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).

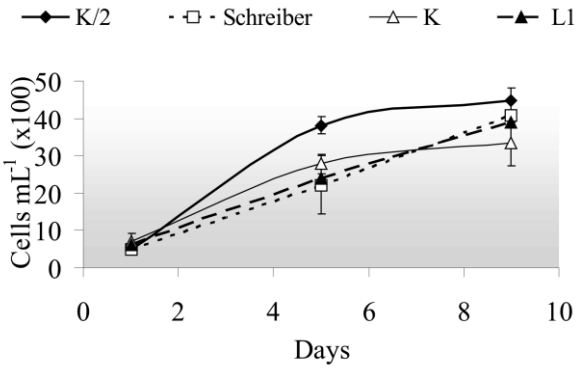

Fig. 1. Growth of *Ostreopsis* cf *ovata* in four different media.

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.

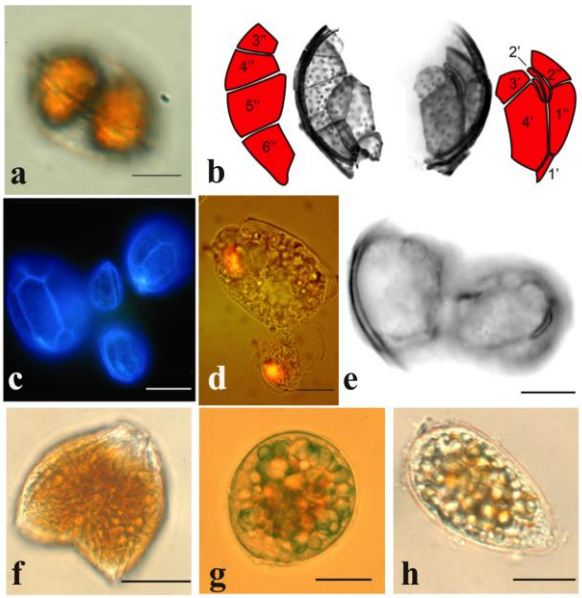

Fig. 2. *Ostreopsis* cf *ovata*. Dividing cell (a). Thecal fission pattern of the epitheca (b). Calcofluor-stained cells (c). Nuclei-stained cells (d). Calcofluor-stained gamete pair (e). Fusing gamete pair (f). Pellicle cyst (g). Thecate cyst (h). Scale=20µm

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.

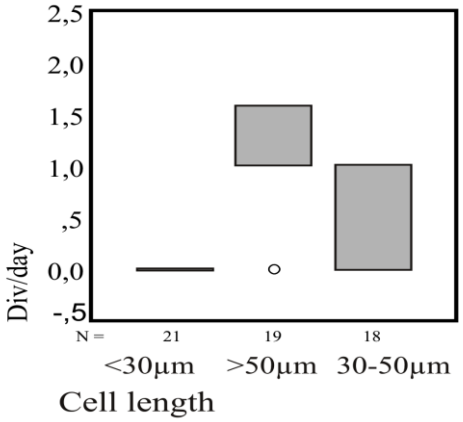

Fig. 3. Division rates of *Ostreopsis* cf *ovata* of different sizes

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

Acknowledgements

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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.

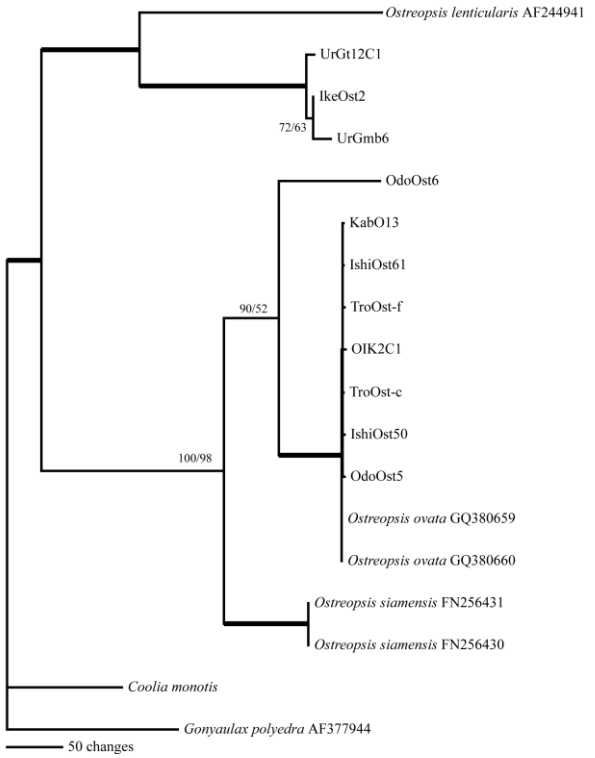

Fig. 2. Phylogenetic tree of *Ostreopsis* spp. inferred from the 28S rRNA gene D1D3 regions (708 bp) of MP tree. The bootstrap values (1000 replicates) are indicated at nodes (MP/NJ). The MP tree was generated by a heuristic search with TBR of the branch-swapping option and a random stepwise-addition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

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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.

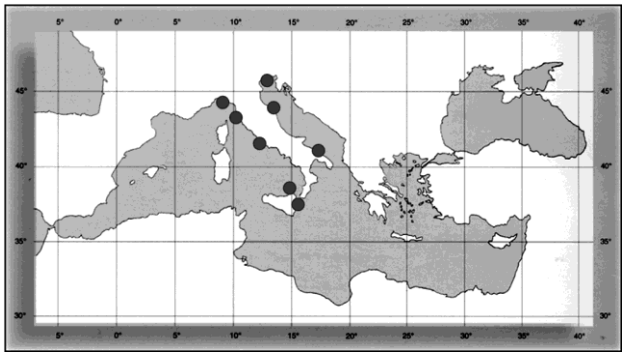

Fig. 1. Sampling stations along Italian coastline.

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.

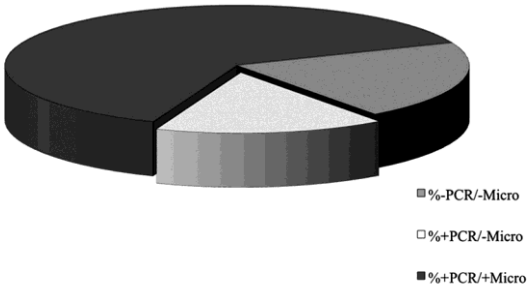

Fig. 2. Comparison of PCR and microscopy of field samples for Ostreopsis sp.. PCR amplifications compared with the corresponding positive and negative microscopy analyses of samples at different coastal sites in 2009 are shown. Data are expressed as % of total positive and negative values obtained with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

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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.

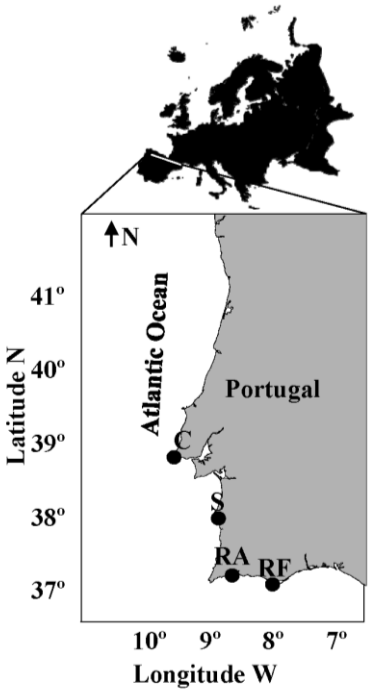

Fig. 1. Location of sampling stations. RF and RA, natural coastal lagoons; C (Cascais) and S (Sines), recreational marinas where *O*.cf. *siamensis* was recorded.

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.

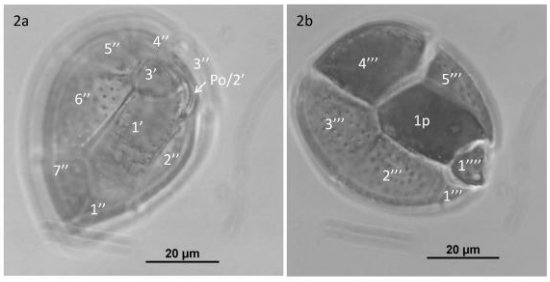

Fig 2. *O.* cf. *siamensis* theca from culture stained with acid lugol. (a) Epitheca and (b) hypotheca with scattered trichocyst pores. Diferential staining of plates in the hypotheca reveals the fission line of vegetative cell division.

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.

Acknowledgements

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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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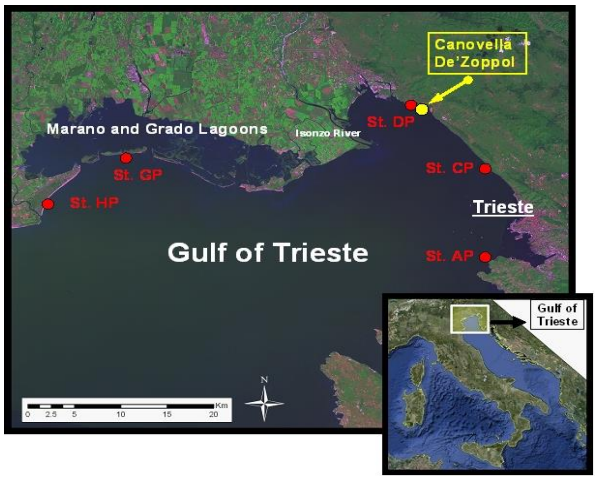

Fig.1. Map of sampling stations.

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.

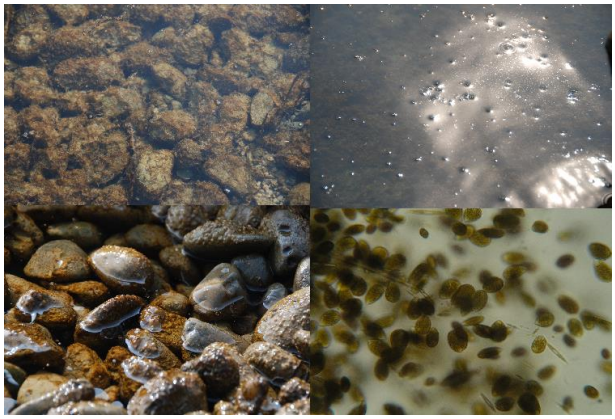

Fig.2. Floating brown macro-aggregates associated with Ostreopsis bloom.

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

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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).

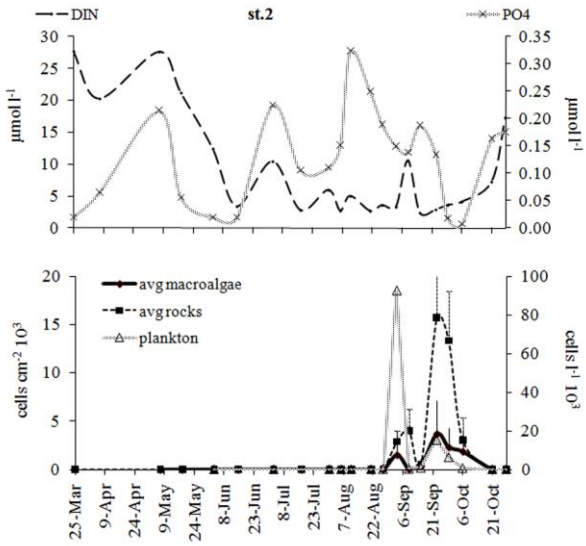

Fig.1. Nutrient concentrations and *Ostreopsis* cf. *ovata* cell densities at st.2. (A) Dissolved inorganic nitrogen (left y-axis) and phosphate (right y-axis). (B) *O.* cf. *ovata* abundance on macroalgae and rocks (left y-axis), and in the water column (right y-axis).

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).

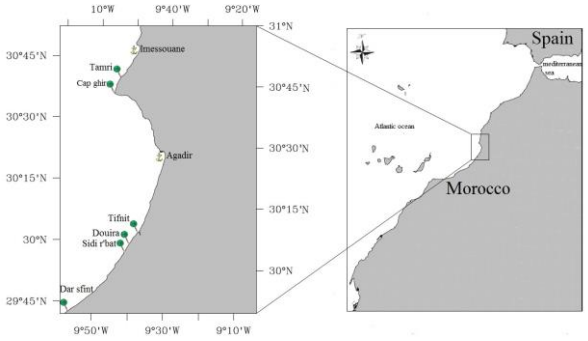

Fig. 1. Sampling area.

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

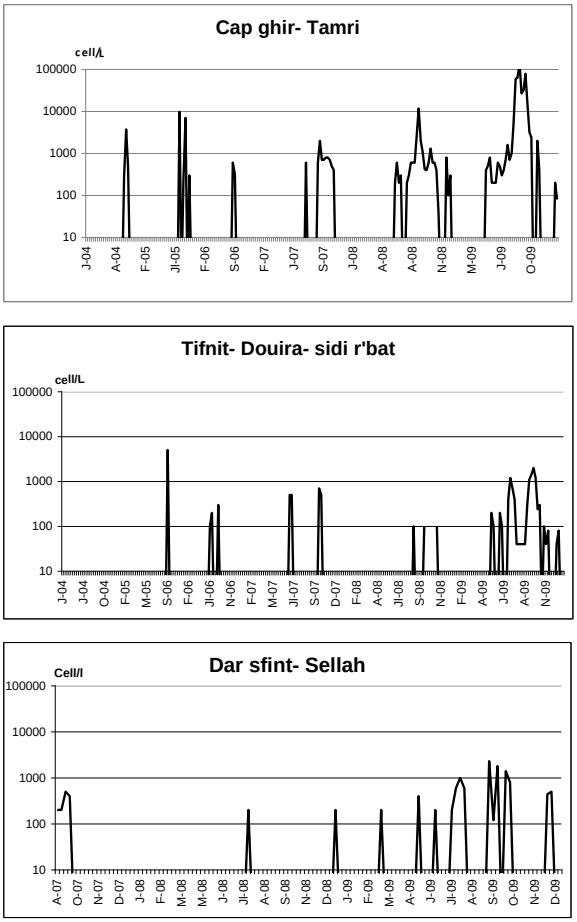

Fig. 2. Time distribution of *Ostreopsis* on the waters monitoring stations

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.

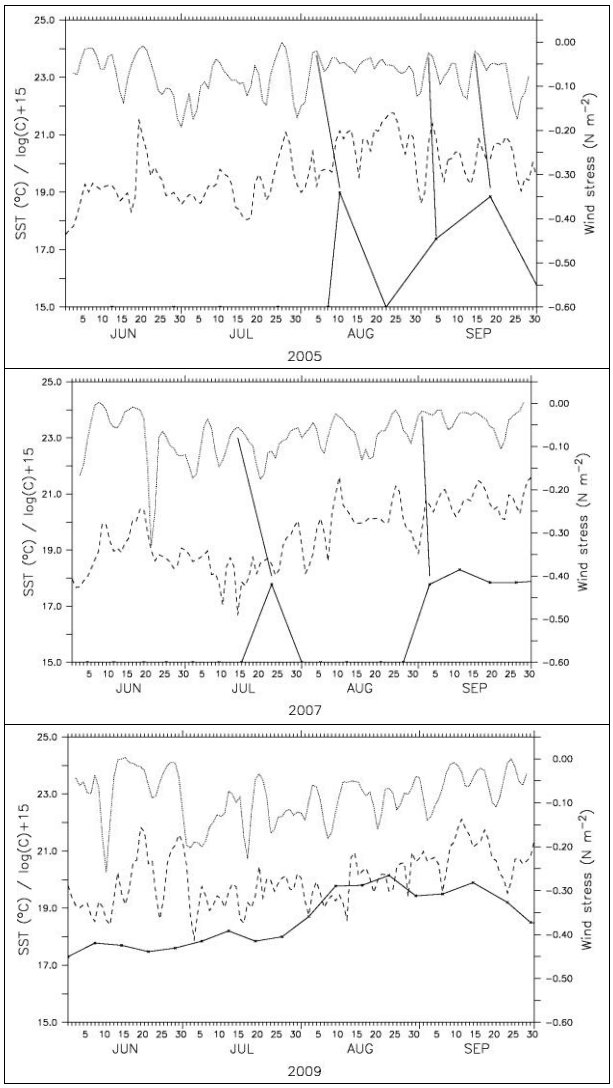

Fig. 3. Environmental conditions off Cape Ghir: satellite-derived sea surface temperature (°C, left axis, dashed line), N/S wind stress (N m^{-2}, right axis, dotted line) and cell concentration (log(C)+15, left axis, solid line) for 2005, 2007 and 2009 during periods of *Ostreopsis* proliferation.

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.

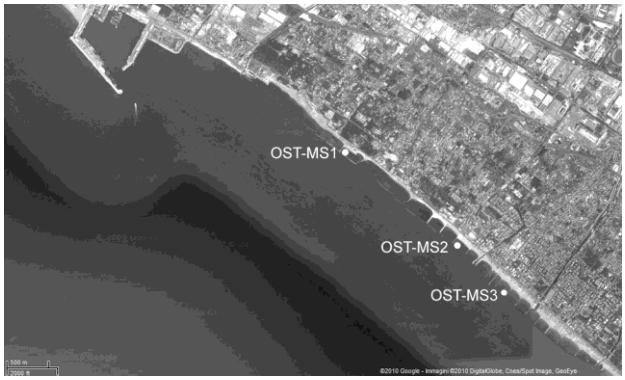

Fig 1. Sampling stations along the coast of Tuscany where blooms of *Ostreopsis ovata* are recurrent. Breakwater barriers parallel with the shoreline and artificial jetties are clearly visible.

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.

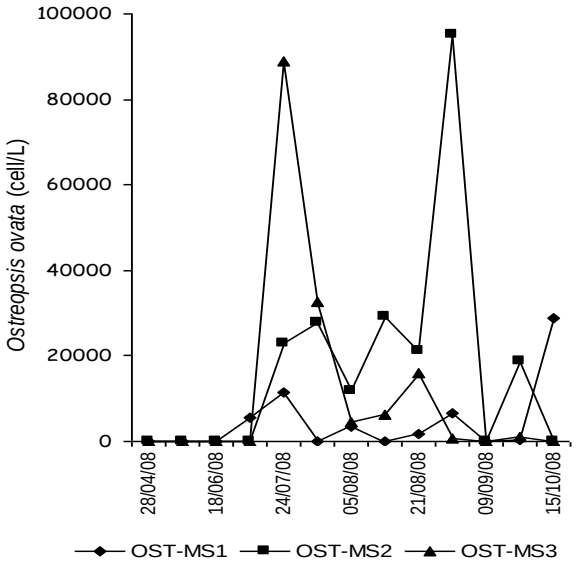

Fig 2. Densities (cell/L) of *O. ovata* along the coast of Marina di Massa (Tuscany).

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

This research was partly supported by the Region of Tuscany (DDRT 6702/2007).

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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

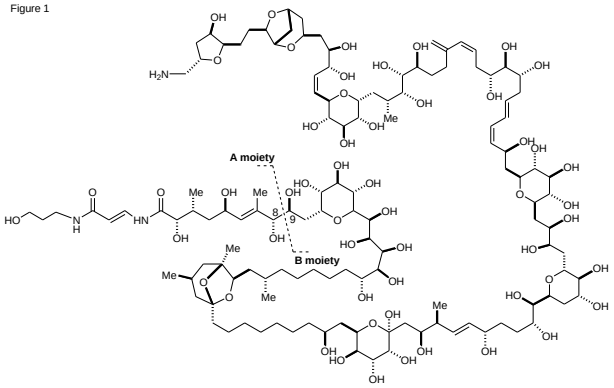

Fig.1. Molecular structure of ovatoxin-A

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

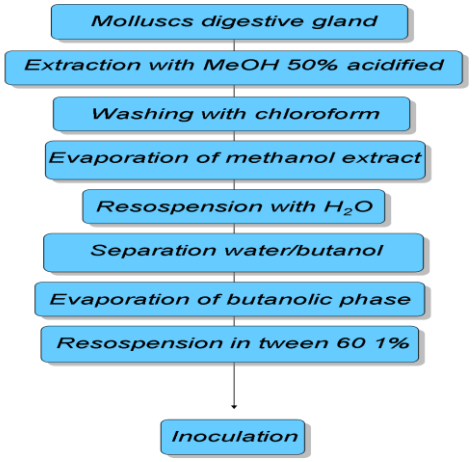

Fig 2. Specific bioassay (MBA PITXs)

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.

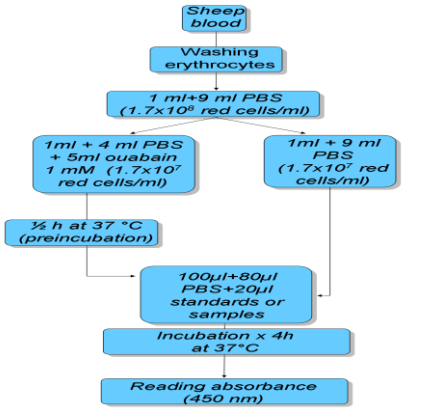

Fig 3. Hemolysis neutralization assay (HNA)

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

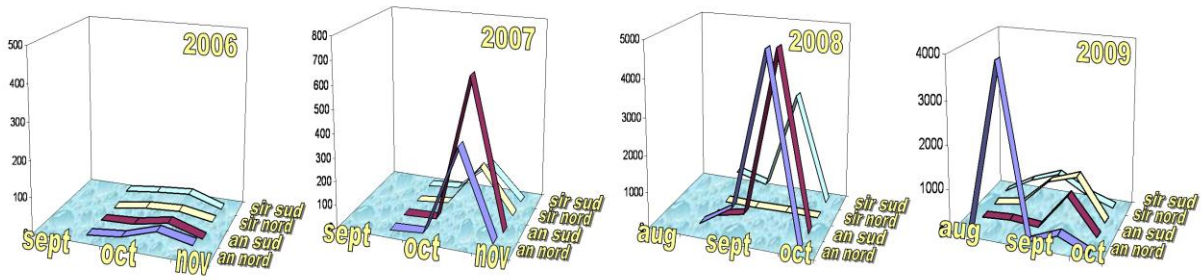

Fig 4. Ostreopsis ovata concentration along the coast of Monte Conero in the years 2006-2009 (cell/L 1000x)

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.

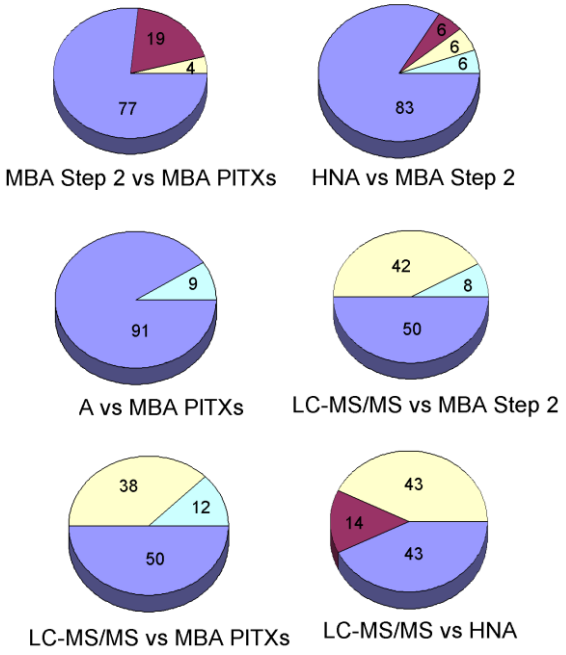

Fig 5. Correlations in percentage between methods tested: blue= +/+, red= +/-, yellow= -/+, light blue= -/-.

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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

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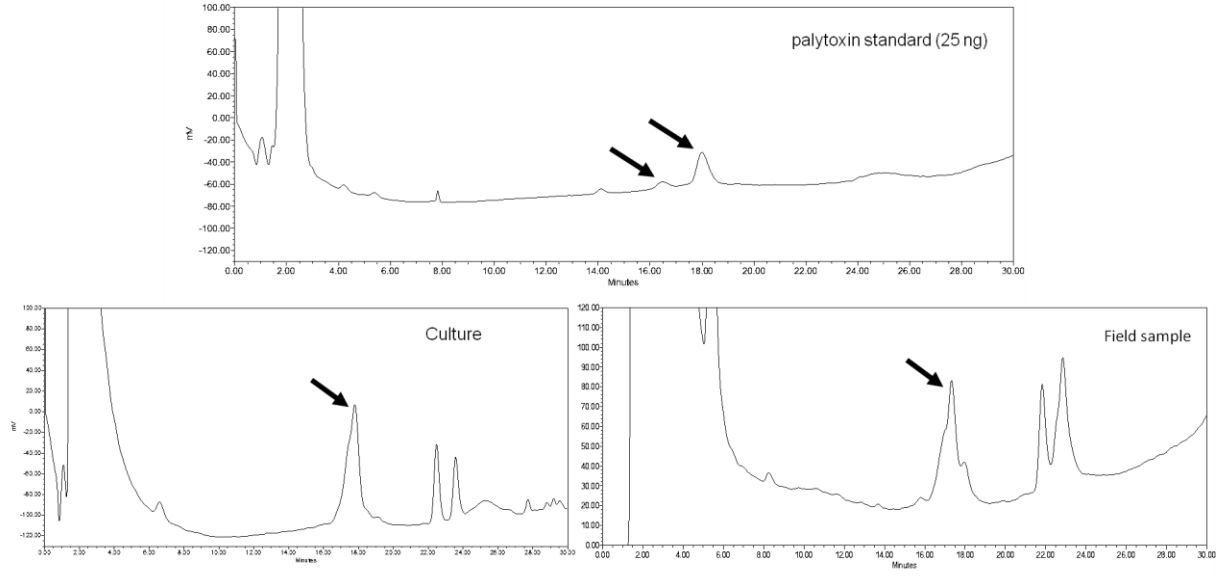

Fig.1 Chromatograms obtained by HPLC with fluorescence detection for palytoxin standard and samples derivatized with ACCQ reagent following the method described by Riobó et al (2006) with slight modifications.

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.

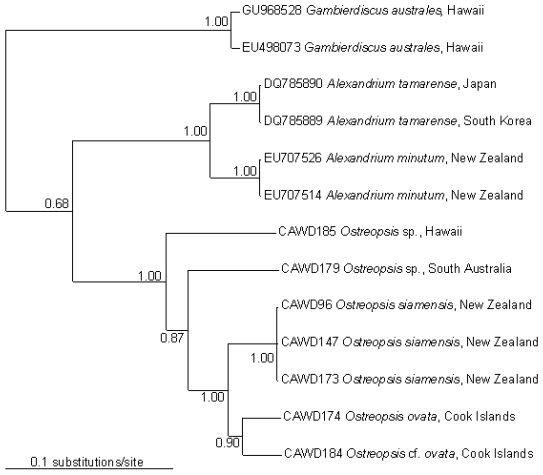

Fig. 1. Bayesian phylogenetic tree of the D8-D10 region of the LSU rDNA from *Ostreopsis* species isolated from the Pacific region with sequences from closely related species in GenBank.

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