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Toxicon 51 (2008) 418–427

TOXICON

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First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece) ☆

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Received 23 October 2007; received in revised form 27 October 2007; accepted 29 October 2007
Available online 6 November 2007

Abstract

In order to investigate the toxicity of *Ostreopsis* species present in Greek coastal waters, cultures of *Ostreopsis* sp. and *Ostreopsis ovata*, mixed *Ostreopsis* field populations and shellfish collected from coastal waters of North Aegean Sea during late summer and autumn periods of 2004, 2005 and 2006 were examined by both mouse bioassay (MBA) and hemolysis neutralization assay (HNA). MBA testing was based on two different extraction protocols, while HNA also included the use of ouabain, a known palytoxin (PLT) antagonist. Results indicated the presence of a compound in both *Ostreopsis* cells and shellfish tissues, which was strongly toxic to mice. This compound exhibited characteristic symptomatology in mice (death, numbness, waddling gait and blindness) to that of PLT, as well as delayed hemolytic activity, which was neutralized by ouabain. HNA indicated that *Ostreopsis* cells contained a PLT-like compound (putative PLT, p-PLT) at concentrations ranging between 0.4 and 0.9 pg/cell, whereas concentration in shellfish tissues was estimated to range from about 33.3 to 97.0 μg p-PLT/kg tissue. To our knowledge, this is the first report of p-PLT contamination of shellfish by natural *Ostreopsis* species populations in European coastal waters and possibly globally, and also the first evidence on *Ostreopsis* cells' toxicity in the Eastern Mediterranean Sea.

Keywords: Aegean Sea; Greece; Hemolysis neutralization assay (HNA); Mediterranean Sea; Mouse bioassay (MBA); *Ostreopsis*; Palytoxin; Shellfish toxicity

1. Introduction

Palytoxin (PLT) is one of the most potent natural non-protein compounds known to date, exhibiting extreme toxicity in mammals (i.v. LD_{50} 10–100 ng/kg; Wiles et al., 1974). The exact toxic dose in humans is not known; however, extrapolation of the available animal toxicity data would correspond to a toxic dose of about 4 μg (Taniyama et al., 2002). Despite its high lethality in terrestrial animals, PLT has also

☆ *Ethical statement*: I certify that the results of the submitted for publication in Toxicon manuscript are original and are not submitted elsewhere for publication.

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0041-0101/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2007.10.016

been detected in crabs (Yasumoto et al., 1986; Lau et al., 1995), various fish (Hashimoto et al., 1969; Fukui et al., 1987; Kodama et al., 1989) and a sea anemone (Mahnir et al., 1992), without causing any deleterious effects. This resistance of marine animals to PLT enables their sequestration and accumulation in the food chain (Gleibs and Mebs, 1999), resulting in numerous cases of human poisoning and lethality (Gonzales and Alcala, 1977; Alcala, 1983; Alcala et al., 1988; Noguchi et al., 1988; Yasumoto, 1998; Onuma et al., 1999).

PLT is a large, very complex molecule possessing both lipophilic and hydrophilic regions, and has the longest chain of continuous carbon atoms known to exist in a natural product (Moore and Bartolini, 1981; Shimizu, 1983). One of the most characteristic properties of PLT is that it can cause delayed hemolysis in erythrocytes, which can be suppressed by ouabain (Habermann et al., 1981; Habermann and Chhatwal, 1982).

PLT was initially isolated in 1971 from marine soft corals of the genus *Palythoa* (Moore and Scheuer, 1971). However, dinoflagellates of the genus *Ostreopsis* have been proposed as possible biogenic origins of PLT (Usami et al., 1995; Taniyama et al., 2003), a hypothesis further supported by the implication of *O. siamensis* in a case of clupeotoxism caused by PLT or its analogs (Onuma et al., 1999).

During the last decade, benthic dinoflagellates of the genus *Ostreopsis* have expanded their distribution from tropical–subtropical to temperate waters, such as the Mediterranean Sea (Tognetto et al., 1995; Vila et al., 2001a, b; Penna et al., 2005; Aligizaki and Nikolaidis, 2006b; Ismael and Halim, 2006; Monti et al., 2007). Furthermore, summer *Ostreopsis ovata* blooms mainly in Italian coasts have been recently associated with human irritations, resulting in considerable economic losses in the tourism industry (Brescianini et al., 2006; Ciminiello et al., 2006).

Since 2003, a number of shellfish samples, collected in the framework of the Greek monitoring program for marine biotoxins in summer and autumn, tested positive by DSP mouse bioassay (MBA) with short death times (Katikou, unpublished data). These positive results, in connection with the absence of any planktic DSP-producing microalgae, resulted in the detection of *Ostreopsis* species in Greek coastal waters (Aligizaki and Nikolaidis, 2006b) and also raised the necessity for clarification of the toxins' origin.

Due to the previously reported connection between PLT analogs and various *Ostreopsis* species (Usami et al., 1995; Taniyama et al., 2003; Lenoir et al., 2004), the presence of a PLT-like compound was suspected to be the cause of the observed mouse toxicity. The purpose of this study was therefore to investigate the presence of PLT-like compounds in *Ostreopsis* cells and shellfish tissue collected from Greek coastal waters.

2. Materials and methods

2.1. Samples collection

Macrophyte and surface water samples were collected from six sites (st. 1–6) along North Aegean coasts (Fig. 1) during late summer and autumn periods of 2004, 2005 and 2006 (Table 1). Three more stations (st. 7–9) were sampled in spring and early summer (Table 1) when *Ostreopsis* species are not usually detectable (Aligizaki and Nikolaidis, 2006a, b).

The surface water samples were used for *Ostreopsis* cell density estimation in the water column above macrophytes. The macrophyte samples, collected from depths up to 1.5 m, were adequately treated in order to detach the epiphytic dinoflagellates. A subsample was preserved for further identification and abundance estimation (abundance expressed as cells/g fresh weight of macrophytes, fwm). Another subsample was collected on GF/F glass microfibre filters (diameter 47 mm, pore size 0.7 mm, Whatman) for toxicological analyses (sample codes 12 and 13).

From the alive field material, cells were isolated by micropipetting (Andersen and Kawachi, 2005). Clonal cultures of *Ostreopsis* species were established in F/2 medium and grown at temperature $19 \pm 1\,^{\circ}C$ and photoperiod 14:10 h light:dark. Illumination was at about 70 $\mu mol/m^2 s$. Cells from the established cultures were also analyzed toxicologically (sample codes 10, 11, 14) after harvesting at approximately the 22nd day since inoculation, which coincides with the end of the exponential phase and the beginning of the stationary phase of growth.

Shellfish samples (mussels: *Mytilus galloprovincialis*, clams: *Venus verrucosa*, hairy mussels: *Modiolus barbatus*) were collected from five stations (1, 3–6) along North Aegean coasts (Table 1 and Fig. 1) at the same period (sample codes 1–9, 15, 16). Upon arrival to the laboratory, shellfish

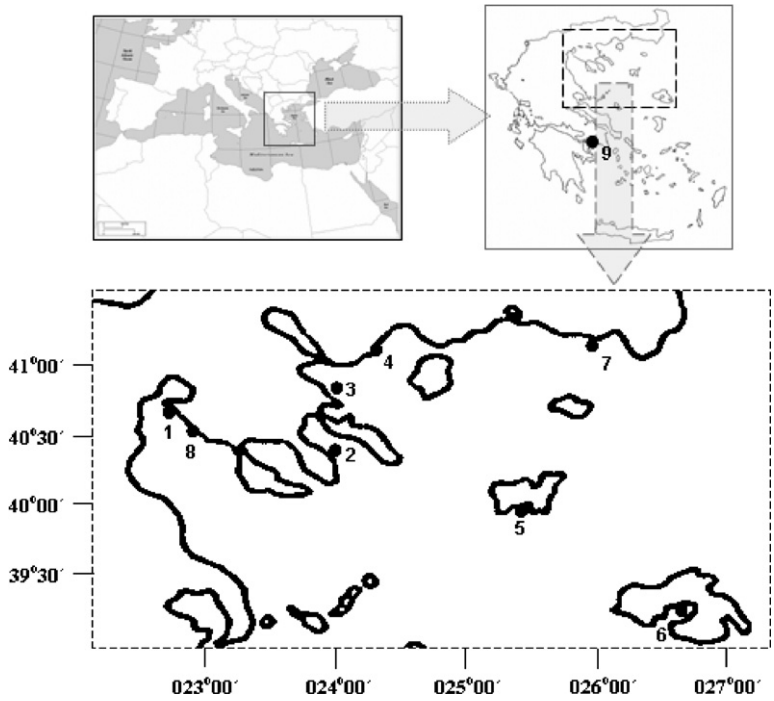

Fig. 1. Maps showing the study area and the sampling sites along North Aegean coasts.

Table 1
The presence of *Ostreopsis* spp. in the sampling sites where shellfish were collected for toxicological analyses

Sample code	Station	Year/month	Shellfish	Epiphytic *Ostreopsis*[a]	Water column *Ostreopsis*[b]
1	Hrakleitsa (st. 4)	2006/12	Mussels	0[c]	0[c]
2	Lesbos (st. 6)	2005/10	Venus clams	66	1240
3	Lesbos (st. 6)	2005/10	Hairy mussels	66	1240
4	Limnos (st. 5)	2005/10	Mussels	273	40
5	Olympiada (st. 3)	2005/9	Mussels	1498	0
6	Olympiada (st. 3)	2005/9	Mussels	1498	0
7	Aggelochori (st. 1)	2005/9	Mussels	33,212	3600
8	Hrakleitsa (st. 4)	2004/9	Mussels	39,493	1000
9	Olympiada (st. 3)	2004/9	Mussels	6051	640
15	Hrakleitsa (st. 4)	2006/8	Mussels	n.d.[d]	40
16	Hrakleitsa (st. 4)	2006/9	Mussels	n.d.[d]	640
17	Megara (st. 9[e])	2006/3	Mussels	0	0
18	Aggelochori (st. 1[e])	2007/4	Mussels	0	0
19	Epanomi (st. 8[e])	2007/4	Mussels	0	0
20	Evros (st. 7[f])	2007/6	Mussels	0	0
23	Hrakleitsa (st. 4[f])	2007/3	Mussels	0	0

[a]Mean epiphytic value in cells/g fresh weight of macrophyte (fwm).
[b]Abundance in the water column above the sampled substrates in cell/l.
[c]*Ostreopsis* spp. were recorded during the preceding 4 months.
[d]n.d. = not determined.
[e]Samples containing OA derived from areas where only *Dinophysis* spp. were present in high abundance levels (Nikolaidis, unpublished data).
[f]These samples were used as negative controls (blank samples).

were washed and shucked; hepatopancreas and whole tissue were separated and homogenized. Moreover, mussel samples from stations 1, 8 and 9 (sample codes 17, 18, 19), which were contaminated by okadaic acid (OA), as previously confirmed by MBA and protein phosphatase inhibition assay (PP2A; results not presented), and blank mussels from stations 4 and 7 (sample codes 20, 23), which tested negative with MBA and PP2A, and with no potential toxic microalgal species detected in the area, were also included in the study in order to investigate the selectivity of the methods.

2.2. Toxin extraction

2.2.1. From Ostreopsis cells

The filters containing the cells were suspended in 50% aqueous methanol (MeOH), homogenized and sonicated (Sonics, Vibra Cell). The material was defatted with diethylether (DEE) and the remaining aqueous fraction was partitioned between water and *n*-butanol; the latter extract was evaporated to dryness and resuspended in water for further analyses.

2.2.2. From shellfish tissue

Two different extraction protocols of the shellfish tissue were employed for conduction of MBAs: (A) the protocol of Yasumoto et al. (1978) involving acetone extraction of 20 g shellfish hepatopancreas followed by evaporation to dryness and (B) a modified protocol based on Taniyama et al. (2002) using 75% aqueous ethanol for extraction of 80 g shellfish whole flesh and defatting with DEE (DEE extract); the remaining aqueous fraction (crude extract) was subsequently partitioned between water (aqueous extract) and *n*-butanol (butanolic extract); the DEE, aqueous and butanolic extracts were then evaporated to dryness and resuspended in standard volumes of water for further use in both MBA and hemolysis neutralization assay (HNA).

2.3. Hemolysis neutralization assay (HNA)

Hemolysis assays were conducted following a method based on a combination of those described by Bignami (1993), Taniyama et al. (2002) and Riobó et al. (2006), using sheep erythrocytes.

The red blood cells were separated from plasma by centrifugation (1800 rpm, 12 min). They were afterwards washed three times with Dulbecco's phosphate buffer saline (D-PBS, Sigma-Aldrich Chemie GmbH, Germany) in a ratio 1:2 and diluted in D-PBS (0.5 g erythrocytes in 100 ml D-PBS); D-PBS contained 1 mM $CaCl_2$ and 0.5 mM H_3BO_3 (pH: 7.0–7.2).

The first experiments were conducted by adding 50 µl of sample or standard (Palytoxin Standard, Wako Chemicals GmbH, Germany) in 950 µl of red blood suspension. In order to reduce to the minimum the sample/standard quantity used, several proportions were used; addition of 20 µl of samples/standard in 180 µl erythrocytes provided comparable results. Incubation for 1, 2, 4, 6, 8 and 24 h in 37 °C followed. After incubation, the samples were centrifuged, the supernatants were transferred to a 96-well plate and measured at 405, 540 and 595 nm (Versa Max UV-Vis microplate reader, Molecular Devices). Preliminary experiments indicated that 4 h incubation time and 540 nm wavelength were adequate in order to conduct the HNA, since hemolysis up to 100% was observed within the 4 h of incubation. Additionally, hemolytic activity was always determined in the first hour in order to investigate whether it was delayed or not.

Total (100%) hemolysis was achieved by adding saponin 1% to the red blood suspension instead of samples.

Ouabain (Wako Chemicals GmbH, Germany), a known PLT antagonist (Habermann et al., 1981), was used to demonstrate the presence of PLT in the tested materials. For this reason red blood cells' suspension was pretreated with 1 mM ouabain.

Concentrations between 1 and 100 ng/ml of PLT standard were used in order to investigate the dose-dependent curve of hemolysis of sheep erythrocytes induced by different PLT concentrations.

2.4. Conduction of MBA

Toxicity in mice was determined using the extract from protocol (A) and both butanolic and aqueous extracts of protocol (B). With regard to protocol (A), the dry organic extract was resuspended in 4 ml of 1% Tween-60. This was also the case with the (organic) butanolic extract of protocol (B), whereas the dry aqueous extract was resuspended in the same volume of water.

In both protocols, each one of three mice (Albino Swiss, 18–20 g b.w.) was injected intraperitoneously with 1 ml of the respective solution. The death of two out of three mice within 24 h after inoculation for protocol (A) and 48 h for protocol (B) was

considered as a positive result. The mice were allowed laboratory chow and water *ad libitum* throughout the observation period. All animal manipulations were performed in accordance with the EU Directive 86/609/EC (1986), under official license from the Prefectural Veterinary Service of Thessaloniki, Greece.

3. Results and discussion

3.1. Hemolysis neutralization assay (HNA)

HNA, using the reference PLT in concentrations up to 15 ng/ml, resulted in the response curve shown in Fig. 2a. At higher concentrations, however, a flattening of the curve was observed (Fig. 2b), making quantification of the toxic compound impossible. For this reason, all samples examined were diluted from 1:2 to 1:100 in order to fit in the 0–15 ng/ml range of the standard curve.

Suppression of hemolytic activity of the samples after pretreatment with ouabain together with the characteristic delayed hemolysis were indicative of the PLT character (p-PLT) of the toxic compound detected in our samples (Table 2). On the other hand, samples contaminated with OA (code samples 17, 18, 19) displayed rapid hemolysis which was not inhibited by ouabain, whereas no hemolysis was detected in blank samples (Table 2, code samples 20, 23).

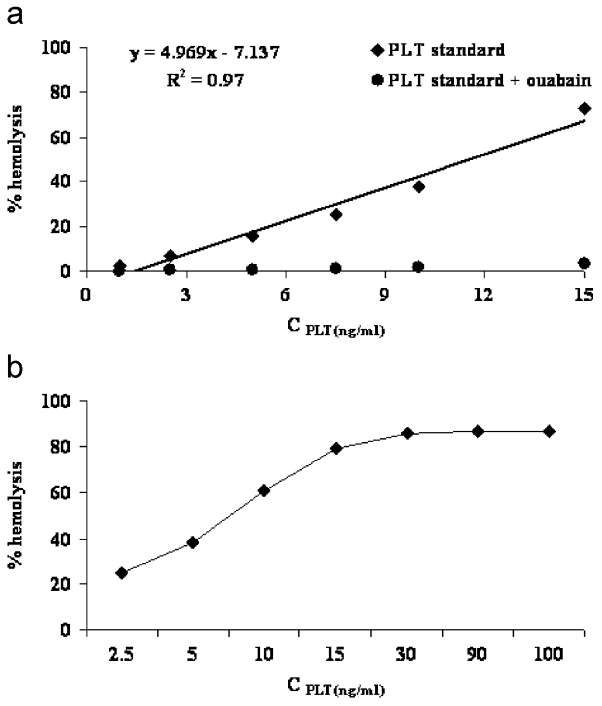

Fig. 2. Hemolysis response curves induced by different PLT concentrations on sheep erythrocytes (incubation 4 h, absorbance 540 nm). (a) Response curve and effect of ouabain pretreatment; (b) flattening of the curve in PLT concentrations >15 ng/ml.

Table 2
Hemolysis and MBA results of shellfish tissue

Sample code	MBA		Hemolytic activity	
	Prot A	Prot B[a]	Type	Ouabain suppression
1	+++[b]	+++	Delayed	Yes
2	−−−	−−−	Delayed	Yes
3	−−−	+++	Delayed	Yes
4	−−−[c]	−−−	Delayed	Yes
5	++−	+++	Delayed	Yes
6	+++	+++	Delayed	Yes
7	++−	++−	Delayed	Yes
8	−−−[c]	+++	Delayed	Yes
9	+++	+++	Delayed	Yes
15	+++	+++	Delayed	Yes
16	+++	+++	Delayed	Yes
17	+++	+++	Rapid	No
18	++−	−−−	Rapid	No
19	++−	−−−	Rapid	No
20	−−−	−−−	None	n.d.[d]
23	−−−	−−−	None	n.d.[d]

[a] *n*-butanol fraction.
[b] +++: 3/3 mice dead, −−−: no deaths.
[c] Symptomatology typical of p-PLT present in all mice.
[d] n.d.: not determined.

Hemolysis was observed in all four fractions (crude, DEE, aqueous and *n*-butanol) of each shellfish tissue sample examined. However, only results from the aqueous and *n*-butanol fractions were used for estimating p-PLT concentrations in these samples. The hemolytic activity observed in the crude and DEE extracts was noticeable but was not completely suppressed by ouabain, and in some cases was partly rapid, a fact that could be attributed to the presence of fatty acids (Kaul and Daftari, 1986) and/or to the simultaneous presence of other lipophilic toxins in the case of shellfish samples.

Based on the *n*-butanol extracts, p-PLT concentration of *O. ovata* was estimated to be around 0.4 pg/cell, while for *Ostreopsis* sp. it ranged from 0.7 to 0.9 pg/cell (Table 3). The above concentrations are within the range and somewhat higher than those reported by other authors (Rhodes et al., 2000). Lenoir et al. (2004) have reported lower values, such as 0.04 pg eq. PLT/cell, for *Ostreopsis mascarenensis* in field bloom populations. In the

Table 3
Hemolysis and MBA results of *Ostreopsis* cells

Sample code	*Ostreopsis* species (and cells number)	Origin of cells	MBA Protocol B[a]	Hemolytic activity		
				Rapidity	Ouabain suppression	C_{PLT} (pg/cell)
10[b]	*O. ovata* (2,964,840)	Olympiada (st. 3)	+++[c]	Delayed	Yes	0.43±0.165
11[b]	*O. ovata* (1,416,780)	Sarti (st. 2)	+++	Delayed	Yes	0.45±0.147
14[b]	*Ostreopsis* sp. (1,392,850)	Limnos (st. 5)	+++	Delayed	Yes	0.78±0.170
12[d]	*Ostreopsis* spp. (1,773,200)	Hrakleitsa (st. 4)	+++	Delayed	Yes	0.31±0.099
13[d]	*Ostreopsis* spp. (535,780)	Aggelochori (st. 1)	+++	Delayed	Yes	0.68±0.230

[a]Based on the *n*-butanol fraction.
[b]Culture material.
[c]+++: 3/3 mice dead, −−−: no deaths.
[d]Field material.

Table 4
MBA (Prot. B) and hemolysis results including estimated p-PLT (μg/kg tissue for shellfish) in different fractions of sample extracts presenting delayed hemolysis

Sample code	MBA			Hemolysis		
	Aqueous	*n*-butanol	DEE	Aqueous	*n*-butanol	Est. total[a]
1	+++[b]	+++	−−−[c]	76.9±3.55	19.9±6.14	96.8
8	+++	+++	−−−[c]	72.7±4.02	24.3±9.63	97.0
9	+++	+++	−−−[c]	18.1±3.54	21.8±1.23	39.9
16	+++	+++	−−−[c]	20.1±6.20	13.2±2.23	33.3

[a]These p-PLT concentrations correspond to an estimated total for each sample in order to approach the actual p-PLT content.
[b]+++: 3/3 mice dead, −−−: no deaths.
[c]Symptomatology typical of p-PLT present in all mice.

present study, however, cell concentrations from mixed field *Ostreopsis* spp. populations were found to contain p-PLT at levels of 0.3 and 0.7 pg/cell in stations st. 4 and st. 1, respectively (Table 3).

The estimated concentrations of p-PLT in the shellfish *n*-butanol extracts ranged between 13.3 and 24.3 μg/kg tissue, whereas in the case of aqueous extracts it ranged between 18.1 and 76.9 μg/kg tissue (Table 4). After adding the p-PLT concentrations of the two extracts (*n*-butanol and aqueous), estimated total levels in the shellfish samples ranged from 33.3 to 97.0 μg p-PLT/kg tissue. Rhodes et al. (2006), using also HNA, reported rather lower levels of p-PLT (1.2–14.5 μg/kg) in bivalve mollusks and sea urchins after feeding with *Ostreopsis siamensis* (46×10^6 cells) in laboratory conditions for a period of 3 days. The exposure of our collected shellfish samples in a natural and high abundant *Ostreopsis* population for a period, which in some cases lasted a few (3–4) months, could account for the higher p-PLT concentrations found in the present study. A further indication for this could be the almost three-fold increase in the estimated (by HNA) total p-PLT concentration in shellfish collected from station 4; the total p-PLT concentration was estimated to be about 33.3 μg/kg tissue in September (sample code 16), while 3 months later (December, sample code 1) the corresponding concentration was 96.8 μg/kg tissue.

The detection of p-PLT in both *n*-butanol and aqueous fractions of tissue samples with the HNA differentiates the results of the present study from earlier ones. Onuma et al. (1999) and Gleibs and Mebs (1999) found toxicity only in the aqueous extract, while Taniyama et al. (2002) reported that only the *n*-butanol fraction was toxic.

HNA has been previously shown (Bignami, 1993) to be an easy and also accurate tool for estimating putative PLT toxicity (Lenoir et al., 2004; Riobó et al., 2006). In the present study, it was possible to detect p-PLT concentrations of about 0.016 pg/μl of sample extract (0.32 pg/well). After extrapolation to

sample size and dilution factor, this concentration corresponds to 1.6 ng p-PLT/kg shellfish tissue. Similarly, Riobó et al. (2006) reported a low detection level of about 0.005 pg/μl (0.8 pg/well), whereas Onuma et al. (1999) found hemolysis to be a sensitive method in detecting PLT at a level as low as 50 pg/well (1 pg/μl).

In the present study, HNA was also found to be a useful discriminative tool between OA and p-PLT. This is considered very important because *Ostreopsis* species usually coexist with benthic *Prorocentrum* species, known producers of OA and derivatives, which in some cases reach high abundance levels (Aligizaki and Nikolaidis, 2006a). In the same context, Onuma et al. (1999) have also used the HNA to discriminate sardine toxin, PLT and ostreocin D from maitotoxin, amphidinol and prymnesin-2.

3.2. Mouse bioassay (MBA)

The majority of the shellfish samples containing p-PLT, as indicated by ouabain suppression in the HNA, also tested positive with MBA protocols (A) and (B) of this study (Tables 2–4). Few samples tested negative, but with mice exhibiting symptomatology from the nervous system typical of PLT, i.e. convulsions, waddling gait, numbness and blindness (Rhodes et al., 2002). The remaining samples were negative with no deaths or nervous symptomatology, but surviving mice showed severe cachexia, a symptom reported to occur in sublethal PLT doses (Ito et al., 1996). On the other hand, all *Ostreopsis* cell extracts containing p-PLT tested positive with the MBA protocol (B), showing the above-mentioned characteristic nervous symptomatology.

In particular, when tested with the MBA protocol (A), almost all samples containing p-PLT, as confirmed by the HNA, tested positive (Table 2) and presented very short death times (<30 min) with asymptomatic mice. However, a similar situation was also observed in the case of samples (sample codes 17, 18, 19) containing increased OA concentrations, making the distinction between samples containing p-PLT or OA extremely difficult by means of symptomatology. On the contrary, MBA protocol (B) proved to be much more selective for the detection of p-PLT, allowing easy discrimination between OA- or PLT-containing samples, both by mouse symptomatology and death times.

In the case of most of the p-PLT-containing samples, mouse toxicity was detected in both butanolic and aqueous fraction, whereas this was not the case with samples containing OA, where toxicity was present only in the butanolic fraction. There is some contradiction in the literature with regard to the presence of mouse toxicity in the aqueous fraction of *Ostreopsis* sp. extracts or extracts from seafood contaminated by suspected PLT. The aqueous fraction of PLT or p-PLT was also found toxic in mice by some research groups (Moore and Scheuer, 1971; Nakajima et al., 1981; Tindall et al., 1990, Onuma et al., 1999), which is in agreement with our results. In contrast, Taniyama et al. (2002, 2003) and Lenoir et al. (2004) did not detect any toxicity in the aqueous fractions. The presence of toxicity both in the aqueous and organic fraction could be justified by the structure of the PLT molecule possessing both hydrophobic and hydrophilic areas (Shimizu, 1983). This is further supported by the chromatographic identification of a PLT-like substance in the butanolic (*Ostreopsis mascarenensis*; Lenoir et al., 2004) and of an extract from *O.* cf. *ovata* in the aqueous fraction (Riobó et al., 2004). It is possible that the origin of the PLT-like substance and/or the involvement of different *Ostreopsis* species/strains could be a determining factor with regard to partitioning between the organic and aqueous layers, since PLT-like substances of different origins are not identical in terms of molecular structure (for a review, see Katikou, 2007).

In the p-PLT-containing shellfish samples, mice presented typical PLT symptomatology (see above), while, almost in all samples, death occurred within 30 min after injection. In the case of samples containing highly elevated concentrations of OA, as confirmed by a protein phosphatase inhibition assay (data not shown), mice showed typical OA symptoms (lethargy) and much longer death times (several hours). This could be attributed to the possibility of a more limited retention of OA in the butanolic fraction, which was the only toxic fraction in these samples, in comparison to the retention of p-PLT. Another possible explanation could also be the partial removal of OA during the step of defatting with diethylether.

3.3. General remarks

Results of this study indicated the presence of a, similar to PLT, toxic compound in both *Ostreopsis*

cells and shellfish tissues collected from Greek coastal waters. The detection of this compound coincided timewise with *Ostreopsis* spp. occurrence (Table 1). In particular, the presence of *Ostreopsis* spp. has been recorded repeatedly in the period between July and the end of November in the years 2003, 2004, 2005 and 2006 (Aligizaki and Nikolaidis, 2006b; present study), while toxicity in the corresponding shellfish samples was usually detected 1 or 2 weeks after the initial *Ostreopsis* spp. appearance and lasted until 2–3 weeks after *Ostreopsis* spp. were not anymore detected on macrophytes and subsequently in the water column (Table 1).

All *Ostreopsis* strains are potentially toxic (Faust et al., 1996; Faust, 1999) and, during the last years, there are increasing reports (Onuma et al., 1999; Taniyama et al., 2003; Rhodes et al., 2000; Lenoir et al., 2004; Penna et al., 2005; Riobó et al., 2006) of many cases of *Ostreopsis* species toxicity by use of different methods (hemolysis, cytotoxicity, LC–MS). For instance, *O. ovata* from tropical waters was considered to be a species of limited toxicity (Nakajima et al., 1981; Tindall et al., 1990); however, *O. ovata* from the Mediterranean Sea have been shown to be equally as toxic as *O.* cf. *siamensis* Mediterranean strains (Penna et al., 2005; Riobó et al., 2006).

This is further supported by the fact that *O. ovata* blooms in Italy have been recently associated with human illnesses; several people have been hospitalized with symptoms such as rhinorrhea, cough, fever, bronchoconstriction and wheezing, after exposure to seaside areas where *O. ovata* blooms occurred (Brescianini et al., 2006; Ciminiello et al., 2006). However, in that case, the occurrence of *O. ovata* in bloom densities has not been yet associated with shellfish poisoning, although heavy consequences on benthic fauna have been reported (Sansoni et al., 2003). On the other hand, *Ostreopsis* blooms in Greece, though strong but mainly epiphytic, have not been associated with human illness, either directly by human exposure (bathers) or by shellfish consumption. The lack of human intoxication reports due to shellfish consumption in Greece could be partly attributed to the fact that since 2003, within the framework of the national biotoxins' monitoring program, both the positive DSP samples detected and the presence of *Ostreopsis* species (Aligizaki and Nikolaidis, 2006b) resulted in repeated restrictions of shellfish-harvesting activity during summer–autumn periods and consequently limited exposure of consumers to p-PLT-contaminated shellfish.

Bivalve mollusks contamination by putative PLT due to the presence of *Ostreopsis* species in Greek coastal waters constitutes the first record for the Mediterranean Sea and Europe and, to the best of our knowledge, worldwide.

Taking into account that 1 MU is equivalent to 9 ng PLT (Onuma et al., 1999), HNA revealed that the shellfish samples contained p-PLT in the range 74–216 MU/kg tissue, whereas in *Ostreopsis* cells this range was $4.7–10.0 \times 10^{-5}$ MU/cell, near to the level of 1.0×10^{-4} MU/cell reported by Taniyama et al. (2003). Moreover, regarding the MBA protocol (B), sample size was chosen in order to set the detection limit for p-PLT to the value of 450 ng/kg, which coincides with the reported LD_{50} value for PLT (Wiles et al., 1974). This limit of detection is well below the recently EU-proposed provisional limit of 250 μg/kg (WG Toxicology, 2005), a fact indicating that this protocol could be an interesting approach for regulatory monitoring purposes by further adjustment of sample size.

Results of this study demonstrate the requirement for further research on the clarification of both the taxonomical identity and toxicological properties of *Ostreopsis* species. In this context, further studies are underway in order to elucidate the chemical structure and properties of the detected compound in both *Ostreopsis* cells and shellfish by liquid chromatography and mass spectrometry. It should finally be pointed out that the expansion of these species during the last years, their occurrence in massive blooms in the Mediterranean Sea and their association with ciguatera poisoning in ciguatera endemic areas constitute them as a potential threat for human health. It is therefore evident that a routine monitoring strategy for these benthic species is essential in susceptible areas.

Acknowledgments

The authors would like to thank the Institute of Infectious and Parasitic Diseases of the Centre of Veterinary Institutes of Thessaloniki for providing the sheep blood used in the hemolysis experiments and all the staff of the National Reference Laboratory on Marine Biotoxins for their assistance in the experiments. Financial support was provided by PENED 2003 (Third Community Support Programme, Operational Programme: Competitiveness, Priority Axis 8: Human Resources, Measure

8.3: Human Resources in Research and Technology, Action 8.3.1: Reinforcement Programme of Human Research Manpower-PENED).

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