included by definition in the harmful algal blooms or HABs) it is considered to have a significant economic impact on the mussel farming industry. However, no well documented assessment of the potential losses exists capable to lead in an objective quantification assisting hence, stakeholders' decisions. Such an assessment should take in account that mussel farming has a seasonal character and is thus differentially affected by timing or length of site closures. Furthermore, there are indirect risks associated with the over or under reaction of the stakeholders involved in the ban decisions (authorities, scientists, farmers) rendering more important the use of a risk assessment approach for the harvest ban rather, than a HAB's actual occurrence. In this study such an approach was adopted for the Greek mussel farming industry to raise a tool and data set that might help the decision makers to promote win-win risk management strategies. Issues pertaining to overstocking due to product harvest stop, overwhelming price drops due to massive offering at site opening, availability of farm space for new stock handling etc., further highlight the need for such an approach. Shellfish farming in Greece is a relatively new industry based almost exclusively on the cultivation of the Mediterranean mussel *Mytilus galloprovincialis*. As the marine environment in Greece is usually oligotrophic few sites are suitable for effective mussel farming, restricted mainly in the delta areas of some big rivers. The industry today involves about 500 operating mussel farms, occupying an area of *ca*. 400 ha structured in average in 1-2 ha each. Although hanging park technology in shallow waters was first to be implemented, due to space competition all farms licensed

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after 90's use the single long-line floating technology in deeper waters with an average of 100 tonnes/ha annual production capacity. For 2010 the industry was estimated to achieve *ca*. 20,000 tonnes with an annual turnover of 9 M € of net product with a near future forecast of up to 45,000-50,000 tonnes based on potential areal capacities (Theodorou *et al.*, 2011). The aim of the present study was to identify the qualitative characteristics of losses in the Greek mussel farming industry resulting from corresponding harvest bans due to HABs, in an effort to aid the development of suitable risk-management policies.

MATERIALS & METHODS

The method used to analyse the consequences of HABs closures on Greek shellfish farming has been adapted from the formal risk assessment process of the Australian & New Zealand Standard Risk Management AS/NZS 4360:1999 and the companion paper on Environmental Risk Management – Principles and Process (HB 203:2000) following the guidelines developed by Fletcher *et al.,* (2004**),** to support an Ecologically Sustainable Development (ESD) reporting framework for aquaculture in Australia. Experts (n=8) with more than 10 years of experience in the shellfish aquaculture industry and farmers (n=48) from different parts of Greece were interviewed between October and December 2008 to give their opinion and personal information regarding the effects of the HABs closures on mussel farming.

Table 1. HAB Incidents Risk Ranking & Likely Management Response. Information on actions needed in the text.

Risk Rankings	***Risk Values***	***Likely Management Response***
Negligible	0	Nil
Low	1 - 6	no specific action needed to achieve acceptable performance
Moderate	7 - 12	specific management needed to maintain acceptable performance
High	13 - 18	possible increase in management activities needed
Extreme	> 19	likely additional management activities needed (e.g. public compensation)

According to their responses consequences of HAB closures were ranked for their severity using a scale from 0 (negligible) to 5 (catastrophic) while the necessity for taking actions was ranked for each severity category according to Table I.

RESULTS & DISCUSSION

The farming of the Mediterranean mussel *Mytilus galloprovincialis* in Greece follows a seasonal scheme starting from seed stocking on ropes with specialised spat collectors during winter to early spring. Then by consecutive transfer to new ropes to provide more suitable space to the animals as they grow out (tubing), harvest occurs during summer to early autumn after shell length reaches 6 cm.

The experts viewed the consequences of a mussel harvest ban depending on the season reflecting thus the growth phase of the Mediterranean mussel. Therefore, the farmers were asked to rank the severity of a harvest ban for each season, and also to give their opinion on likelihood of occurrence and the degree of necessity to take actions in order to compensate for the probable losses. Results are given in Table II.

The most probable period for high losses was between March to August. A harvest ban exceeding 6 weeks in March to April or even 4 weeks in May to August was considered in general catastrophic with irreversible consequences that could not be confronted and could only be compensated via support from the government (if any). In contrast, a harvest ban of less than 2 weeks year round did not seem to cause any noticeable damage to the farms. Moreover, any ban from November to December was considered completely indifferent by the farmers. A site closure due to HABs for more than 2 weeks from January to October requires special actions from the farmers to prevent losses. If the ban lasts more than 4 weeks in March to April or 3 weeks in May to August or 6 weeks in September to October then an intensive management plan was required. This plan consists of harvesting, de-clumping, grading, cleaning and packing of the marketable mussels in 10 kg netting bags, finally re-immersing them into the farm's waters. This way the mussels could first

recover from treatment and then survive for up to 2 weeks without problems in the hope that the ban could meanwhile be lifted.

A 2 weeks closure in high season between March to August was considered affordable. A 2-6 weeks closure during fall and winter calls only for optional proactive responses such as preparations for the intensive management plan and may be some packing of a reasonable volume as backup plan. During spat collection or planned tubing operations an excessive closure (over 4 weeks) was considered also dangerous calling for intensive management plans. In conclusion, the consequences of an imposed harvest ban on mussel farming were considered by the Greek farmers different between seasons, rating their losses or necessary countermeasures high during the harvest period of late spring to late summer for a length exceeding 3 weeks.

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Table 2. Risk Matrix. Numbers indicate risk value as in Table I. Shades indicate risk rankings (shade ranking lighter to deeper as risk increases).

Consequences			***Minor***	***Moderate***	***Severe***	***Major***	***Catastrophic***
Likelihood	**Levels**		**1 - 2 wks** ***1***	**2 - 3 wks** ***2***	**3 - 4 wks** ***3***	**4 - 6 wks** ***4***	**< 6 wks** ***5***
Remote	**Nov-Dec**	***1***	1	2	3	4	5
Rare	**Jan-Feb**	***2***	2	4	6	8	10
Unlikely	**Sep-Oct**	***3***	3	6	9	12	15
Possible	**Mar-Apr**	***4***	4	8	12	16	20
Occasional	**May-June**	***5***	5	10	15	20	25
Likely	**July-Aug**	***6***	6	12	18	24	30

Production and harvesting management of Greek cultured mussels linked to toxic episodes of lipophilic toxins: optimization possibilities

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Abstract

Management of mussel culture production based on previous data regarding time period, duration and factors affecting lipophilic biotoxins episodes can significantly contribute to economic losses' reduction. Data from mussel culture areas representing ca. 95% of Greek production (Saronikos, Thermaikos, Maliakos, Gulf of Kavala and Amvrakikos Gulf) during 2003-2008 were subjected to statistical analysis. These data included periods when mussel harvesting in production areas was banned due to lipophilic biotoxins, as well as abundances of *Dinophysis*. In combination with climatological parameters (wind direction and intensity, rain precipitation and temperature) during the same periods, useful conclusions were obtained concerning factors which can affect growth of *Dinophysis* spp. *Dinophysis* spp. maximum growth coincided with temperatures between 10-20 ºC, while its major abundances were recorded at 4-20ºC and with low intensity (up to 4 beauforts) winds, blowing mainly from north (NW, N and NE) and south (SE and S) directions, and low rainfalls. Such features contribute towards clarification of DSP toxic episodes and more efficient management of mussel production.

Introduction

One of the most important group of lipophilic toxins is the OA group of Diarrhetic Shellfish Poisoning (DSP) toxins. Presence of DSP toxins is connected to blooms of toxic *Dinophysis* and to a lesser extent *Prorocentrum*. Seasonal periodicity of *Dinophysis* spp. during the year has been common in Greece (Koukaras and Nikolaidis, 2004; Nikolaidis *et al.* 2005), other European countries (Bernardi Aubry *et al.* 2000; Vale and Sampayo, 2003; France and Mozetič, 2006; Ninčević-Gladan *et al.* 2008), and worldwide (Koike *et al.* 2000; Morton *et al.* 2009; Swanson *et al.* 2010). This could be partly attributed to regional climatological parameters able to influence nutrient availability and/or water column conditions, which can largely affect *Dinophysis* spp. growth patterns (Estrada and Berdalet, 1997; Vale and Sampayo, 2003). Such climatological parameters include wind intensity and direction, temperature and rain precipitation. During 2000-2008 in Greece, according to available data of the National Reference Laboratory for Marine Biotoxins (NRLMB), marine biotoxin episodes are almost exclusively due to lipophilic toxins of the DSP group (Mouratidou *et al*. 2004; Prassopoulou *et al*. 2009; Louppis *et al*. 2010). These toxins occur in production areas on a yearly basis. Duration and time of toxicity, however, does not always coincide, even between adjacent areas. The possibility of narrowing or avoiding coincidence of regulatory closure periods due to lipophilic toxin episodes with periods of shellfish commercialization could significantly contribute towards more efficient time management of regional shellfish production. The aim of this study is the investigation of temporal and spatial distribution of *Dinophysis* spp. and lipophilic toxins in the whole of Greece together with the influence of climatological parameters.

Data and methods

Data were obtained during implementation of the "National Program for Monitoring of Bivalve

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Molluscs' Production Areas for the presence of Marine Biotoxins", coordinated by the Ministry of Rural Development and Food (MRDF). The laboratories involved in this monitoring program are the NRLMB (MRDF) conducting the shellfish toxicity analyses and Laboratory Unit of Marine Toxic Microalgae, School of Biology, Aristotle University of Thessaloniki, responsible for counting toxic/ potentially toxic microalgae in sea water, among which *Dinophysis* spp. cell counts. The study covered the period of 2003-2008, whereas sampling frequency was at least weekly or more frequent whenever abundances of toxic and/or potentially toxic phytoplankton exceeded established surveillance limits. The area includes the five most important Greek gulfs of mussel culture activity: (1) Saronikos, (2) Thermaikos, (3) Gulf of Kavala, (4) Amvrakikos and (5) Maliakos Gulfs (Fig 1).

Fig 1. Production areas covered in the study.

Fig 2. Location of the meteorological stations

Climatological data (temperature, wind direction and intensity, rain precipitation) were obtained from the Hellenic National Meteorological Service (HNMS) from stations near the areas of interest (Fig 2). Statistical methods included one-way Analysis Of Variance (ANOVA), with mean confidence interval set at 95% and calculated by the ANOVA error and Principal Component Analysis (PCA). Statistical analysis was by Minitab® (v.15). Data categorization was required for processing certain parameters, e.g. *Dinophysis* spp. abundances (Table 1).

Table 1. Categories of *Dinophysis* cell abundances according to Greek National Monitoring Program

Category	*Dinophysis* spp. (cell/l)	Regulatory status of area
1	0-199	Open
2	200-999	Under surveillance
3	≥1000	Closed

Results

Dinophysis spp. abundance levels exceeding those leading to closures (≥1000 cells/l) were connected to climatological parameters as follows: a) Temperature: ≥1000 cells/l were recorded more frequently at temperatures 4-20°C, the whole range being between -2ºC and 31°C (Fig 3a).; b) Rain: levels coincided more frequently with no or little rain and less frequently with more intense rain (Fig 3b); c) Wind direction: Increased *Dinophysis* were recorded with most wind directions, while the highest cell densities coincided with NW winds (Fig 3c).; d)Wind intensity: ≥1000 cells/l were encountered exclusively with weak (1-2 Beauforts) to moderate (3-4 Beauforts) winds (Fig 3d). There existed an inverse relationship between them.

When examining concentrations of *Dinophysis* versus time of the year, expressed on fortnightly basis, and temperature, a clear increasing trend was observed in the temperature range 10-20ºC which coincided in the time period between early March and early May (Fig 4). According to results of the PCA with regard to factors influencing occurrence of DSP (Fig 5), temperature and time seem inversely related to *Dinophysis* counts, accounting for episode seasonality and periodicity. There was evident periodicity in *Dinophysis* spp. presence with the highest cell counts recorded in winter-spring months, while their presence was negligible in summer-autumn months (Fig.6). Reduction of *Dinophysis* spp. counts was possibly connected with SW (225º) winds, although this could not be safely concluded.

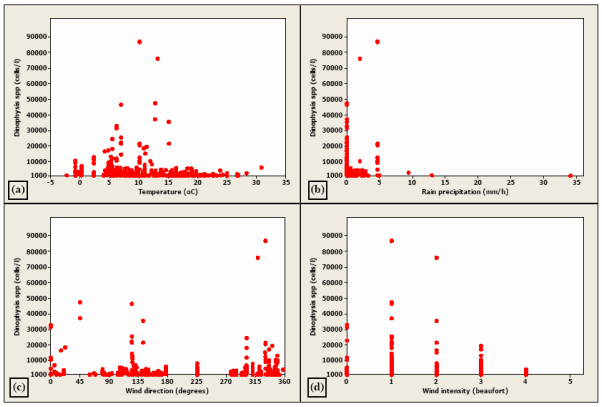

Fig 3. *Dinophysis* abundance levels of ≥1000 cells/l in relation to: (a) temperature, (b) rain precipitation, (c) wind direction and (d) wind intensity.

Conclusions

This is the first study on the relationship of *Dinophysis* with climatological parameters to include 6 years (2003-2008) and covering the main Greek production areas. Maximum *Dinophysis* counts were mostly recorded at temperatures between 4-20^{o}C and in conjunction with low intensity winds (up to 4 Beauforts), blowing mainly from north (NW, N and NE) and south (SE and S) directions

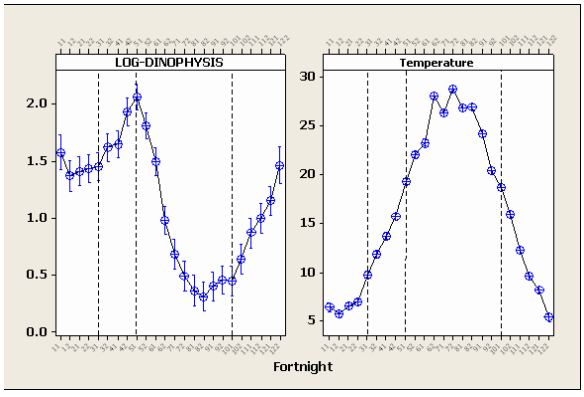

Fig 4. *Dinophysis* spp. concentrations versus time of the year (fortnight) and temperature.

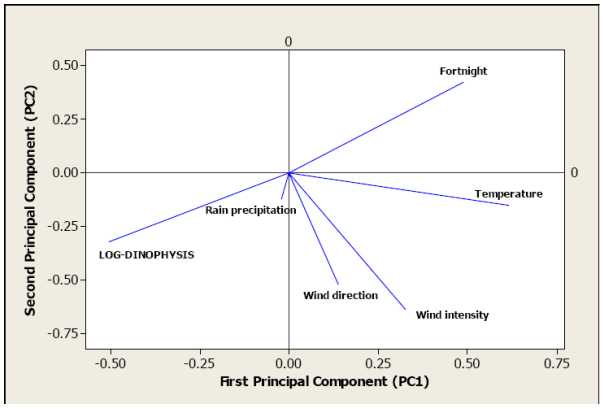

Fig 5. Principal Component Analysis of factors influencing DSP toxic episodes

and low rainfalls. The most probable combination of climatological parameters for occurrence of *Dinophysis* blooms seems to be when temperature ranges between 0-20^{o}C, most importantly 10-20^{o}C, with no or low rainfall and no or low intensity winds from north and south directions

Dinophysis blooms showed a clear periodicity, occurring in winter and spring and repeated every year. These observations can contribute towards effective management of mussel culture production.

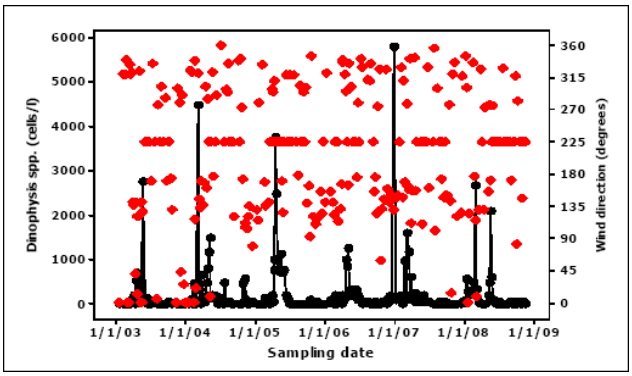

Fig 6. *Dinophysis* spp. counts versus sampling date (years 2003-2008) and wind direction.

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Biofouling Tunicates on Aquaculture Gear as Potential Vectors of Harmful Algal Introductions

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Abstract

Biofouling tunicates are ubiquitous in coastal systems and among the main overgrowers of aquaculture gear. Our study tests the hypothesis that the proliferation of abundant tunicate fouling species and their subsequent transport, removal and transfer by aquaculturists provide mechanisms for concentration and distribution of harmful algal cells to new areas. Wild-caught species of common, biofouling ascidians (= tunicates; sea squirts) (*Styela clava, Molgula manhattensis, Bottryloides violaceus, Didemnum vexillum* and *Botryllus schlosseri*) were exposed individually to cultured strains of harmful algae (*Prorocentrum minimum, Alexandrium fundyense,* or *Heterosigma akashiwo*) at simulated bloom densities of each HAB. After feeding, ascidians were transferred and kept for 48h in ultrafiltered seawater. After 24 and 48h, biodeposits were collected and observed microscopically for the presence of intact and possibly viable cells. Subsamples of biodeposits were transferred into algal culture medium and monitored for algal growth. Thus far, cells of three HAB species have been found to pass intact through the digestive system and remain viable. Potential mitigation strategies associated with transporting harmful algal species through movement of shellfish aquaculture gear and disposal of biofouling material are being investigated.

Introduction

Both harmful algal blooms (HAB) and biofouling cost the shellfish aquaculture industry millions of dollars annually. Economic impacts of HABs worldwide have been estimated to be in the range of $50M (Anderson et al. 2000). Biofouling by invasive tunicates (= sea squirts, ascidians) causes extreme economic damage in aquaculture by fouling man-made structures and by overgrowing and out-competing resource species, e.g. oysters, mussels, scallops (Greene and Grizzle 2007; Braithwaite et al. 2000). A recent study by Piola and Johnston (2008) concluded that small-scale disruptions (i.e. fouling) are a potential risk for the translocation of new species even to "highly protected" marine areas.

Translocation in bulk of biofouling material is unregulated and in many places common practice during the cleaning of aquaculture gear. Other aquaculture practices, such as the relocation of shellfish, have already been shown to be a potential vector of HAB introductions (Hégaret et al 2008). Hégaret et al. (2008) conclusively proved that HAB cells could pass intact though the digestive system of different bivalved mollusc species and remain viable, thus posing a potential threat for new introductions. We propose that biofouling ascidians may also serve as a potential vector of HAB introductions, as persistent species are known to co-occur with biofouling tunicate species.

Materials and Methods

Ascidians (*Styela clava, Molgula manhattensis, Bottryloides violaceus, Didemnum vexillum* and *Botryllus schlosseri*) were collected from local waters in Long Island Sound, CT and maintained in 0.22-µm filtered seawater (FSW) for 24h prior to

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experiments. Exposure assays were performed according to methods outlined by Hégaret et al. (2008) with minor modifications. Briefly, each individual animal was exposed to a bloom concentration of a cultured co-occurring harmful algal species obtained from the NOAA, Milford laboratory collection (10^4 cells ml^{-1} for *Prorocentrum minimum*, 10^3 cells ml^{-1} for *Alexandrium fundyense*, or 10^4 cells ml^{-1} for *Heterosigma akashiwo*). Each species was exposed to a different harmful algal species (Table 1) for a period of 24h in a 1L basin. Each animal was used once per exposure and all experiments were conducted at 20ºC. 500µL sub-samples of the water were taken at T_0 and T_{60} of the exposure assay to calculate feeding rates using flow cytometric techniques. After exposure, fecal samples were collected and animals were placed in 0.22-µm FSW for a 48h depuration period. Fecal samples were collected at 24h and 48h during this time. Fecal pellets were observed under a fluorescence microscope for the presence of potentially viable cells. Collected feces were cultured in autoclaved FSW at 18ºC with a 12:12 photoperiod and monitored for cell motility and growth.

Table 1. Ascidian-HAB interactions tested to date. First column represent the sea squirt species used, top row represent the HAB species in the exposure assay. White boxes show combinations that have not yet been tested. Grey boxes show combinations that resulted in re-establishment of the population of HAB cells incubated 24 h post-ingestion.

	Prorocentrum minimum	***Alexandrium fundyense***	***Heterosigma akashiwo***
Styela clava		***N=7***	***N=7***
Molgula manhattensis	***N=10***	***N=9***	***N=8***
Bottryloides violaceus	***N=11***	***N=9***	***N=12***
Didemnum vexillum	***N=2***	***N=3***	***N=3***
Botryllus schlosseri	***N=11***	***N=10***	***N=10***

Results

Mean filtering rates for the various tunicate species ranged from 4 to 23 L h^{-1} g dry wt^{-1} (data not shown), higher than what has been reported for most bivalve molluscs at this temperature. Fecal pellets from all of the exposures were found to have some fluorescent cells, indicating the presence of potentially viable HAB cells (Figure 1). Some HAB cells were viable up to 48h post-ingestion for at least one of the ascidian species. These cells were able to re-establish a population post-ingestion.

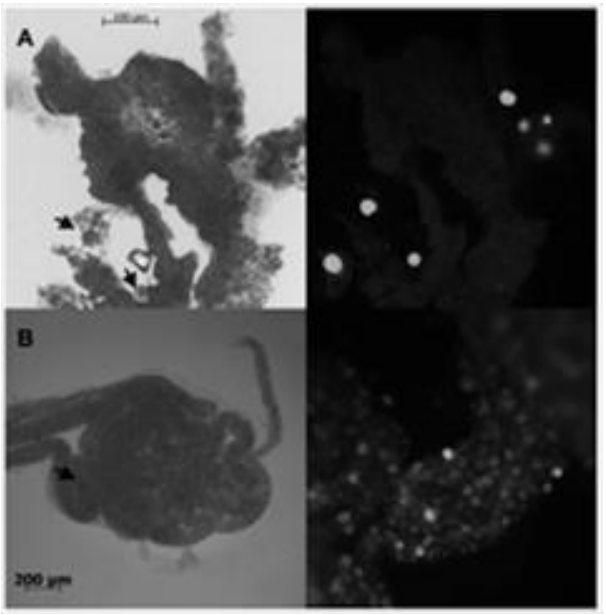

Fig. 1. Microscopic images of tunicate fecal pellets. Left panels are fecal pellets under light microscopy from A) *B. violecus* and B) *M. manhattensis.* Arrowhead indicates *A. fundyense* cells in the feces. Right panels are the fecal pellets using red fluorescence filter. Fluoresence indicates presence of chlorophyll a and potentially viable cells.

Thus far, *P. minimum* has been the most resistant to digestion, blooming when exposed to three of the four sea squirt species. These cells were able to re-establish a population in the tubes inoculated with the feces from the 24h post-ingestion period (i.e. immediately post-exposure) in as little as 6 d. Tubes inoculated with the 48h post-ingestion fecal samples established a population anywhere from 19 to 34 d after inoculation of the tubes. The only ascidian species to date that has produced no viable cells in the feces is *Didemnum vexillum*. All HAB species fed to

M. manhattensis were viable after gut passage, regardless of post-ingestion time. *A. fundyense* was able to re-establish a population from 19 to 34 d after inoculation. *H. akashiwo* was able to re-establish a population 15 to 32 d after inoculation.

Discussion

Our results demonstrate that some ascidians may serve as vectors of viable HAB cells. HAB cells can be found intact in the feces of various ascidian species and in some cases return to bloom concentrations 48h post-ingestion. Ascidian species have been reported to capture particles ranging from 0.6µm to 100µm with high efficiency (See review by Hughes et al. 2005). Some workers, however, have reported that most of the assimilated material in one tunicate species is 3-5 µm in size, with the rest passing undigested through the animal (Seiderer and Newell 1988; Hughes et al. 2005). This would help to explain why most HAB cells were able to remain viable in the feces. These results represent only a few of the potential interactions between ascidians and HAB species. In Long Island Sound for example, biofouling species exist in mixed assemblages of up to nine different species. We plan to assess interactions between at least one more species of ascidian and at least four more co-occurring HAB species. Experiments are also ongoing to determine viable gut residence time and assess potential mitigation strategies. Given the mixed assemblages of sea squirts commonly found in biofouling communities, the high filtering capacity of the animals, and the extended length of time that cells have been found to remain viable in the guts, sea squirts may pose a greater threat than bivalve molluscs in the potential distribution of HAB species

Acknowledgements

The authors thank J. Alix and M. Dixon for providing HAB cultures and other technical support. The Milford Laboratory provided space and equipment for all experiments and the Ward lab provided help in collection of ascidians. This work was supported by NOAA grant # NA09NMF4270075 to SES and SB.

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Nets and mussels as cysts captors during the decline phase of a *Alexandrium catenella* bloom

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Abstract

In late summer 2009, an intense *Alexandrium catenella* bloom occurred at the mouth of Aysén fjord, Chile (45º 06' S, 73 º 30' W). The concentration found in the first 10 m depth in peak phase was 396. 10^3 cells l^{-1}. Three sampling sites were established to study the potential of nets and mussels as *A. catenella* cysts captors. During the bloom decline, 20 days after, commercial size mussels (*Mytilus chilensis*) and nets taken both from a place free of this dinoflagellate were placed in the water column. At each site three nets were suspended at 5, 10 and 15 m depth and the three net cages with 35 mussels each were placed at 10 m depth. Both were moored for 30 days. At the three stations, the net cyst mean number by depth was 2,761; 975 and 258 cysts at 5, 10 and 15 m, respectively. The cysts mean content in mussel digestive glands for was 14 cysts g^{-1} (wet weigth). Since nets and mussels are widely utilized in fishing and aquaculture activities, they could constitute potential dispersant agents of *A. catenella* to free areas of this declared pest species in Chile.

Introduction

The geographical distribution of *Alexandrium catenella* in Chile extends between Calbuco and the Beagle Channel (Guzmán *et al.* 2010). Present regulation prevent dispersal of this microalga beyond its current northern border. The species has gradually been advancing towards lower latitudes, passing by Aysén (Lembeye *et al.* 1997) and reaching the island of Chiloé (Fig. 1) (Lembeye et al., 1998) since it was first reported at the Magellan Strait in 1972 (Guzmán & Campodonico 1975). Since 2005 *A. catenella* has been declared plague species in Chile. In late summer and early autumn 2009 (March-April), an intense *A. catenella* bloom occurred in the mouth of Aysén fjord (Fig. 1). The maximum cell concentration was 396,100 cells l^{-1} in the upper 10 m depth at the end of the exponential growth phase. The bloom decline (Fig. 2, see Guzmán *et al.* 2010 about relative abundance *vs* cell density) was an opportunity to evaluate the capacity of *A. catenella* (cysts and/or vegetative cells) to attach *in situ* on the fishing gear and live molluscs, substrates considered potential dispersion elements for the microalgae.

Material and Methods

In situ experiment. Commercial size mussels (*Mytilus chilensis*) and nets (mean length 2.5 m and width 0.09 m; 3 cm mesh size) taken from a place free of *A. catenella* (* in Fig. 1) were suspended in the water column during bloom decline (bar in Fig. 2) at three field stations: Julián Island (A30), Huichas Islands (A31) and Vergara Island (A32) (Fig. 3). Three nets per site were suspended at 5, 10 and 15 m depth, respectively, and three net cages with 35 mussels each were placed at 10 m depth. Both were moored for 30 days (March 22nd-April 22nd,2009).

Cyst extraction from the mussel digestive glands. Digestive glands of ten mussels per net cage were weighted and homogenized with filtered sea water (1 µm) using an Ultra-Turrax for 1 min. The homogenate was sieved through a 106 µm onto a 20 µm sieve. The remaining fraction on the 20 µm sieve was transferred to a beaker, sonicated for 0.5 min, and newly

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sieved with a 20 µm sieve. The remnant was poured into a clean beaker and the volume completed with filtered sea water up to 10 ml (Matsuoka & Fukuyo 2000).

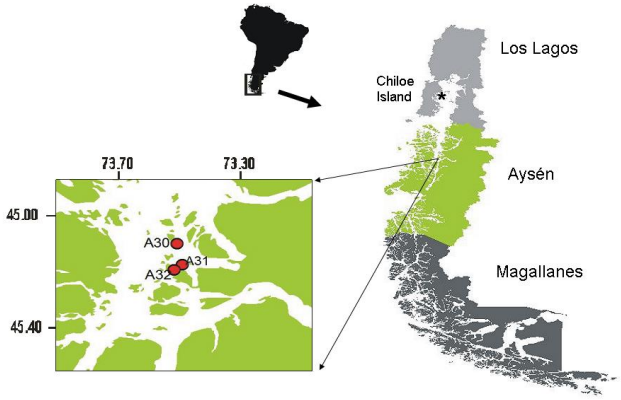

Fig. 1. Experimental stations: Julián I. (A30), Huichas I. (A31) and Vergara I. (A32).

Cysts extraction from nets.
Each net was immersed in a plastic recipient with filtered sea water and sonicated for 5 min. The nets were shaken to drain excess of water. The washing-water was sieved through 106 µm and 20 µm. The remaining fraction on the 20 µm sieve was transferred to a glass disc and mixed by hand until the particulate material was separated in the central circle. The lighter material was pipeted into a clean beaker. Then the volume was made up with filtered sea water to 10 ml (Matsuoka & Fukuyo 2000).

Resting cysts analyses.
Resting cysts in good conditions were identified and quantified from three 1 ml aliquots taken using a Sedgwick Rafter chamber under a light microscope. The results are the mean of three counts.

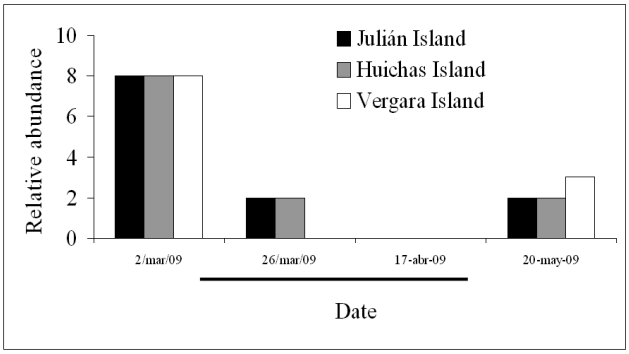

Fig. 2. Relative abundance of *A. catenella* bloom (see Guzmán *et al.* 2010 for relative abundance *vs* cell density). Bar: experiments *in situ*.

Results and discussion

Only resting cysts in good physiological conditions were found (Fig. 4) in mussel digestive glands and on nets after exposure during the decline of an intense *A. catenella* bloom. Empty resting cysts or temporal cysts were not detected. Mean cyst number in digestive glands of mussel ranged between 160 and 227 cysts g^{-1} (Table 1) and on nets, between 214 and 5,334 cysts net-$month^{-1}$ (Table 2). Mean cyst number contained in the digestive glands of the mussels placed at 10m depth, similar between stations, were significantly lower (P=0.05, Mann-Whitney)

Table 1. Resting cyst number found in mussel digestive glands suspended at 10 m depth at the three experimental stations.

Exp. stations	10 m
Julián Island (A30)	200
Huichas Islands (A31)	160
Vergara Island (A32)	227
Mean ± SD	**196 ± 34**

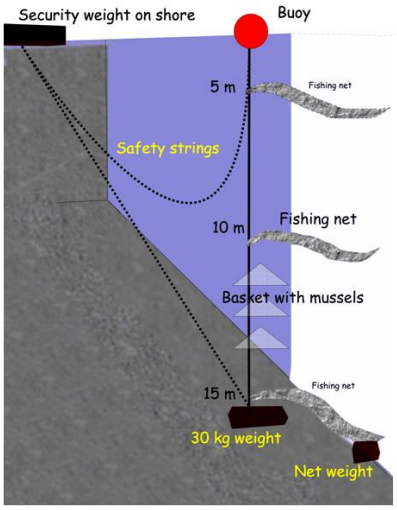

Fig. 3. Mussels and nets experimental mooring at each station.

than those observed on nets exposed at the same depth. Mean cyst numbers found on the nets were significantly different between stations (P<0.05, ANOVA two way) and depths (P<0.001). Vergara Island was significantly higher than Julián Island and similar to Huichas Iislands, while 5 m depth

was higher than 10 m and 15 m depths. Considering a digestive gland mean wet weight of individual mussel (1.4 g) and a net mean area (2,250 cm^2), the cyst number reached 274 cysts $mussel^{-1}$ and for the nets between 0.1 and 2.4 cysts cm^2.

The results show the differences between active (mussel) and passive (net) adhesion means for cysts. The first substrate shows adhesion depending on the mussel filtration –and elimination by feces (Hégaret *et al*. 2008) and the nets reflect environmental differences in cyst adhesion between sites and depths.

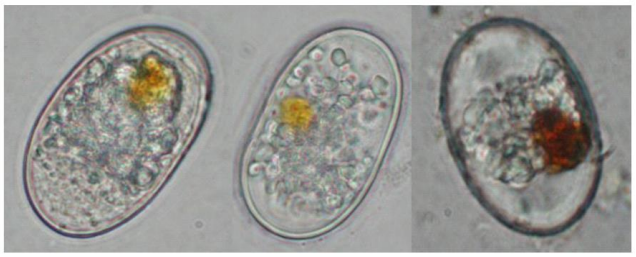

Fig. 4. *A. catenella* resting cysts found in the mussel digestive glands and nets moored in the water column for one month period. Bar: 10µm

Conclusions

Mussels and nets are potential dispersal vectors for *A. catenella* resting cysts after an intense bloom period. The higher amount of resting cysts found at 5 m depth serves as a warning for wellboats that transport alive organisms and usually take water at this depth and renew sea water content during transport. Measures should be taken by the regulatory authority to prevent potential spread by this means.

Acknowledgement

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Table 2. Mean resting cyst number (± SD) found in nets suspended in the water column

Exp. Stations	5 m	10 m	15 m	Mean
Julián Island. (A30)	343 ± 66	1,602 ± 1,099	244 ± 219	730
Huichas Islands (A31)	2,607 ± 1389	843 ± 527	214 ± 311	1,221
Vergara Island. (A32)	5,334 ± 603	480 ± 438	315 ± 332	2,045
Mean	**2,761**	**975**	**258**	

One HAB, Many Angles: The Florida Story

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Abstract

As Harmful Algal Blooms (HABs) are spreading in intensity and duration around the world, stakeholders are increasing pressure on the scientific community to identify potential control strategies and mitigation techniques. The State of Florida allocated funds from 2007 to 2009 specifically for the exploration of environmentally acceptable approaches to control the harmful algae, *Karenia brevis*, which blooms almost annually in Florida coastal waters, or to mitigate the effects of *K. brevis* blooms. The Red Tide Control and Mitigation Grant Program administered by the Florida Fish and Wildlife Conservation Commission (FWC) was the first US-funded program specifically for HAB Control and Mitigation research. A 2 ½ day public workshop was held in February, 2010 at Mote Marine Laboratory in Sarasota, FL to present the results of 12 projects funded through the FWC Red Tide Control and Mitigation Grant Program. The workshop was sponsored by the Gulf of Mexico Alliance, Solutions to Avoid Red Tide (START) and the Marine Policy Institute at Mote Marine Laboratory. In addition to the 12 principal investigators from the FWC program, workshop participants included scientists with previous control and mitigation experience, advocacy groups, government officials, and resource managers. A summary of workshop sessions and discussions are presented.

Introduction

As Harmful Algal Blooms (HABs) are spreading in intensity and duration around the world, stakeholders are increasing pressure on the scientific community to identify potential control strategies and mitigation techniques (HAB RDTT, 2008). Florida experienced a particularly long *Karenia brevis* bloom in 2005, lasting over 13 months. This extended bloom raised many concerns to citizens and decision makers alike, in particular concern over developing control and/or mitigation strategies. In response, the State of Florida allocated funds from 2007 to 2009 specifically for the exploration of environmentally acceptable approaches to control the harmful alga, *Karenia brevis,*which blooms almost annually in Florida coastal waters, or to mitigate the effects of *K. brevis* blooms. The Red Tide Control and Mitigation Grant Program administered by the Florida Fish and Wildlife Conservation Commission (FWC) was the first US-funded program specifically for HAB Control and Mitigation research.

The Grant Program

The FWC program was a competitive grant program to explore environmentally acceptable techniques or technologies to minimize the size, intensity, or duration of Florida red tides (control) or reduce the environmental, economic, social, or public health impacts of red tides (mitigation). Twenty two proposals were received in 2007, and 12 projects were funded. The breakdown of the types of these 12 projects is shown in

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Figure 1. Proposals were evaluated by the Red Tide Control and Mitigation (RTCM) Panel, an independent panel of partners and stakeholders representing state agencies, non-governmental agencies, universities, local governments, and advocacy groups.

The Workshop

Day 1 of the workshop included 8 presentations on different HAB control strategies, including 4 projects that were funded through the FWC grant program. The control projects ranged in scope from initial laboratory investigations using stock cultures to one small-scale open water application. Discussion surrounding control projects included the evaluation of environmental impacts, permitting processes, and the willingness of stakeholders' to accept various risks and costs associated with individual HAB control methods. Discussion also covered the need for a stakeholder consensus on the scientific and socioeconomic 'soundness' of a particular control strategy - and at what point those discussions on these issues should occur prior to testing or implementation.

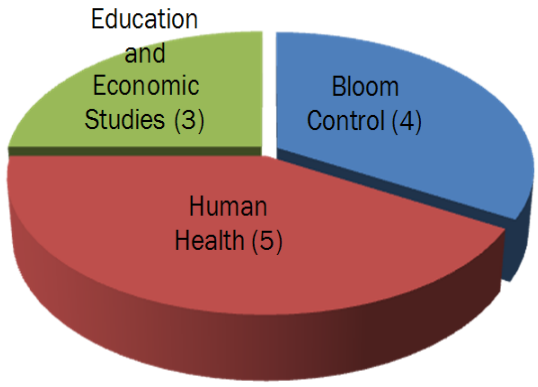

Fig. 1. Type and number of grants awarded

As an example, if the application of XYZ agent appears to be environmentally sound but produces a non-toxic but putrid odor, when should stakeholder feedback be assessed regarding whether they would accept the odor to control the bloom. In addition, group consensus was that it is unlikely that there will be one control strategy that will fit *K. brevis* blooms in all geographical situations.

For example, applications specifically to control blooms around shellfish or fish aquaculture regions require a different strategy than controlling a bloom along a 5 km stretch of beach. The temporal and spatial scales of effort still remain an issue with most control strategies, and several times the 1954 application of copper sulfate off St Petersburg, FL beaches to control a *K. brevis* bloom was cited as a sentinel bloom control attempt where scale of the issue was a major limitation.

Several of the FWC control projects focused on changing phytoplankton community structure or altering the nutrient regime to provide increased algal competition. Workshop participants speculated that these types of more 'natural' manipulations would be more readily supported by stakeholders over chemical or physical agents not found naturally.

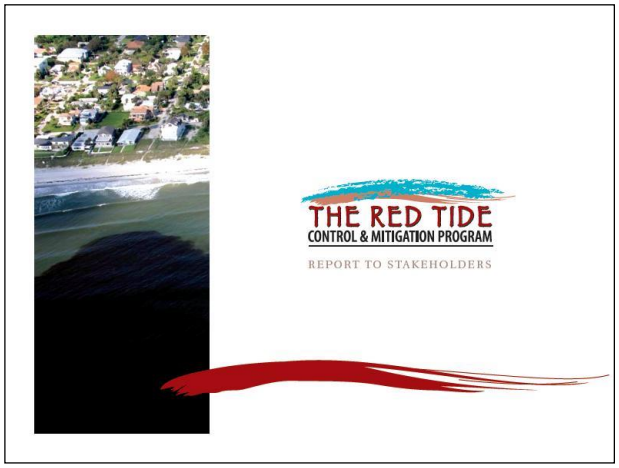

Fig. 2. Cover of Stakeholder's Report

Days 2 and 3 of the workshop included presents of 12 mitigation projects, 8 of which were funded through the FWC program. Discussion surrounding the mitigation projects included effective presentation and translation of project results to stakeholders, particularly for HAB-related human health studies. Again natural products found within blooms such as brevenal and cysteine appeared to the group to be more palatable to stakeholders than other chemical agents. Group consensus was that the scientific community needs to collaborate with graphic artists and communication specialists

to improve outreach efforts. A product of the FWC program was a "Report to Stakeholders" produced jointly by Solutions to Avoid Red Tide (START) and the FWC's Fish and Wildlife Research Institute. Workshop participants agreed that it is an excellent example of accurate translation of science to the public in a visually attractive booklet. The Report to Stakeholders can be accessed at

http://research.myfwc.com/features/view_article.asp?id=27877.

The cover of the document is displayed in Figure 2. The "Report to Stakeholders" is an important outreach product to use as governmental leaders change positions and new decisions makers need to be informed of prior progress/success. Although many of the individual projects have peer reviewed publications as a product of the grant, policy makers and other stakeholders need materials in a 'quick to read' format. This product fulfils those needs. Contact information of each project lead is given in the back of the report if readers want more information on a specific project.

Conclusions

The State of Florida FWC conducted its first grant program for research projects investigating control and mitigation strategies for *Karenia brevis* blooms. The workshop that followed raised important concerns from stakeholders, particularly if funds had been available to move the projects from the laboratory to the field. These lessons learned should be considered in future control and mitigation programs.

Acknowledgements

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Communicating Science: Video as a Medium for HAB Outreach

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Abstract

Communicating science to the public in a clear and effective manner is challenging. Now consider how difficult it is to attempt to communicate a complex scientific topic that is *also* a potential threat to human and animal health and rife with controversy, and political and economic implications. That scenario describes the intrinsic difficulty in harmful algal bloom (HAB) outreach. As part of two separate grant-funded research projects into HABs, two outreach videos were created. The two videos will be used to illustrate various considerations that go into the creation of outreach videos. We will briefly describe the video production process, discuss lessons learned, and provide information on inexpensive, often free, resources for video content and dissemination. The lessons learned from the production of the videos may be helpful to other scientists as they consider multimedia products to communicate their research.

Introduction

Communicating science to the public in a clear and effective way is challenging. It requires an understanding of science and the scientific process, as well as the ability to convey that science to the scientific community, public and policy makers in an understandable manner. Now consider how difficult it is to attempt to communicate a complex scientific topic that is *also* a potential threat to human and animal health and rife with controversy, and political and economic implications (Nierenberg in prep, Hoagland 2009). Potential outcomes could produce public fear or worse, apathy. That scenario describes the intrinsic difficulty in harmful algal bloom (HAB) outreach, considerations we took into account in the creation of two outreach videos on the human health aspect of two harmful algal blooms. We will briefly describe the video production process, discuss lessons learned, and provide a few tips for researchers interested in engaging in video as a medium for outreach.

Why Choose Video to Disseminate Science? In a recent article in the journal Science, researchers who used video to communicate their curriculum found that video was a crucial tool in conveying complex concepts in an understandable way; providing a level of understanding that would have otherwise been next to impossible to deliver using academic lectures or textbooks (Schneps *et al.* 2010). For instance, "Reef or Madness", a short film we created on the controversial diagnosis of chronic ciguatera fish poisoning, gives attention to gaps in research and traditional medical literature about the subject.

Pre-Production Considerations. There are a number of questions to consider before beginning any actual filming or video production. Answers to these questions can help create an effective, targeted outreach product, and potentially save dozens of hours of labor and hundreds to thousands of dollars in expenses for rewrites of the script or revisions of the video.

Defining the audience. The first step, whatever the medium, should be defining the *primary* audience. Will outreach be directed toward the public in general or a specific segment of the population? Perhaps outreach is geared toward policy makers or agency personnel. Are they within the subject discipline or outside? If students are the intended audience, what level? K-12, undergraduate, or graduate? It is extremely important to determine the *primary* audience because this dictates the knowledge level at which the subject matter will be aimed.

Purpose of the video. The next step involves determining the *purpose* of the video. Will the video be used to educate students, recruit field study participants, or engage policy makers? Perhaps the purpose is just to get the science out

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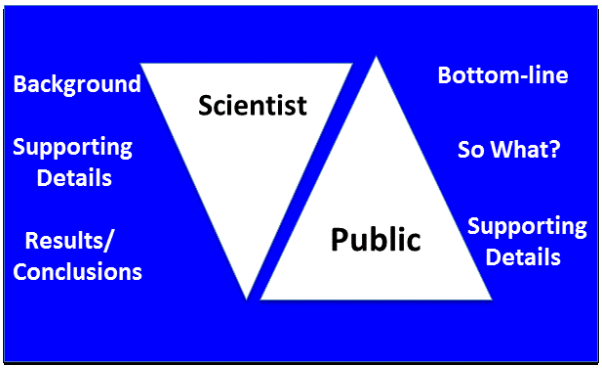
there. Regardless, the purpose should be specific and narrow. For example, when we created the outreach video "Red Tide Research in Paradise", as part of our NIEHS P01 aerosolized Florida Red Tide Research grant, the purpose was to recruit asthmatics to participate in a field study. An outreach product that deals with multiple purposes is often too long and has either an unclear or watered down message.



Supporting Messages. To keep the video concise and impactful, choose three-five messages (AAAS 2008) to support the purpose of the video. Referring again to "Red Tide Research in Paradise", we used three messages within the video to show prospective asthmatic participants 1) what they could expect should they decide to volunteer; 2) why their participation was important to our research; 3) and that participating in the study was safe.

Production Process. Creating an outreach video is a lot of work; experienced outreach and education professionals do not enter into it lightly. A short, well-produced video, even a few minutes long, can take months to produce. Consider that creating an outreach video is like planning a research study and carrying it out. Develop the idea (conduct background research); write a proposal (synopsis/treatment); carry out the study (film/interview); analyze the data (review footage); prepare the publication (script); and communicate the research (editing/post-production)

When producing videos, there is a rule of thumb that will help guide video length:

30 sec – 2 min: simple, single subject clips
3 – 5 min: how-to/process-oriented videos
5 – 7 min: informative content, no narrative
Up to 15 min: narrative-style where the story drives viewer interest.

Expect to "shoot" 10-30 minutes of video for every 1-minute used in the final product. Depending on a number of variables, such as the creation of complex animations, graphics and music/sound effects, narration, multiple shoots, and scheduling issues, production time can run anywhere from one-two weeks per 1 minute of video, up to 6 months to a year for a 15-20 minute piece. Writing and editing (and re-editing) the script can take up to half of the total production time. The entire process is time-consuming, especially if filmmaking is unfamiliar, but it can be extremely rewarding, providing a product for posterity.

Engaging the Public.A simple technique to engage the public in an outreach video, getting them to *care* about the research or a complex topic, is to "bookend" the video with a personal story or element that demonstrates how the research personally affects their lives. "Reef or Madness" was bookended with a very engaging victim of ciguatera. The film began with him and ended with him, with science and additional personal stories in the middle. Bookending the video with his personal story invested the public in the narrative and they were compelled to watch the entire video to find out what happened to him.

Lessons Learned. Even though our production team included a journalist and documentary filmmaker, as well as a scientist who was also an experienced outreach and education communicator, the endeavor of creating scientific outreach videos posed a few challenges.

Opposing communication styles: An important point to consider is that the method of communicating science *to scientists* is opposite how scientific outreach should be communicated to the public (Fig 1: source AAAS 2008). Researchers present background information, then supporting facts, and finally close their scientific presentation with results and conclusions. However, the public expects a story to lead with the main point (conclusion), followed by the "so what", to establish why they should care about the details to follow. Writing the script for "Red Tide Research in Paradise", exposed how the differing communication styles can impair the traditional collaborative scientific writing process.

Implications HAB outreach: The "Red Tide" script was carefully word-smithed to avoid unduly alarming tourists and locals about the human health effects of Florida Red Tide.

Release forms: Everyone involved in the research, the film and the filmmaking process must sign a release form to avoid potential ownership or fiscal liability problems.

Set realistic expectations: People interviewed on camera for 30-40 minutes and included in only 15 seconds of the video, have a tendency to get quite disappointed. Set their expectations low and surprise them later.

Set a deadline: A definitive deadline compels all parties involved in the production to adhere to a timeline. For "Reef " our deadline was a film festival; for "Red Tide", it was a scientific conference.

Resources. There are numerous resources for acquiring inexpensive (often free) graphics, photographs and video to supplement a HAB outreach video, especially if the video is for educational purposes or created by students. New mediums like You Tube™, and many other video content websites can provide a connection to filmmakers who are often very willing to give their content for free provided they are credited properly and the outreach video is educational and not-for-profit. We found an animated cartoon illustrating how the dinoflagellate *Gambierdiscus toxicus* affects the food chain from fish to humans on YouTube™, and used it in "Reef". We contacted the animator who created the video and he allowed us to use the cartoon for free provided he was given credit in the end credits. It was also valuable to ask around. Many of the photographs and video clips used in the two HAB videos were provided for free by colleagues, students, researchers from around the world, interested only in getting their research out there.

Leveraging Resources. Video production can cost upwards of $1000 per minute in production expenses. However, both HAB videos were created for under $200 each. Institutional resources were leveraged to make this happen. For "Reef", film school students from the University of Miami did much of the filming and editing. Using university equipment saved hundreds of dollars in equipment purchases or rental fees.

Outlets for Video Outreach. YouTube™, Vimeo™ and Facebook™, are just a few video content websites who offer free posting of videos. However, there are others means of getting outreach out there. Science Centers, aquariums, museums, and scientific conferences may all offer options for presenting a video. Consider using home institutional websites or "piggy-backing" on an agency websites, many are looking for well-made, scientific video content to supplement information or increase traffic on their websites.

Conclusion. Video as a medium for HAB outreach is challenging, but often well worth the effort. HAB scientists are perfectly poised to show the public that research into HABs directly affects them, and could actually help protect them and their families, whether it is through avoidance of HABs or potential beneficial medical treatments and pharmaceuticals some HAB species may provide. There can be helpful news in harmful algal blooms!

At a time when funding agencies increasingly require scientists to bring their research directly to the public, researchers can choose to go beyond press releases and publications (AAAS 2008), creating short but effective outreach videos that communicate science while educating and engaging the public. To view "Reef or Madness" and a narrated Powerpoint movie of this presentation, visit the Univ. of Miami NSF NIEHS Oceans and Human Health website: www.rsmas.miami.edu/groups/ohh To view "Red Tide Research in Paradise", visit NIEHS Red Tide Research Group website: http://isurus.mote.org/ niehsredtidestudy/ video.htm

Acknowledgements

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Relative abundance as a tool to increase the certainty of temporal and spatial distribution of harmful algal species

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Abstract

The patchy distribution of phytoplankton determines biases in quantitative distribution results. Relative abundance (RA) represents more appropriately species distribution, especially when densities are low, and provides insight into species abundance and temporal variations. RA is a semi-quantitative estimator of abundance for a given species based on net samples and is expressed in a nominal scale. It is defined for an ample geographic area and considers interannual variability. RA is referred to a particular species and not to its relationship to the other species assemblage. RA in comparison to density estimations for four harmful species (*Alexandrium catenella, Dinophysis acuminata, D. acuta, Protoceratium reticulatum*) and a potentially noxious species (*A. ostenfeldii*) from southern Chile are presented. Data were collected between 41° to 55° S along 151 fixed sampling sites from May 2006 to May 2010. There exist no straightforward relationship between RA and density although both are statistically correlated. Results have practical meanings for harmful species in boundary areas of distribution. RA for *A. catenella* is correlated to PSP toxicity. RA for the other species, excluding *A. ostenfeldii*, is significantly correlated to DSP mouse bioassay results.

Introduction

In Chile, fjords are the most affected area by harmful algal blooms. *Alexandrium catenella* was detected in 1972 in the Strait of Magellan associated to PSP and its current distribution is from Calbuco (41°48'S; 73°10'W) to Seno Ponsonby (55°08'S; 68°43'W) (Guzmán et al., 1975a,b, 2010). *Dinophysis acuta* was detected in 1970 and *D. acuminata* in 1996, both associated to DSP (IOC, 1995; Zao et al., 1995). *Protoceratium reticulatum* has been associated to yessotoxins (Pizarro et al., 2006). *A. ostenfeldii* has not being linked to bloom and neither to toxins (Guzmán et al., 2010). The density of microalgae does not properly represent the species distributions. RA is evaluated as a semi-quantitative estimator of abundance in comparison to density and as an indicator of species distribution.

Material and Methods

In southern Chile, 151 sampling sites were sampled monthly (Fig. 1) excluding winter season (~40 days). In each site three net samples (23 μm) at two different points from 20 m depth to surface were collected. The six net hauls were integrated into a single sample. RA was estimated from sedimented samples. On a glass slide 3 replicates of 0.1 ml with coverslips of 18x18 mm were placed. The motile stage of each species was counted by microscopy. The average count per sample was assigned to the corresponding level of the RA scale (Table 1). Quantitative phytoplankton was sampled with a 2.5 diameter hose, from surface to 10 m and from 10-20 m depths. Density was estimated by Utermöhl (1958) method. Results are presented as cells l^{-1}. Paralytic (PSP) and Diarrhetic Shellfish Poisoning (DSP) toxins

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Table 1. Relative abundance scales. (1) *Dinophysis acuta* and *D. acuminata;* (2) *Alexandrium catenella, A. ostenfeldii* and *Protoceratium reticulatum.* (3) Counts usually does not exceed the hyper-abundant level.

	SCALE	*Dinophysis* (1)	*Alexandrium, Protoceratium* (2)
Absent	0	0	0
Rare	1	1 – 5	1 – 2
Scarce	2	6 – 15	3 – 10
Regular	3	16 – 35	11 – 42
Abundant	4	36 – 75	43 – 170
Very-Abundant	5	76 – 155	171 – 682
Extremely-Abundant	6	156 – 315	683 – 2.730
Hyper-Abundant	7	316 – 635	2,731 – 10,922
Ultra-Abundant(3)	8	636- 1,275	10,923 – 43,690
Mega-Abundant(3)	9	1,276-2,555	43,691 – 174,762

were estimated in *Aulacomya ater*, *Mytilus chilensis* and *Venus antiqua*. Analyses were conducted at the laboratories of each Ministerial Regional Secretariat for Health. PSP was evaluated following A.O.A.C. (1990) and DSP according to Yasumoto et al. (1984).

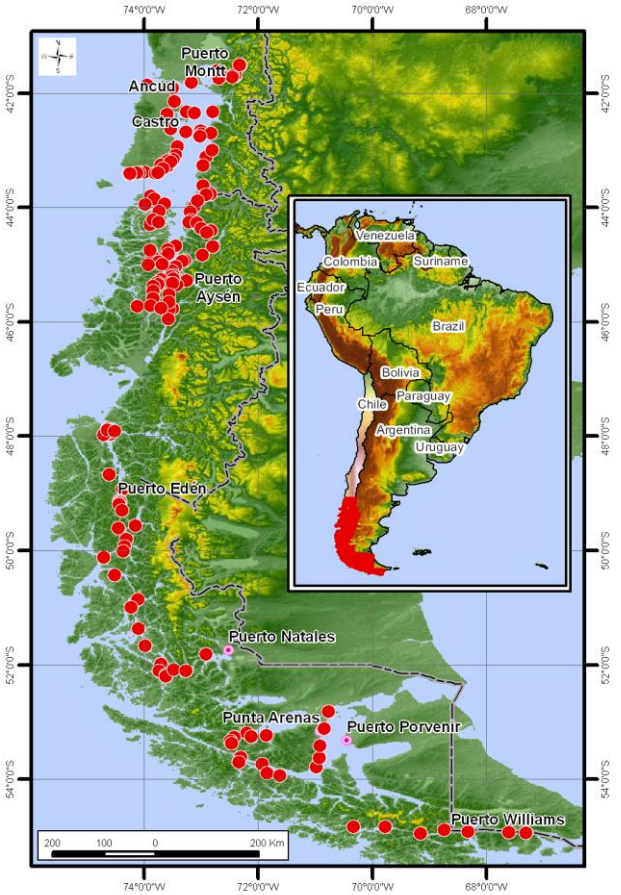

Fig. 1. Sampling sites in the study area.

Results

RAs are correlated with density estimates from each stratum (Spearman, P<0.05) but no model relates both variables in any of the studied species. For each RA estimate there is a wide range of density estimates, being the greatest for *A. catenella* (Fig. 2).

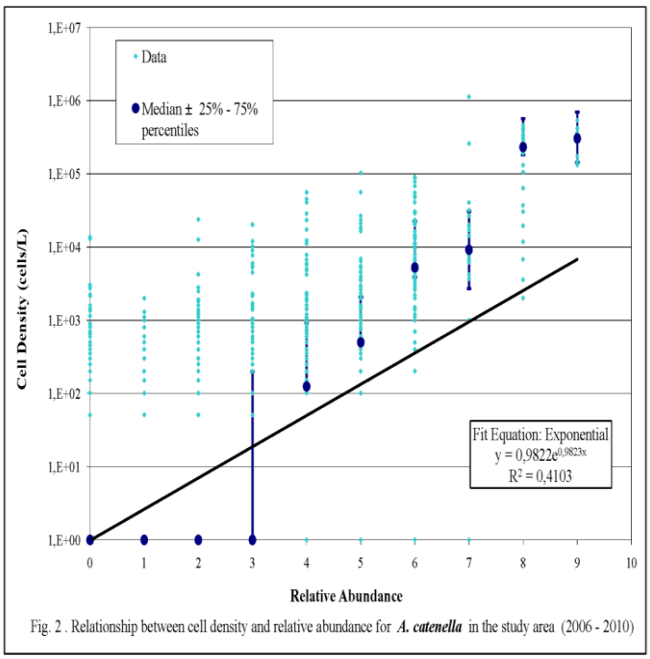

Fig. 2 . Relationship between cell density and relative abundance for ***A. catenella*** in the study area (2006 - 2010)

In comparison to density data, RA reflects more precisely the geographic distribution of species (Fig. 3). RAs estimates showed interannual, seasonal and geographical variations (Fig. 4), including differences in local distributions (Fig. 2). PSP data correlated to RA of *A. catenella* (P<0.05) and density estimates (P<0.05). The sum of RA and

density for *D. acuta*, *D. acuminata* and *P. reticulatum* and the sum of positive DSP assays are correlated (P<0.05).

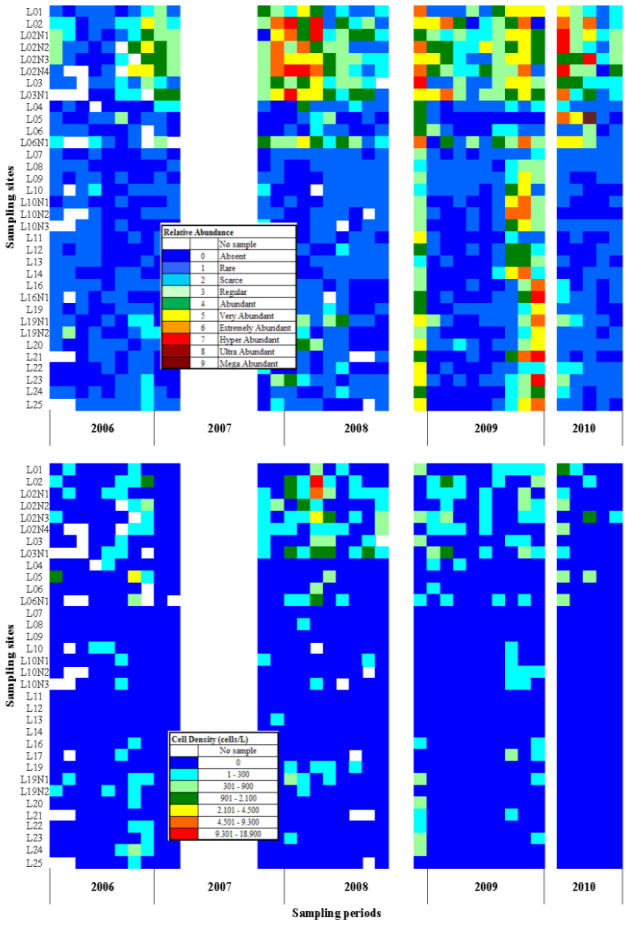

Fig. 3. Relative abundance and cell density for *D. acuminata* in the region of Los Lagos.

Discussion

Notwithstanding patchiness, RA represents more precisely species geographical distributions and temporal variations, including for low density species as *A. ostenfeldii*. This higher precision is due to better sampling by net hauls than with hoses or bottles. For any of the studied microalgae, high densities determine a higher precision in abundance estimation, but usually these microalgae are present with low densities.For *A. catenella*, a declared pest species in Chile, since RA defines more precisely its distribution, the northern boundary of this species is better defined. In these areas a sampling frequency increase is recommended, when over a number of sampling sites an increment of spatial frequency occurs or RA estimates show an increase tendency, in order to strengthen control measures.

Conclusions

RA represents more precisely the geographic distributions than cell densities, constituting an early warning tool. RA also reflects geographical and temporal variations. For *A. catenella* it is important to monitor its current northern natural boundaries. For each species there is a positive correlation between RA and density, but there is no single model that defines the relationship between both variables.

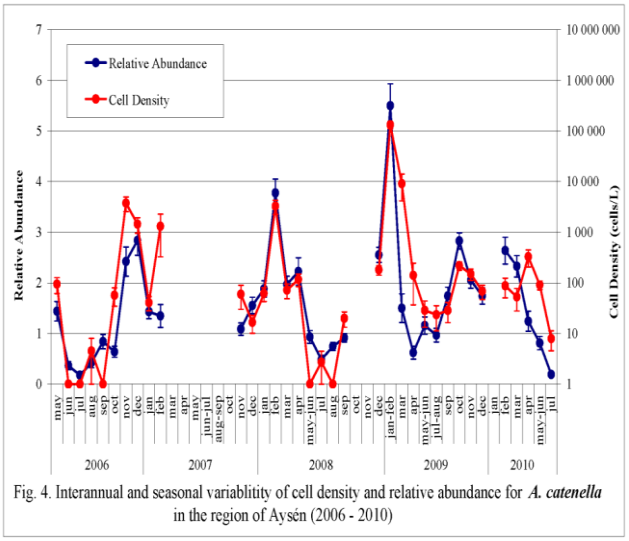

Fig. 4. Interannual and seasonal variablitity of cell density and relative abundance for *A. catenella* in the region of Aysén (2006 - 2010)

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Evolution of European monitoring systems for shellfish toxins – an increased need for the surveillance of phytoplankton

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Abstract

Monitoring systems for shellfish toxins in Europe have evolved over the last 20 years. An overview of systems, as implemented by member states, is given through the 2007 survey of NRLs. Recent legislative changes have resulted in the need for further evolution of sanitary monitoring systems. The switch from mouse bioassay to chemical testing for lipophilic toxins requires increased vigilance in the monitoring of toxicity in shellfish. Experiences from surveillance systems are outlined for the French surveillance system over the period from 2003 to 2008, and its recent implementation of chemical testing. Beside the requirement for novel techniques in the detection of emerging and unknown toxins, the increased need for vigilance also puts a stronger emphasis on possible changes in the composition and toxicity of micro-organisms in the coastal environment surrounding shellfish growing areas. The paper reviews recent developments in the detection of harmful algae and the description of phytoplankton community, including satellite imagery, modelling approaches, automated shape recognition in microscopy, bio-molecular techniques and flow cytometry. The integration of data from environmental surveillance with those from sanitary programs is suggested for the detection of changes in coastal ecosystems.

Introduction

European monitoring systems

Monitoring systems for shellfish toxins constantly evolve as new toxic algae, new toxins or new analogues of known toxins emerge in a given region. The 2007 survey on monitoring systems was conducted by the Irish national reference laboratory (NRL) as an EU NRL activity. Of the 26 member states (MS) contacted, 20 replied and 14 are shellfish producers. While the legal requirement in the EU prescribed mouse bioassay (MBA) as a test for lipophilic toxins, five of 14 MS already used liquid chromatography coupled to mass spectrometry (LC-MS) in the monitoring of shellfish samples. In the EU, phytoplankton monitoring is legally prescribed although its use is not specified in contrast to shellfish flesh testing. In 2007, 8 MS used phytoplankton results to trigger shellfish flesh testing. Hence, it is clear that phytoplankton monitoring plays a major role in the consumer protection system in those countries. A combination of political events and the summary report of the European Food Safety Authority on regulated marine biotoxins (EFSA, 2009) have resulted in legislative changes in the EU in 2010. Thus, the reference test for the detection of lipophilic toxins in shellfish flesh changes from the MBA to LC-MS. While the MBA had been shown to perform according to expectations for azaspiracids (Hess et al., 2009), the test does not meet required detection limits for other toxin groups, e.g. the OA group (Aune et al., 2007; EFSA 2009). In France, the rate of false negative MBA results was approximately 10% over a six-year period from 2003 to 2008 (Belin et al., 2009). In France, shellfish monitoring of each area is divided into two periods: a low risk and a high risk period; during the high risk period, shellfish are tested weekly, irrespective of the results of phytoplankton monitoring, while during low risk periods phytoplankton is used as the primary monitoring tool and only when toxic algae are detected, shellfish are monitored. As some areas were heavily affected by false positive MBA

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results, risk periods will reduce significantly with the change from MBA to LC-MS, thus clearly increasing the need for efficient monitoring of phytoplankton during low risk periods. Hence, this review focuses on available techniques and approaches to increase the efficacy of phytoplankton monitoring.

Use of satellite imagery for harmful algal bloom alerts. Satellite imagery has been successfully used for the description of water quality through the monitoring of chlorophyll-a in surface waters. In particular, the calibration of *in-situ* measurements of chlorophyll-a compared to satellite images allowed for sufficiently accurate prediction of chlorophyll-a levels in a 6-year study to establish water quality parameters for the European Water Framework Directive using SeaWIFS and MODIS satellite data (Gohin et al., 2008). However, satellite imaging has several major pitfalls as an alert tool for the now-casting of harmful algal blooms: (i) there is no guarantee that images can be obtained due to possible cloud cover, (ii) satellite images can only be used for surface waters limiting the technique to algal blooms at the surface, (iii) only dominating algal components can be detected.

Modelling approaches for prediction of harmful algal occurrences. Up to now, realistic ecological models of the marine shelves have focused on biogeochemical fluxes between nutrients and bulk phytoplankton compartments (diatoms, dinoflagellates, nanoflagellates.). The need for going deeper into the modelling of phytoplanktonic diversity first arose in areas exhibiting mass occurrences of a harmful species: *Alexandrium fundyense* in the Gulf of Maine (Anderson et al., 2005), *Phaeocystis globosa* in Belgian waters (Lancelot et al., 2005), *Karenia mikimotoï* in the western English Channel (Vanhoutte et al., 2008). As ecological models of the coastal zone have been intensively used to model eutrophication, they can be used to simulate harmful species commonly linked to enrichment of the coastal zone, especially in nitrogen. In the case of Brittany (France), where nitrate fluxes to coastal waters have increased 10 times during the last four decades, eutrophication is suspected to have triggered the toxicity of some phytoplankton species by increased N:Si ratio in the coastal sea. Diatoms belonging to toxic species of *Pseudo-nitzschia* have been regularly observed in some areas off Brittany, along with high domoic acid contents in scallop flesh, which may induce closure of shellfish harvest areas. A specific sub-model of *Pseudo-nitzschia* and its domoic acid production has been coupled to an operational model of the primary production of the Brittany coastal waters (Pénard, 2009). Since June 2008, this real-time tool provides, on the previmer.org website, d and d+1 day maps of *Pseudo-Nitzschia* biomass and domoic acid content of the water column. The model produces brief but intense bursts of toxicity in the western and the southern Brittany areas, in which the REPHY monitoring network of domoic acid content in scallops also show the highest contaminations. To increase reliability of this real-time warning tool, a sub-model of scallop gonado-somatic growth and contamination should be added, and field measurements must be extended more offshore to validate some simulated high toxicity events. A new release of the previmer.org website will provide in 2011 forecast maps of two another harmful phytoplankton species over the Bay of Biscay-English Channel area: the ichtyotoxic dinoflagellate *Karenia mikimotoï* and the foam-producing *Phaeocystis globosa*. On the other hand, very little work has been completed to develop biological models of phytoplankton community structure down to minor components, e.g. *Dinophysis* spp. (Anderson, 2005; Finkel et al, 2010).

Automated imaging techniques for detection of harmful algae. Identification of phytoplankton is necessary to monitor the occurrences of HAB events, providing a rapid assessment of the potential for public health hazards from contaminated shellfish and fish. Some toxic species are detectable by light microscopy while other species require electron microscopy for complete identification. As these methods are time consuming, automated detection techniques have been developed during the last decade to improve the early detection of HABs. As early as 1995, Culverhouse declared:«There are no robust techniques that may be deployed in the field for the routine assay of harmful algae in water samples». Between 1996 and 2010, however, attempts were made worldwide to improve existing methodology in the context of automatic methods for the detection of harmful algae. At least 3 different methods were explored, with various results:

• Pattern recognition: some laboratory-based or field-based systems were developed. The highest discrimination levels were obtained with neural networks, but they generally require a pretty good

reference data set (image captures) for classifier training.

• Flow or solid-phase cytometry: this method is based upon particle size and fluorescence (chlorophyll) detection and can be coupled with other specific probes. Some limitation is due to the difficulty to tag cells with chemical labels without applying high dilution factors.

• Molecular probes: numerous RT-PCR or immunochemical probes need to be developed and validated for routine automated detection ?

Pattern recognition devices are generally supported by image captures followed by discriminant analysis of morphometric parameters. Users need a microscope and a video camera + PC. It was tested, for instance, on *Dinophysis* spp. (Truquet et al., 1996; Lassus et al., 1998) but with a very limited use for routine monitoring. Artificial neural networks represent a real improvement of the previous method but they need a more specific software. They can reach a higher level of accuracy by implementation of a high resolution 3D rotatable images reference data set (Culverhouse et al., 2006). Once included in a field-based system (HAB-Buoy) equipped with high speed camera and automatic specimen labelling software (DICANN) they were tested on different dinoflagellate species. Flow or solid-phase cytometry is more and more developed. When using a solid-phase cytometer an automated counting of fluorescently labelled cells on a membrane filter is performed and molecular specific probes are applied later on. This method was tested on *Prymnesium* spp (Töbe et al., 2006; West et al., 2006) and a commercial device was also implemented (CHEMSCAN technique). In the case of flow cytometry, size and fluorescence of targeted cells are detected in a fluid stream, either directly or using nucleic acid-specific dyes. This technique can be combined with monoclonal antibodies (Stauffer et al., 2008) or with real time RT-PCR (Bowers et al., 2010), or with video technologies (Campbell et al., 2010). It was tested on either selected cell detection (*Dinophysis* spp, *Aureococcus anophagefferens*) or on mixotrophic behaviour (*Prymnesium parvum* feeding on *Rhodomonas*). More recently, a new technology merging flow cytometry and imaging microscopy was successfully developed and labelled. With the FLOW CAM system digital images capture in a fluid stream is followed by PC-assisted post-processing (morphological parameters) and fluorescence emission detection. It was tested on *Karenia brevis* enumeration (Buskey and Hyatt 2006) and on copepods feeding (Ide et al., 2008). A recent international intercalibration exercise has shown promising results at operational level (Denis et al., 2010).

Biosensor approaches for detection of harmful algae. The opportunities to import innovations from non-ocean technology sectors and the recent development in "omics", molecular biological methods, novel sensing technologies and nanotechnology opens up a range of opportunities for the development of automated intelligent monitoring of natural waters using emerging biological sensors. Although the development of these tools is gradually increasing, operational systems in order to simultaneously identify and enumerate different harmful algal bloom species is quite limited and still under progress. No cost effective device for the specific detection of toxic algae is still designed for the market. Numerous publications (Diercks *et al.* 2008, E. Turrell *et al* 2008, Rodriguez-Mozaz *et al.* 2005, Metfies *et al* 2009, Ahn *et al* 2006, C. Scholin *et al* 2009, Sako *et al*., 2004, Anderson *et al.* 2006) are focused on the use of oligonucleotide probes to identify HAB species using short synthetic DNA strands or rRNA probes and hybridized PCR fragments and on the use of antibodies (Gas *et al* 2010). A variety of detection methods from fluorescence, chemiluminescence, electro-chemistry to optical techniques are used. The sandwich hybridization assay is a frequently used method but requires tagging. The target rRNA or DNA in the sample is hybridized to specific capture probes grafted on a solid support, followed by a second hybridization with a signal probe or an antibody conjugated to an enzyme which generates a signal. These methods based on genetic information are generally used in laboratories and take advantages of the development of microarrays (biochips) or portable device (Metfies 2009 and Diercks 2008). But this technique is also used on the near real-time detection system developed by Scholin (Scholin *et al* 2009). An alternative method exploits the change of the refractive index without labelling requirements. One of this system developed at Ifremer is based on an *in situ* Surface Plasmon Resonance (SPR) biosensor (Laurent *et al.* (2009), Colas *et al.* (2009)). This optical technique is highly sensitive and measures refractive index changes as biomolecules bind to the surface of the sensor. The sensor shows high sensitivity with a lower limit of detection of ~0.1ng.mL^{-1} domoic

acid and a dynamic range of 0.1 to 1.0 ng/ml in PBS (Colas *et al* 2010). It is versatile and may be used for DNA hybridization detection, cells and toxins detection, and can be deployed on a platform, a buoy or a profiler in a early warning system. One of the challenges will be the further expansion of specific molecules and sensitive layers used as selective interfaces to detect phytoplankton and toxins. Steady progress is being made on algal genome level and antibodies production (Gas *et al* 2009, Litaker *et al.*2008).

Real-time detection of change in phytoplankton community structure. Very few studies have allowed for the generic detection of in situ changes in phytoplankton community structure. In principle, such detection methods could be based on metabolomic or metagenomic techniques, however, only morpho-taxonomic studies, e.g. Campbell et al., 2010, have so far convincingly demonstrated that detection of such changes is possible in real-time.

Conclusions

Clearly, the change from biological screening to chemical testing of algal toxins in shellfish results in an increased need for the occurrence of harmful and emerging algae in shellfish production areas. Significant progress has been made in the areas of satellite imagery, modelling, biosensor technology and automated detection of algae using microscopic imaging techniques. As these techniques also have limitations, a combination of approaches is most likely to provide greatest success in complementing shellfish monitoring programs with effective algal alert programs. An additional need is seen for generic tools to detect changes in phytoplankton community structures in real-time. Among possible approaches, including the omics technologies, a high-throughput morpho-taxonomic approach appears to be most operational. Further integration of environmental monitoring with food safety monitoring is strongly recommended. To achieve such integrated monitoring programs in a reasonable time-scale, additional efforts involving the IOC would be helpful.

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