

# Warm Seawater Microalgae: Growth and Toxic Profile of *Ostreopsis* Spp. From European Coasts

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## Abstract

Palytoxin (PLTX) is a complex marine toxin synthesized by the soft coral *Palythoa toxica* and by species of the benthic dinoflagellate *Ostreopsis* spp. The toxin binds to the active Na,K-ATPase pump in the cellular membrane. This interaction changes the protein conformation and produces a non-specific cation channel. Using the Fluorescent Polarization (FP) technique to quantify the PLTX concentration, the production of PLTX-like products was measured in several cultures of *Ostreopsis ovata* and *Ostreopsis siamensis*. The cultures were grown under different conditions to study the optimal parameters to grow and to produce toxins. Serious difficulties were found to quantify the number of cells at the end of the exponential phase. In order to avoid any toxin loss, the weight of the pellet obtained after careful filtration was used as reliable parameter to calculate the growth of the cultures. Also, this parameter was used to refer the results of toxin concentration. In addition the toxicity of the cultures was measured by mouse bioassay. In these conditions, the optimal parameters to grow these strains are 24°C, 37‰ of salinity and 16:8 h light-dark photoperiod. Within these parameters high amounts of PLTX-like compounds with different toxin profiles were obtained.

**Keywords:** Palytoxin; Na,K-ATPase; Ouabain; *Ostreopsis* spp.; Fluorescent polarization; Cleaning method

## Introduction

Palytoxin (PLTX) is a toxic compound initially isolated from marine soft corals of the genus *Palythoa* [1] but also produced by tropical benthic dinoflagellates from *Ostreopsis* genus [2]. This toxin has been involved in human poisoning after the ingestion of food contaminated, but also skin irritations and respiratory effects by contact with contaminated spray seawater have been reported [3]. The PLTX-group of toxins includes complex polyhydroxylated compounds with a lipophilic and a hydrophilic area. Different molecules related with the PLYX structure (Figure 1) have been described: palytoxin-b, homopalytoxin, bishomopalytoxin, neopalytoxin, deoxypalytoxin, 42-hydroxypalytoxin, ostreocin-d, ovatoxin-a, -b, -c, -d, -e and -f and mascarenotoxin-a, -b and -c. However only the chemical structures of PLTX, ostreocin-d, ovatoxin-a and 42-hydroxypalytoxin have been characterized [4-11]. PLTX is a large molecule, with a molecular weight of 2659-2680 daltons depending on the origin [12,13].

The target of PLTX is the Na<sup>+</sup>,K<sup>+</sup>-ATPase pump from the plasma membrane. This pump exchanges three Na<sup>+</sup> ions from the cytosol to the extracellular space for two K<sup>+</sup> ions from the outside of the cell into the cytoplasm, expending one ATP molecule [14]. The toxin produces a free flow of ions, and as consequence the cationic permeability of the cells is altered. This imbalance in excitable cells causes symptoms like scratching, jumping, paralysis of hind limbs, respiratory distress, cyanosis also brings nausea, tiredness, diarrhoea and vomiting followed by dizziness, in animals, but also intestinal symptoms, muscle spasms, breathing difficulties followed in some cases by dead in humans [15-17]. This type of human poisoning is called palytoxicosis or clupeotoxicosis [18].

*Ostreopsis* spp. are unicellular algae. The habitat and the growth of these dinoflagellates are very important in toxin production [5]. Temperate tropical coastal waters were the original localization of these species [19], however these blooms have been described in other localizations far away from tropical waters, as the Tyrrhenian and Adriatic Seas [19,20] Spain [21,22] Greece [23] and more recently in

Portugal [24]. Therefore the general rise of water temperatures may be modifying the *Ostreopsis* spp. world distribution, and therefore these dinoflagellates can appear in new places where they never have been seen before, becoming PLTXs compounds a serious public health problem. *Ostreopsis* spp. grows better in calm, shallow, warm waters and it seems that high salinity concentration increases their development [25-27]. But it is not clear the importance of the salinity and nutrients in their blooms [26]. Therefore it is essential to know the optimal conditions for the growth of these cells and the link with toxin production. Therefore, in this context, the aim of this work is to estimate the production of PLTX-like compounds in different cultures of *Ostreopsis* spp. growing in several biological conditions by using a Fluorescence Polarization (FP) technique recently developed [28].

## Materials and Methods

### Chemicals

PLTX was obtained from Wako Pure Chemical Industries (Neuss, Germany) and it was dissolved in MeOH:H<sub>2</sub>O (1:1). Adenosine 5-triphosphatase (Na,K-ATPase) from porcine cerebral cortex and ouabain was obtained from Sigma-Aldrich Química, S.A. (Spain); Fluoro-Spin 498 Protein Labeling Kit was obtained from EMP Biotech (Germany).

Methanol, n-hexane and n-butanol were obtained from Panreac (Spain). LC-18 SPE tubes 6 mL and 2 mL Empty PP Rev Tube Kit with silica (600mg) was obtained from Sigma-Aldrich Química, S.A.

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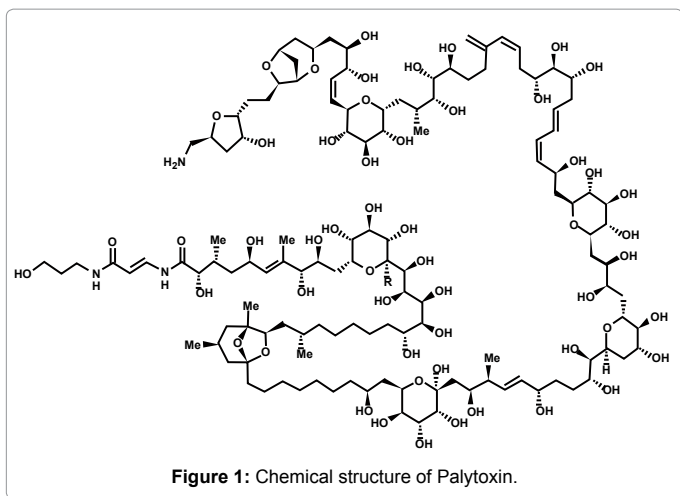


Figure 1: Chemical structure of Palytoxin.

(Spain). Ultrafree-CL filters (0.45  $\mu\text{m}$  pore diameter and low-binding regenerated cellulose as membrane) were purchased from Millipore (Spain). The composition of PBS is: NaCl 137 mM;  $\text{Na}_2\text{HPO}_4$  8,2 mM;  $\text{KH}_2\text{PO}_4$  1,5 mM; KCl 3,2 mM y  $\text{MgCl}_2$  1 mM.

Dionized water (Milli-Q) was used for all experiments. PBS chemicals were obtained from Panreac Quimica S.A.U. (Spain), except  $\text{KH}_2\text{PO}_4$  which was purchased from Merk (Spain). Saline solution was obtained from Laboratorio Grifols S. A. (Spain) and Tween® Monostearate (Tween 60) from Sigma-Aldrich Química, S.A. (Spain).

### Protein labeling and fluorescence polarization measurements

To label Na,K-ATPase, a reactive succinimidyl-ester of carboxy-fluorescein was used. This molecule forms a stable covalent linkage between the protein and the dye. This conjugation has fluorescent excitation and fluorescence emission maxima at 498 nm and 522 nm respectively. The Fluoro Spin 498 Protein labeling kit contains all of the chemicals and tools needed during the labeling and purification processes.

The protein was mixed with a sodium bicarbonate solution, then the dye was added, and the mixture reacted for 1 h at room temperature while protected from light. Afterward, a hydroxylamine solution was added to stop the labeling and incubated for 30 min. The final conjugate (Na,K-ATPase - F) was purified (separated from the non attached dye) by using spin columns. The conjugates can be stored for a long time in frozen aliquots at  $-20^\circ\text{C}$  while protected from light. With this protocol 2 mg/mL of adenosine 5-triphosphatase (Na,K-ATPase) from porcine cerebral cortex were labeled. The labeling ratio was 17 molecules of dye per molecule of protein. The final conjugate was dissolved in PBS. To select the correct dilution factor to make the experiment, six different dilutions, ranging from 1:100 to 1:7500, were tested in a final volume of 250  $\mu\text{L}$ . The dilution 1:5000 was chosen. With this dilution factor the final protein label concentration in each well was 2.5 ng/mL. This concentration remained constant in all experiments.

The FP is a spectroscopic technique based on exciting a fluorescent molecule with plane-polarized light and measuring the polarization degree of the emitted light. An important application of this technique is the study of molecular interactions since the FP value of a molecule will be different if a binding with another molecule occurs [29]. In each experiment as control, the cardiac glycoside ouabain 1mM, a known Na,K-ATPase blocker, was used. This drug binds to Na,K-ATPase in the same mode than PLTX. In this way, a fall of 150 millipolarization (mP)

units was used as control in each experiment for the ouabain wells.

In each well 100  $\mu\text{L}$  of PBS solution, 25  $\mu\text{L}$  of sample (culture extract or toxin) and 125  $\mu\text{L}$  of Na,K-ATPase-F were added. Then the microplate was shaken for 10 minutes at 300 rpm at room temperature, before measuring. In the control, the quantities were 100  $\mu\text{L}$  of PBS solution, 25  $\mu\text{L}$  of ouabain 1 mM and 125  $\mu\text{L}$  of Na,K-ATPase-F. In each plate PLTX calibrations curve with concentrations of: 10 nM, 25 nM, 50 nM, 75 nM and 100 nM were used to calculate the unknown concentration of samples.

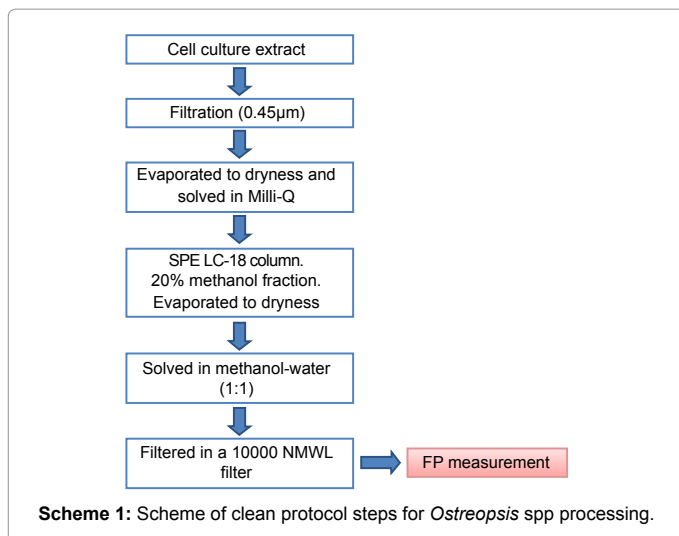
The Synergy 4 instrument from Biotek was used to measure the FP. The measurements were done in black polystyrene 96-well microwell plates (Costar, Spain). The light source was a Tungsten lamp. The emission light was detected by photomultipliers. Filters and polarization filters (for FP) were used to select the correct wavelength. In this case, the filters of 485 nm for excitation and 528 nm for emission were used to measure the fluorescence intensity, and the polarization filters of the same wavelength were used to measure the FP in mP units. The sensitivity was set at 100. The FP results were calculated with the Gen 5 1.07 software.

### Dinoflagellate cultures

The cultures used were: *Ostreopsis ovata*, (A and B), *Ostreopsis siamensis*, *Coolia monotis* and *Protoceratium reticulatum*. The cultures were grown in seawater enriched with L1 medium, without silicates and a salinity level of 37%. Also some cultures were grown at 42% salinity. The salinity was adjusted by the addition of freshwater removing chlorine by aeration. The experiments were carried out at  $19^\circ\text{C}$  and  $24^\circ\text{C}$  and the cultures were subjected to photoperiod with day-light lamps on a 16:8 h light-dark photocycle. Cells were gently shaken manually twice a day and its growth was divided in steps, increasing the volume progressively. 300 cells/ml was always used as initial inoculum and the profile and conditions of growth for each strain were previously established. In these cultures the beginning of the stationary phase of growth was reached 15-18 days after inoculation [25]. The dinoflagellates were kept under these conditions for 30 days, then the whole culture volume was carefully filtered, 20  $\mu\text{m}$  mesh, and the residue obtained was weight. Finally methanol-water (1:1) was added and the extract was sonicated to their complete homogenization and toxin release and was stored frozen until measurements were to be done. The added volume was three times the residue weight. To count the number of cells, the cultures were carefully homogenized, 25 ml pipette shaking, and 5 ml aliquots were collected. In some experiments chemical disaggregation by adding HCl to a final concentration of 4 mM was done. Cells were fixed with Lugol's solution and were counted by optical microscopy in a Sedgewick-Rafter chamber [30].

### Cleaning steps

The cultures cleaning procedure is shown in Scheme 1. Briefly, 500  $\mu\text{L}$  of the cellular extract solved in methanol-water (1:1) were centrifuged for 10 minutes at 5000 rpm at  $4^\circ\text{C}$ . The supernatant was filtered through a 0.45  $\mu\text{m}$  cellulose filter. The solution filtered was evaporated to dryness and diluted in 2 mL of deionized water and charged into SPE (Solid Phase Extraction) cartridge (LC-18). Previously the cartridge was conditioned with methanol and water. Then, after loading the sample, methanol 20% was added and the toxin was eluted. This fraction was evaporated to dryness and solved in 500  $\mu\text{L}$  of MeOH- $\text{H}_2\text{O}$  (1:1). This protocol previously described was extended with a filtration step, through a 10000 NMWL filter [28,29]. The filtered extract was diluted 1:1000 to the final volume of 250  $\mu\text{L}$  to check the



toxin concentration by FP.

### Mouse bioassay

The lethal potency of culture samples was estimated by intraperitoneal (i.p.) injection of the extracts or PLTX standard re-suspended in saline solution with 1% Tween 60 into healthy male Swiss mice (SPR), 19-23 g body weight. The toxicity was determined by the time of death of six mice, according to the standard dose-lethal time plot prepared by using several dilutions of PLTX standard. The culture extract was centrifuged at 5000 rpm for 10 minutes at 4°C, and the supernatant was diluted at least 1:100 in the saline solution with 1% Tween 60. Therefore less than 0.5% of methanol was injected. 1 mL of this preparation was administrated to mice. Controls of 0.5% methanol and non-toxic strains were done and no effects were observed. Mice were observed during 24 hours, the symptoms were described and also the death time.

### Data analysis

All experiments were carried out at least three times using duplicates. Data were normalized and results were expressed as mean ± SEM. Results were analyzed using the Student t test. A probability level of 0.05 or less was used for statistical significance.

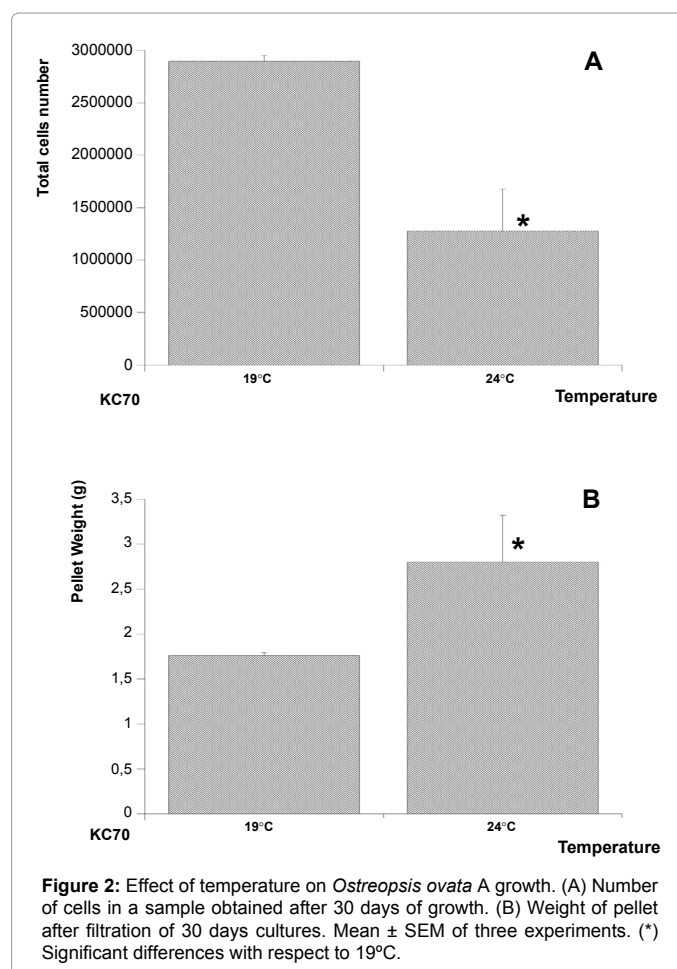
### Results

The aim of this work was to study the effect of different biological conditions in growth and toxin production of several strains of *Ostreopsis ovata* and *Ostreopsis siamensis*. Initially *Ostreopsis* spp. growth at 19°C and 24°C was studied. All cultures were grown in a total volume of 800 mL until the end of stationary phase, 30 days of growth. Then, the cultures were gently homogenated and filtered and the residues obtained were weighed and re-suspended in methanol-water. The cells were counted before filtration. With these data the growth could be represented as the number of the cells at the end of the exponential phase and also as the weight of the pellet after the whole culture volume was filtrated. Since *Ostreopsis* spp. are growing in lumps and glued to the edge of the container involved in a mucous substance it is very difficult to break the lumps and to do a reliable and representative counting. *O. ovata* A growth at 19°C and 24°C is represented in Figure 2.

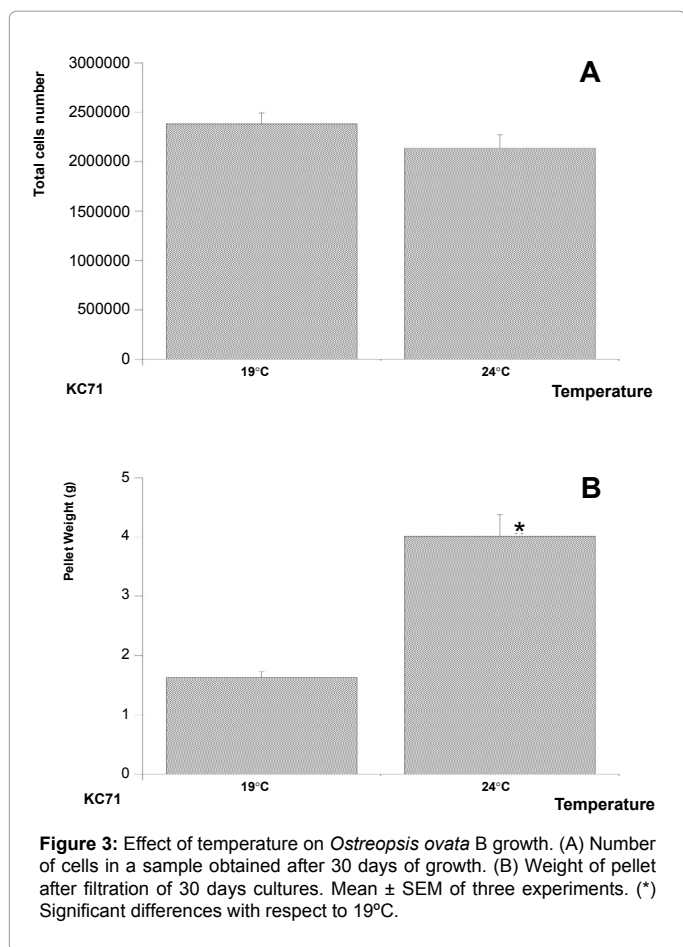
After 30 days of culture a total of  $2.89 \pm 0.057 \times 10^6$  cells at 19°C and

$1.28 \pm 0.4 \times 10^6$  cells at 24°C were obtained (Figure 2A). Therefore, from this a significantly decrease of cells number at 24°C could be concluded. However, when the weight is represented, a significant increase of cell weight at 24°C respect to 19°C is showed,  $1.76 \pm 0.03$  g versus  $2.8 \pm 0.52$  g (Figure 2B). Therefore, the results seem to be opposite taking into account the weight or the cell number. In the case of *O. ovata* B strain (Figure 3) the number of cells also decreases from  $2.38 \pm 0.1 \times 10^6$  cells at 19°C to  $2.14 \pm 0.13 \times 10^6$  cells at 24°C. Attending to the weight of the pellet, there is a significant increase in grams of the pellet, from  $1.63 \pm 0.1$  g at 19°C to  $4 \pm 0.36$  g at 24°C. When *O. siamensis* was studied the results were equal, either representing cell number or the pellet weight (Figure 4); the cell number is significantly higher at 24°C,  $1.5 \pm 0.3 \times 10^6$  cells, than at 19°C,  $0.28 \pm 0.08 \times 10^6$ , (Figure 4A), and the cells weight was also significantly increased from  $1.07 \pm 0.12$  g at 19°C to  $2.3 \pm 0.14$  g at 24°C, (Figure 4B). Therefore, in general and taking into account pellet weight, 24°C is the optimal temperature to grow *Ostreopsis* spp. cultures.

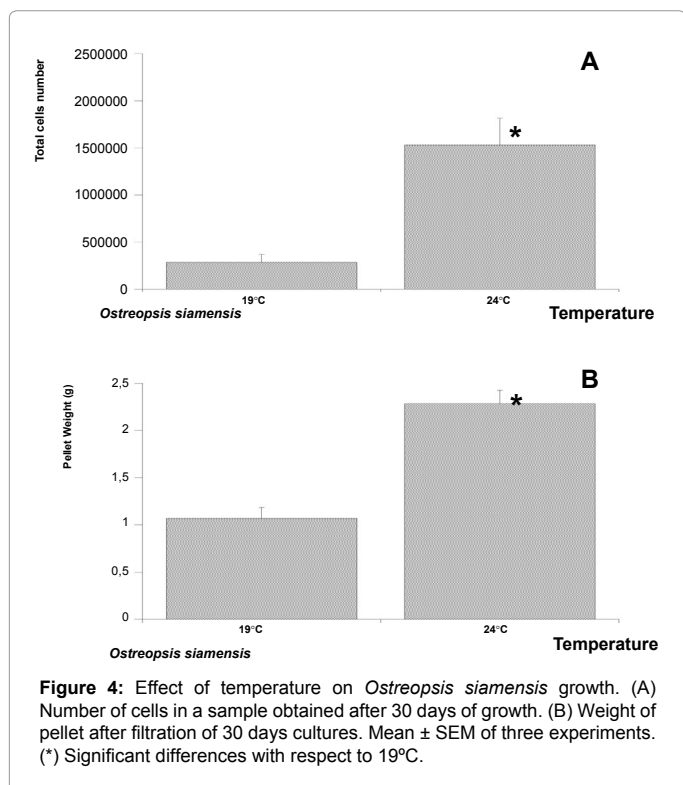
To check if the increase in pellet weight is only due to the mucus presence or to the increase in cell number the counting was repeated after cellular chemical disaggregation by adding HCl 4 mm final concentration [25,31]. Several *O. ovata* a cultures were grown in a total volume of 200 mL for 30 days at 19°C and 24°C. Then in some flasks HCl was added and the cell number was counted. As Figure 5 shows, the number of cells counted with and without HCl is different at any temperature checked, however after HCl addition the number of cells at 24°C was significantly higher and therefore the increase in pellet weight







**Figure 3:** Effect of temperature on *Ostreopsis ovata* B growth. (A) Number of cells in a sample obtained after 30 days of growth. (B) Weight of pellet after filtration of 30 days cultures. Mean  $\pm$  SEM of three experiments. (\*) Significant differences with respect to 19°C.

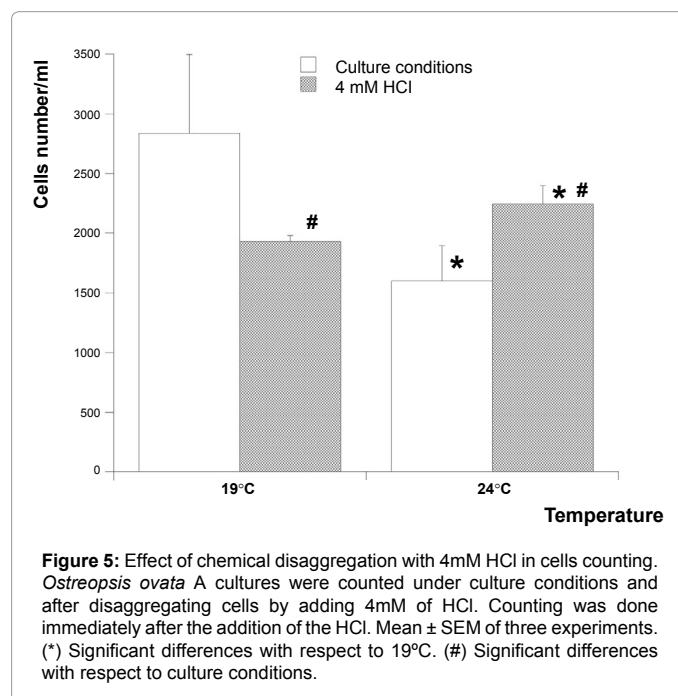


**Figure 4:** Effect of temperature on *Ostreopsis siamensis* growth. (A) Number of cells in a sample obtained after 30 days of growth. (B) Weight of pellet after filtration of 30 days cultures. Mean  $\pm$  SEM of three experiments. (\*) Significant differences with respect to 19°C.

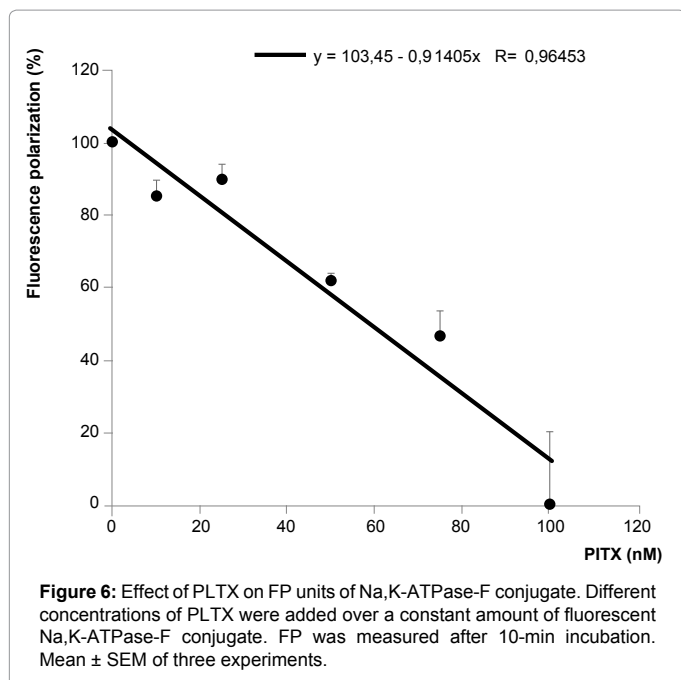
match with an increase in cells number. It is important to bear in mind that HCl addition decreases seawater pH from 8.3 to 3 and cell viability after 15 minutes was 25% lower, in addition motionless cells and several debris were observed. Therefore, the following studies about toxin concentration were always referred to pellet weight since it is a reliable parameter and the cells integrity is an important issue in order to avoid any toxin leakage to the medium in the next experiments.

The next point was to check the PLTX-like compounds production in these conditions. The method to quantify the PLTX is based on the FP methodology. In several studies, the FP technology was used to quantify toxins [29,32,33] and recently it was applied to develop a sensitive PLTX detection method [28]. In general, the FP units of a Na,K-ATPase-F conjugate decreased in the presence of PLTX and/or PLTX-like compounds with the same mechanism of action. By using a calibration curve with FP units versus PLTX concentration, the amount of PLTX-equivalent in a sample can be estimated (Figure 6). To check the toxin concentration in the dinoflagellates cultures, several cleaning steps to avoid a matrix effect were done, then *Ostreopsis* spp. cultures and two non-PLTX producer cultures were tested as negative control, *Coolia monotis* and *Protoceratium reticulatum*. These two cultures were used to check the matrix interference and the recovery of the cleaning procedure. As Table 1 shows, neither *Coolia monotis* nor *Protoceratium reticulatum* produce any change in Na,K-ATPase-F conjugate FP units. However, when *Ostreopsis* spp. cultures were analyzed, different PLTX-equivalent amounts were obtained, 113.41  $\pm$  32.7  $\mu$ M PLTX-equivalent for *O. ovata* A strain, 67.65  $\pm$  12.8  $\mu$ M PLTX-equivalent for *O. ovata* B strain and 20.2  $\pm$  8.4  $\mu$ M PLTX-equivalent for *O. siamensis*. These strains were also analyzed by mouse bioassay and were found positives after mouse injection.

Then the PLTX-like compounds production in different culture conditions was checked. First, the effect of salinity was studied (Figure 7); the rest of the parameters were 24°C and 16:8 h light-dark photoperiod. In these conditions, 526.4  $\pm$  24.3  $\mu$ g PLTX-equivalent/g pellets were produced by the *O. ovata* A strain at 37% of salinity, while at 42% the amount was significantly decreased to 265.45  $\pm$  44.8  $\mu$ g



**Figure 5:** Effect of chemical disaggregation with 4mM HCl in cells counting. *Ostreopsis ovata* A cultures were counted under culture conditions and after disaggregating cells by adding 4mM of HCl. Counting was done immediately after the addition of the HCl. Mean  $\pm$  SEM of three experiments. (\*) Significant differences with respect to 19°C. (#) Significant differences with respect to culture conditions.



Species	PLTX equivalent concentration ( $\mu$ M)
<i>Protoceratium reticulatum</i>	0
<i>Coolia monotis</i>	0
<i>Ostreopsis ovata</i> A	113.41 $\pm$ 32.7 (positive mouse bioassay)
<i>Ostreopsis ovata</i> B	67.65 $\pm$ 12.8 (positive mouse bioassay)
<i>Ostreopsis siamensis</i>	20.2 $\pm$ 8.4 (positive mouse bioassay)

**Table 1:** PLTX-like compounds concentration in different cultures extracts

PLTX-equivalent/g pellet. For the *O. ovata* B strain, the production was 351.5  $\pm$  129.4  $\mu$ g PLTX-equivalent/g pellet at 37% and 336.7  $\pm$  92.7  $\mu$ g PLTX-equivalent/g pellet at 42%. On the other hand, high mortality was observed when *O. siamensis* was grown in a 42% salinity medium. Therefore, 37% salinity was chosen to keep *Ostreopsis* spp. cultures. The second parameter studied was the volume of growth. The cultures were grown in a total volume of 1800 mL or 800 mL, both starting from the same initial inoculums, however no significant differences were found even when lower pellet weights were obtained with 1800 mL (data not shown).

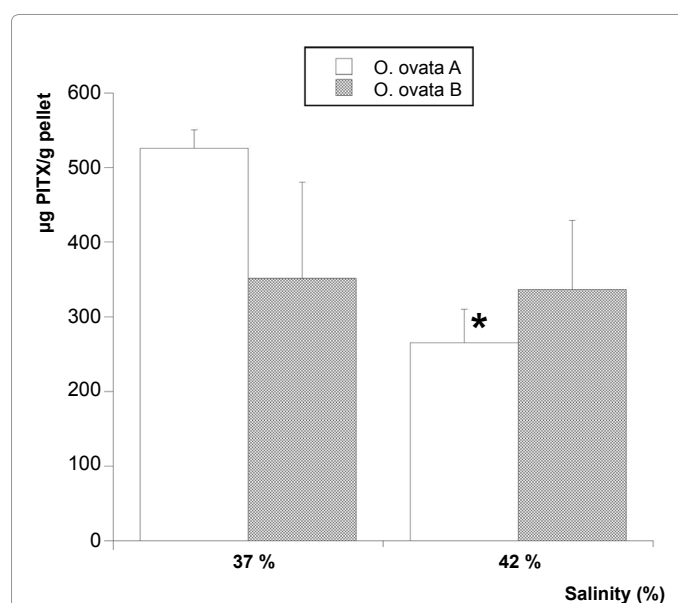
Then, the influence of temperature in PLTX-like compounds production was studied. The cultures were grown at 19°C and at 24°C and the rest of parameters were 37% salinity and 16:8 h light-dark photoperiod. As (Figure 8) shows, in all cases the toxin production is higher at 24°C. *O. ovata* A produces 216.75  $\pm$  151.4  $\mu$ g PLTX-equivalent/g pellet at 19°C and 768.6  $\pm$  115.5  $\mu$ g PLTX-equivalent /g pellet at 24°C. *O. ovata* B produces 415.25  $\pm$  24.8  $\mu$ g PLTX/g pellet at 19°C and 868.26  $\pm$  76.5  $\mu$ g PLTX-equivalent/g pellet at 24°C. In addition *O. siamensis* produces 45.5  $\pm$  20.5  $\mu$ g PLTX-equivalent/g pellet at 19°C and 256.6  $\pm$  26.3  $\mu$ g PLTX-equivalent/g pellet at 24°C. The production at 24°C is at least double than at 19°C and in all cases. Hence the optimal temperature for these dinoflagellates to produce PLTX is 24°C.

Finally, mouse bioassay was used to check the total toxicity of the *Ostreopsis* spp. cultures. Table 2 shows LD<sub>50</sub> of the PLTX standard control and also the lethality of different extracts. Several concentrations of PLTX standard, from 2000 to 500 ng/kg mouse weight, diluted in 1 mL of the saline solution with 1% Tween 60 were injected to mice.

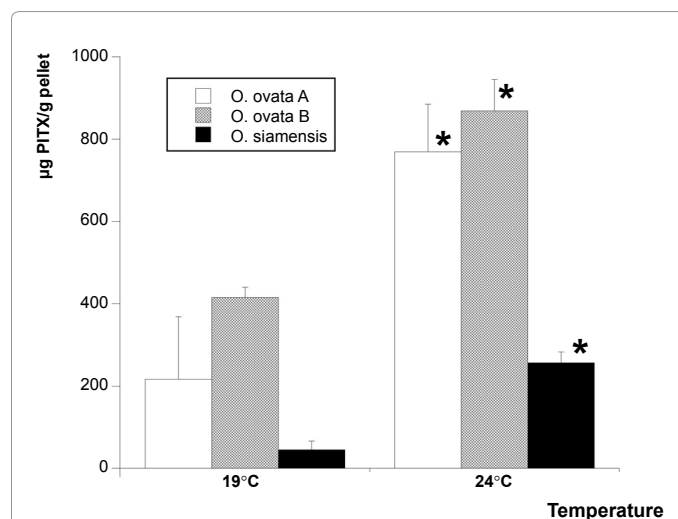
From these experiments, 750 ng PLTX/Kg mice weight was calculated as the LD<sub>50</sub> for PLTX. Then, the toxicity of the *Ostreopsis* spp. cultures was checked. Several mice were injected with 5 decreased quantities of *O. ovata* A culture, 139  $\mu$ M of PLTX-equivalent, *O. ovata* B culture, 56  $\mu$ M of PLTX-equivalent, and culture 12.72  $\mu$ M of PLTX-equivalent. From these experiments, 46.5  $\mu$ g of PLTX equivalent/kg mice weight, 187.5  $\mu$ g of PLTX equivalent/kg mice weight and 136.5  $\mu$ g of PLTX equivalent/kg mice weight respectively were obtained as LD<sub>50</sub> for the *Ostreopsis* spp. cultures indicating a higher toxicity in the case of the *O. ovata* A culture.

## Discussion

The production of toxins *in vitro* plays an important role to obtain



**Figure 7:** Toxin production of cultures of *O. ovata* A and B, growing at 37‰ and 42‰ of salinity and 24°C. Mean  $\pm$  SEM of three experiments. (\*) Significant differences with respect to 37‰ of *O. ovata* A strain.



**Figure 8:** Toxin production of cultures of *O. ovata* A and B and *O. siamensis* at 19°C and at 24°C. Mean  $\pm$  SEM of three experiments. (\*) Significant differences with respect to 19°C.

Extract	Percentage of death mouse (%)			LD <sub>50</sub> (µg/kg)	Symptoms
	0%	50%	100%		
PLTX standard	0.5 µg/kg	0.75 µg/kg	1 µg/kg	0.75	Ataxia, spasms, dyspnea, scruffy
<i>O. ovata</i> A	18.6 µg/kg	46.5 µg/kg	93 µg/kg	46.5	
<i>O. ovata</i> B	112.5 µg/kg	187.5 µg/kg	375 µg/kg	187.5	
<i>O. siamensis</i>	68 µg/kg	136.5 µg/kg	170.5 µg/kg	136.5	
Methanol control	0,50%	-----	-----	-----	No symptoms observed
	0,25%	-----	-----	-----	
	0,125%	-----	-----	-----	
Matrix effect ( <i>Coolia monotis</i> )	0,50%	-----	-----	-----	
	0,25%	-----	-----	-----	

**Table 2:** Mouse bioassay data (PLTX-equivalents) after i.p. injection of *O. ovata* A, *O. ovata* B strains and *O. siamensis*. Injections were made diluted in 1 mL of saline solution with 1% Tween 60. A range of 5 concentrations, 0.05-1 µg/Kg, of PLTX standard were used. The mice were observed the first hour and monitored for 24 hours and death mice were registered in addition with symptoms. One lower concentration than those that produce 0% of mouse death and one higher concentration than those that produce 100% of mouse death were also tested in *Ostreopsis* strains but only three mice were used in each case with the same result than previous concentration. Controls with different methanol percentage and matrix effect were done (three mice per condition) and no effects were observed

standards to develop toxicological and pharmacological studies and more accurate detection methods. More important, the knowledge of the optimal conditions for these species to grow better and produce more toxin help us predict where and when their blooms can suddenly appear. As several studies demonstrate, *Ostreopsis* spp. are changing their world distribution [20-23] and therefore it is necessary to know the strains and species arriving, as well as their toxin profile in order to clarify if any environmental variation can affect their presence.

It was described that only the Japanese *O. siamensis* produces ostreocin [34], however other studies demonstrate that also Mediterranean *O. siamensis* synthesizes this toxin [13,35,36]. In addition PLTX or putative PLTX (depending on the author) have been identified in this specie [4,37,38]. Although the term PLTX-like compound is used to designate toxins coming from *Ostreopsis* spp. [13]. This controversy could be due to the lack of standards that prevent the clarification of the toxins produced by each strain. In this sense, many times the results are shown as PLTX-like compounds because although several analogs have been identified, as far as we know, certified material or standards for these products are not yet available. Therefore the identification studies published are based on theoretical chemical approaches from a PLTX standard [5,7-10,18].

The first observation from these results is the fact that to quantify the growth of cultures, the pellet weight is as accurate as the cells number. Since *Ostreopsis* spp. are benthic dinoflagellate that grow in lumps and segregate a mucous substance the homogenization of the suspension is really hard and some chemical disaggregation is necessary [25,31]. If only the number of cells is measured, the chemical disaggregation with HCl is a useful tool, but when the cultures integrity is important the acidification could be a problem. Although the weight is increased due to the mucous presence, this increase should be proportional with the cells number. In any case, the way these cells grow up and the counting procedure is important since these species are regularly monitored in the water column in some countries to avoid human intoxications. In this sense, a large number of cells could be present in the water but the cells number could be underestimated since most of them are growing in the rock surface or in the seabed making lumps. Therefore, it is very important to bear in mind these data to develop a representative sampling procedure in order to avoid an underestimation in the cell number.

*O. ovata* produces more PLTX-like compounds than *O. siamensis*. It is known that these two species produce different PLTX analogues, *O. ovata* produces ovatoxin-a [36,39] while ostreocin-d is synthesized by *O. siamensis* [34,40]. Probably these two PLTX-like compounds have

different affinity for the Na,K-ATPase. From our data, it seems that ostreocin-d, or the analog produced by this *O. siamensis* strain, could show less bind to the pump, providing a lower FP signal, and therefore lower PLTX-like concentration. But attending to the growth at 19°C and 24°C, it seems that in any case, *O. siamensis* growth is lower than *O. ovata*, therefore maybe the toxin synthesis is also lower.

Different approaches have been published about the optimal temperature of *Ostreopsis* blooms occurrence. A temperature of 23°C has been proposed as the optimal temperature to these cells after a bloom in the Adriatic Sea [19]. On the other hand, high abundance of *O. ovata* has been described when the seawater temperature was 26-30°C in The Tyrrhenian Sea and in the Genoa coast, while 28-29°C were registered along the Italian coastline of Lazio in 1994 during an *Ostreopsis* bloom [26,41,42]. Recently, in Japan's coast a temperature range between 22-30°C depending on the *Ostreopsis* spp. strain studied has been suggested [43]. Under controlled conditions high growth was observed at 20°C although higher toxin production was obtained at 25°C [31], the same as *Ostreopsis lenticularis* that produces more toxin at highest temperatures [44]. In addition, it is not clear the influence of the salinity in the toxin production [26,31]. Our results suggest that the toxin production and the growth are higher at 37% of salinity and 24°C. This is in accordance with some blooms of *Ostreopsis* spp. in the Mediterranean Sea when the salinity was around 37-38% [22,42]. However 33% was described as the optimal salinity [45]. In any case, the same species or strains can grow in different ecological conditions.

Furthermore, the lethal dose of the cultures was checked by bioassay, to know the toxicity of PLTX-like compounds produced by *Ostreopsis* spp. cultures. The European Food Safety Authority (EFSA) establishes the LD50 after i.p. injections of PLTX around 150-720 ng/Kg of mice body weight [46]. Our positive PLTX standard control was lethal to the mice at the concentration of 0.750 µg/kg. *Ostreopsis* spp. mouse bioassay data show that the cultures can be between 40 and 200 times less toxic than the PLTX standard indicating that other toxin with low toxicity and probably lower Na,K-ATPase affinity than PLTX is present in the cultures. From our results, *O. ovata* A strain is more toxic than *O. ovata* B and *O. siamensis*, since around 4 times more extract is necessary to induce the mice death.

In addition, different toxicity as well as different results after FP detection was shown by both *O. ovata* strains. Therefore, depending on the strain and the culture conditions, a larger amount or more toxic compounds can be produced. Due to the lack of standards, it is very difficult to establish the toxin profiles of the samples. By using different cultures and according to the toxin profile identified in each, the range



of toxin potency proposed in the bibliography was PLTX>ostreocin-d>mascarenotoxin-a, and ovatoxin-a was not classified [13,47]. Taking into account our results and based on this classification, if ostreocin-d is present in *O. siamensis*, the compound produced by *O. ovata* A, probably ovatoxin-a, is more toxic and shows higher affinity by the Na,K-ATPase as FP results shows. However several analogs could be present in each strain and probably depending on the conditions maybe one can be prevalent. Therefore the identification of compounds as well as the equivalence of bioassay and Na,K-ATPase affinity should be a further approach when standards become available in order to monitor and predict the presence of toxic strains in temperate regions.

In summary, *O. ovata* produces more PLTX-like compounds than *O. siamensis*. A salinity of 37‰, 24°C, and 16:8 h light-dark photoperiod are the optimal conditions to grow *Ostreopsis* spp. cultures and produce PLTX-like compounds. In addition, another relevant conclusion of this work is that sampling of the algae for a monitoring program can not be done directly on the water given their growth habitat.

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## References

- Moore RE, Scheuer PJ (1971) Palytoxin: a new marine toxin from a coelenterate. *Science* 172: 495-498.
- Taniyama S, Arakawa O, Terada M, Nishio S, Takatani T, et al. (2003) *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. *Toxicon* 42: 29-33.
- Prandi S, Sala GL, Bellocci M, Alessandrini A, Facci P, et al. (2011) Palytoxin induces cell lysis by priming a two-step process in mcf-7 cells. *Chem Res Toxicol* 24: 1283-1296.
- Riobó P, Franco JM (2011) Palytoxins: biological and chemical determination. *Toxicon* 57: 368-375.
- Rossi R, Castellano V, Scalco E, Serpe L, Zingone A, et al. (2010) New palytoxin-like molecules in Mediterranean *Ostreopsis* cf. *ovata* (dinoflagellates) and in *Palythoa tuberculosa* detected by liquid chromatography-electrospray ionization time-of-flight mass spectrometry. *Toxicon* 56: 1381-1387.
- Lenoir S, Ten-Hage L, Tuquet J, Quod JP, Bernard C, et al. (2004) First evidence of palytoxin analogies from an *Ostreopsis mascarensis* (Dinophyceae) benthic bloom in southwestern Indian Ocean. *J Phycol* 40: 1042-1051.
- Ciminiello P, Dell AC, Dello IE, Fattorusso E, Forino M, et al. (2009) Stereostructure and biological activity of 42-hydroxy-palytoxin: a new palytoxin analogue from Hawaiian *Palythoa* subspecies. *Chem Res Toxicol* 22: 1851-1859.
- Ciminiello P, Dell AC, Dello IE, Fattorusso E, Forino M, et al. (2011) LC-MS of palytoxin and its analogues: State of the art and future perspectives. *Toxicon* 57: 376-389.
- Ciminiello P, Dell AC, Dello IE, Fattorusso E, Forino M, et al. (2012) Stereochemical studies on ovatoxin-a. *Chemistry* 18: 16836-16843.
- Ciminiello P, Dell AC, Iacovo ED, Fattorusso E, Forino M, et al. (2012) Unique toxin profile of a Mediterranean *Ostreopsis* cf. *ovata* strain: HR LC-MS(n) characterization of ovatoxin-f, a new palytoxin congener. *Chem Res Toxicol* 25: 1243-1252.
- Ciminiello P, Dell AC, Dello IE, Fattorusso E, Forino M, et al. (2012) Isolation and structure elucidation of ovatoxin-a, the major toxin produced by *Ostreopsis ovata*. *J Am Chem Soc* 134: 1869-1875.
- Katikou P (2008) Palytoxin and analogues: ecobiology and origins, chemistry, metabolism, and chemical analysis. *Seafood and freshwater toxins*. CRC Press, Taylor and Francis Group.
- Ramos V, Vasconcelos V (2010) Palytoxin and analogs: biological and ecological effects. *Mar Drugs* 8: 2021-2037.
- Tosteson MT, Thomas J, Amadottir J, Tosteson DC (2003) Effects of palytoxin on cation occlusion and phosphorylation of the (Na<sup>+</sup>,K<sup>+</sup>)-ATPase. *J Membr Biol* 192: 181-189.
- Tubaro A, Del Favero G, Beltramo D, Ardizzone M, Forino M, et al. (2011) Acute oral toxicity in mice of a new palytoxin analog: 42-hydroxy-palytoxin. *Toxicon* 57: 755-763.
- Alcala AC, Alcala LC, Garth JS, Yasumura D, Yasumoto T (1988) Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. *Toxicon* 26: 105-107.
- Yasumoto T (1998) Fish poisoning due to toxins of microalgal origins in the Pacific. *Toxicon* 36: 1515-1518.
- Amzil Z, Sibat M, Chomerat N, Grosseil H, Marco-Miralles F, et al. (2012) Ovatoxin-a and palytoxin accumulation in seafood in relation to *Ostreopsis* cf. *ovata* blooms on the French Mediterranean coast. *Mar Drugs* 10: 477-496.
- Accoroni S, Romagnoli T, Colombo F, Pennesi C, Di Camillo CG, et al. (2011) *Ostreopsis* cf. *ovata* bloom in the northern Adriatic Sea during summer 2009: ecology, molecular characterization and toxin profile. *Mar Pollut Bull* 62: 2512-2519.
- Tognetto LB, Moro I, Andreoli C (1995) Occurrence of *Ostreopsis ovata* (Dinophyceae) in the Tyrrhenian Sea during summer 1994. *Botanica Marina* 38: 291-295.
- Vila M, Camp J, Garcés E, Masós M, Delgado M (2001) High Resolution Spatio-temporal Detection of Potentially Harmful Dinoflagellates in Confined Waters of the NW Mediterranean. *J plankton res* 23: 497-514.
- Vila M, Garcés E, Masó M (2001) Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. *Aquat Microb Ecol* 26: 51-60.
- Aligizaki K, Nikolaidis G (2006) The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. *Harmful Algae* 5: 717-730.
- Silva A, Brotas V, Orive E, Neto A (2010) First records of *Ostreopsis heptagona*, *O. cf. siamensis* and *O. cf. ovata* - in the Azores archipelago, Portugal. *Harmful Algae News* 42: 1-3.
- Guerrini F, Pezzolesi L, Feller A, Riccardi M, Ciminiello P, et al. (2010) Comparative growth and toxin profile of cultured *Ostreopsis ovata* from the Tyrrhenian and Adriatic Seas. *Toxicon* 55: 211-220.
- Pistocchi R, Pezzolesi L, Guerrini F, Vanucci S, Dell AC, et al. (2011) A review on the effects of environmental conditions on growth and toxin production of *Ostreopsis ovata*. *Toxicon* 57: 421-428.
- Simonini R (2011) Is the toxic dinoflagellate *Ostreopsis* cf. *ovata* harmful to Mediterranean benthic invertebrates? Evidences from ecotoxicological tests with the polychaete *Dinophilus gyrociliatus*. *Mar Environ Res* 1-4.
- Alfonso A, Fernández AA, Alfonso C, Caramés B, Tobio A, et al. (2012) Palytoxin detection and quantification using the fluorescence polarization technique. *Anal Biochem* 424: 64-70.
- Alfonso C, Alfonso A, Vieytes MR, Yasumoto T, Botana LM (2005) Quantification of yessotoxin using the fluorescence polarization technique and study of the

- adequate extraction procedure. *Anal Biochem* 344: 266-274.
30. Paz B, Vázquez JA, Riobó P, Franco JM (2006) Study of the effect of temperature, irradiance and salinity on growth and yessotoxin production by the dinoflagellate *Protoceratium reticulatum* in culture by using a kinetic and factorial approach. *Mar Environ Res* 62: 286-300.
  31. Pezzolesi L, Guerrini F, Ciminiello P, Dell AC, Dello IE, et al. (2012) Influence of temperature and salinity on *Ostreopsis cf. ovata* growth and evaluation of toxin content through HR LC-MS and biological assays. *Water Res* 46: 82-92.
  32. Vilarinho N, Fonfría ES, Molgó J, Araújo R, Botana LM (2009) Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. *Anal Chem* 81: 2708-2714.
  33. Fonfría ES, Vilarinho N, Espiña B, Louzao MC, Alvarez M, et al. (2010) Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization using a receptor-based assay in shellfish matrixes. *Anal Chim Acta* 657: 75-82.
  34. Ciminiello P, Dell AC, Iacovo ED, Fattorusso E, Forino M, et al. (2013) Investigation of toxin profile of Mediterranean and Atlantic strains of *Ostreopsis cf. siamensis* (Dinophyceae) by liquid chromatography-high resolution mass spectrometry. *Harmful Algae* 23: 19-27.
  35. Penna A, Vila M, Fraga S, Giacobbe MG, Andreoni F, et al. (2005) Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. *J Phycol* 41: 212-225.
  36. Cagide E, Louzao MC, Espiña B, Vieytes MR, Jaen D, et al. (2009) Production of functionally active palytoxin-like compounds by Mediterranean *Ostreopsis cf. siamensis*. *Cell Physiol Biochem* 23: 431-440.
  37. Onuma Y, Satake M, Ukena T, Roux J, Chanteau S, et al. (1999) Identification of putative palytoxin as the cause of clupectoxism. *Toxicon* 37: 55-65.
  38. Honsell G, Bonifacio A, De Bortoli M, Penna A, Battocchi C, et al. (2013) New insights on cytological and metabolic features of *Ostreopsis cf. ovata* Fukuyo (Dinophyceae): a multidisciplinary approach. *PLoS One* 8: e57291.
  39. Ciminiello P, Dell AC, Fattorusso E, Forino M, Tartaglione L, et al. (2008) Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian coasts during the 2006 toxic outbreak. *J Am Soc Mass Spectrom* 19: 111-120.
  40. Rhodes L (2011) World-wide occurrence of the toxic dinoflagellate genus *Ostreopsis* Schmidt. *Toxicon* 57: 400-407.
  41. Granéli E, Vidyarthna NK, Funari E, Cumararatunga RT, Scenati R (2011) Can increases in temperature stimulate blooms of the toxic benthic dinoflagellate *Ostreopsis ovata*? *Harmful Algae* 10: 165-172.
  42. Mangialajo L, Bertolotto R, Cattaneo VR, Chiantore M, Grillo C, et al. (2008) The toxic benthic dinoflagellate *Ostreopsis ovata*: quantification of proliferation along the coastline of Genoa, Italy. *Mar Pollut Bull* 56: 1209-1214.
  43. Tanimoto Y, Yamaguchi H, Yoshimatsu T, Sato S, Adachi M (2013) Effects of temperature, salinity and their interaction on growth of toxic *Ostreopsis* sp. 1 and *Ostreopsis* sp. 6 (Dinophyceae) isolated from Japanese coastal waters. *Fisheries Sci* 79: 285-291.
  44. Ashton M, Tosteson T, Tosteson C (2003) The effect of elevated temperature on the toxicity of the laboratory cultured dinoflagellate *Ostreopsis lenticularis* (Dinophyceae). *Rev Biol Trop* 51 Suppl 4: 1-6.
  45. Morton SL, Norris DR, Bomber JW (1992) Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. *J Exp Mar Biol Ecol* 157: 79-90.
  46. (2009) Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish - Palytoxin group. *The EFSA Journal* 7: 1-38.
  47. Ito E, Yasumoto T (2009) Toxicological studies on palytoxin and ostreocin-D administered to mice by three different routes. *Toxicon* 54: 244-251.