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Toxicity assessment of *Amphidinium carterae*, *Coolia* cfr. *monotis* and *Ostreopsis* cfr. *ovata* (Dinophyta) isolated from the northern Ionian Sea (Mediterranean Sea)

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A B S T R A C T

In many coastal areas the abundant proliferation of microalgae producing biotoxins determines the occurrence of Harmful Algal Blooms (HABs). Their presence in temperate waters is well documented and often associated with marine toxin-derived disease. The occurrence and toxicity of three harmful microalgae (*Amphidinium carterae*, *Coolia* cfr. *monotis* and *Ostreopsis* cfr. *ovata*) from the northern Ionian Sea (Mediterranean Sea) is hereby reported. The three dinoflagellates were sampled both on macroalgae and water and their morphology and occurrence were compared to those of other Mediterranean sites. The toxicity of the three cultured strains was tested by *Artemia salina* and hemolysis tests and their effects on the first stages of the sea urchin development was also evaluated. The contemporary presence of the three species inhibited the *in vitro* sea urchin embryonic development. But this action could be ascribed to the sole *Ostreopsis* as the addition of the single species to the sea urchins embryos evidenced no effects in presence of *Amphidinium* or *Coolia* cells, and an irregular segmentation in presence of *Ostreopsis*. In particular, this latter species exerted a cytotoxic effect in a dose-dependent manner, with a production of deformed embryos even at very low cell concentration (42 cells mL^{-1}). Nevertheless, when algal cell lysate was added, some effects on the sea urchin development was detected for each dinoflagellate, and also in this case *Ostreopsis* has proved to be the most toxic species. However, the lysate of *Amphidinium* and *Ostreopsis* strongly affects the *A. salina* nauplii vitality, while the hemolytic activity was very low for *Amphidinium* and *Coolia* lysate and very strong for *Ostreopsis*.

Our results highlight the importance to monitoring the presence of these dinoflagellates whose effects may also be reflected on the early life stages of marine organisms, especially those species that are important from both an ecological and economic point of view, as the sea urchins are.

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1. Introduction

Microalgae, existing either in unicellular forms or in colonies, are invisible organisms playing a key role in the aquatic ecosystems: as producers of organic material, they are the first ring of the aquatic food chain. In many coastal areas the abundant proliferation of microalgae producing biotoxins determines the occurrence of Harmful Algal Blooms (HABs) with important consequences on the trophic cascade. An important influence on fishery industry with consequent economical losses has been evidenced (Lewis, 2001). The phenomenon, is more frequent than in

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the past and it is due to some dinoflagellates, which have exceeded their own biogeographical boundaries (tropical and sub-tropical areas). Their presence in temperate waters is well documented (e.g. Fraga et al., 2008; Munday, 2011; Rhodes, 2011). Some dinoflagellates are associated with ciguatera fish poisoning, which is the most frequently reported marine toxin-derived disease worldwide and resulting in illness for more than 50,000 people each year (Friedman et al., 2008). Ciguatera syndrome is caused by different toxins (ciguatoxin, maitotoxin, palytoxin, gambiertoxin, etc.) produced by different dinoflagellates and having different effects on humans (Botana, 2008). The dinoflagellates, responsible for this syndrome, form epiphytic communities on hard substrates or are associated with macroalgae. These assemblages may vary in species composition and cell concentration among sites (Tindall and Morton, 1998). The mixed association of toxic dinoflagellates may contribute to the polymorphism of the clinical features of ciguatera syndrome (Vila et al., 2001; Yasumoto et al., 1987). Among these harmful microalgae the most common species in Mediterranean waters are *Ostreopsis ovata*, *Coolia monotis*, *Amphidinium carterae*, and *Prorocentrum* spp. (Zingone, 2010) whereas *Garbierdiscus toxicus*, the primary causative agent of ciguatera (Yasumoto et al., 1977), has been actually detected only in Greece coastal waters (Aligizaki et al., 2010).

Recently in the northern Ionian Sea (eastern Mediterranean Sea) benthic assemblages of toxic dinoflagellates have been detected. The main components of these assemblages were *A. carterae*, *Coolia* cfr. *monotis*, and *Ostreopsis* cfr. *ovata* (Caroppo and Bisci, 2010).

A. carterae Hulburt 1957 (Gymnodiniaceae), besides being involved in ciguatera poisoning (Hallegraeff, 2003), is known to cause red tides and has been implicated in fish mass mortality (Yasumoto, 1990). This effect seems to be related to the hemolysins, low molecular weight compounds, produced by this dinoflagellate (Tindall and Morton, 1998; Yasumoto, 1990). Toxicity tests carried out on Arabian strains of *A. carterae* evidenced its potential to produce toxins, being toxic towards albino mice but not towards *Artemia salina* larvae (Baig et al., 2006).

C. monotis Meunier 1919 (Ostreopsidaceae) is a producer of a yessotoxins analogue (cooliatoxin, Holmes et al., 1995), polyethers of very high molecular weight like palytoxins, but without any known toxicological relation with them (Riobò et al., 2004). Mouse bioassay revealed the existence of toxic and non toxic strains of *C. monotis* (Rhodes et al., 2000) of which probably different species exist, all referred as *C. monotis* (Penna et al., 2005).

O. ovata Fukuyo, 1981 (Ostreopsidaceae) produces a palytoxin analogue, the ovatoxin-a (Ciminiello et al., 2008) and others new compounds (Ciminiello et al., 2010; Rossi et al., 2010) that can cause, as in Italy, severe health problems also by inhalation. Respiratory illness (rhinorrhea, cough, fever and broncho-constriction) in beachgoers who often required hospital treatment (Durando et al., 2007) and irritations due to contact (skin irritations) have been also recorded (Tichadou et al., 2010).

O. ovata is responsible of blooms which are becoming more common in the Mediterranean Sea (Munday, 2011). Besides the toxic effect on humans, the *Ostreopsis* blooms induce relevant alterations in water quality, causing the death of benthic invertebrates (Mangialajo et al., 2011). Massive mortalities of marine invertebrates and macroalgae (Vale and Ares, 2007), and visible impacts on sessile (cirripeds, bivalves, gastropods) and mobile (echinoderms, cephalopods, little fishes) epibenthos were observed after summer blooms of *O. ovata* (Ciminiello et al., 2006; Totti et al., 2010). In particular, mortality of sea urchins during *Ostreopsis* blooms have been reported from some authors (Granéli et al., 2002; Sansoni et al., 2003; Shears and Ross, 2009). So, blooms of harmful algae are a threat to sea urchins that, as herbivores are a key species in the marine food chains. Indeed, the disturbance induced by poisoning effects on a single ecologically important species may have repercussions in the whole ecosystem. Furthermore, the preservation of the species depends also on the ability they have to overcome the critical stage of embryonic development. In this view, the effect of toxic species (i.e. harmful algae) could be more significant during the first stages of the life cycles. So far, the effects of ciguatera and other toxins in respect to developmental aspects have been poorly investigated.

The aims of this study were to evaluate: i) the presence of harmful dinoflagellates as *A. carterae*, *C.* cfr. *monotis* and *O.* cfr. *ovata* in the northern Ionian Sea; ii) the toxicity of the three isolated strains by *A. salina* and hemolysis tests and their effects on the first stages of the sea urchin development.

2. Materials and methods

2.1. Sampling and analyses of microalgae

During July and August 2009, with 15-days frequency, samplings were carried out in three sites, located in sheltered areas along the coastline of the northern Ionian Sea (Mediterranean Sea): Mar Piccolo (St 1), Mar Grande 5 (St 2) and Lido Bruno (Gulf of Taranto) (St 3) (Fig. 1). Seawater and macroalgae were collected. Surface water samples were immediately fixed with a Lugol's iodine solution and its temperature, salinity and pH were measured with an Idronaut Ocean Seven 501 multiprobe.

The sampled macroalgae included *Padina pavonica*, *Dictyota dichotoma* and species belonging to Gracilariales, Ulvales and Rhodymeniales. Macroalgae samples (50–150 g wet weight) were collected between 0.5 and 1.5 m depths by diving, carefully cut, placed in plastic bottles with seawater filtered through 0.22 μm pore-size Millipore filters (FSW) and kept in the dark (Totti et al., 2010). In laboratory, the macroalgae samples were vigorously shaken in sterile seawater to detach the cells that were filtered through 250 and 100 μm mesh sieves to remove large particles. The macroalgae were dried and then weighed. The abundance of the harmful species was expressed as cells g^{-1} dry weight of macroalgae. The wild filtrate containing the harmful algae was used for both the species' isolation and for the identification and quantitative analyses after fixation in Lugol's iodine solution.

All the samples were examined and cells were counted according to the Utermöhl method (1958) by using an
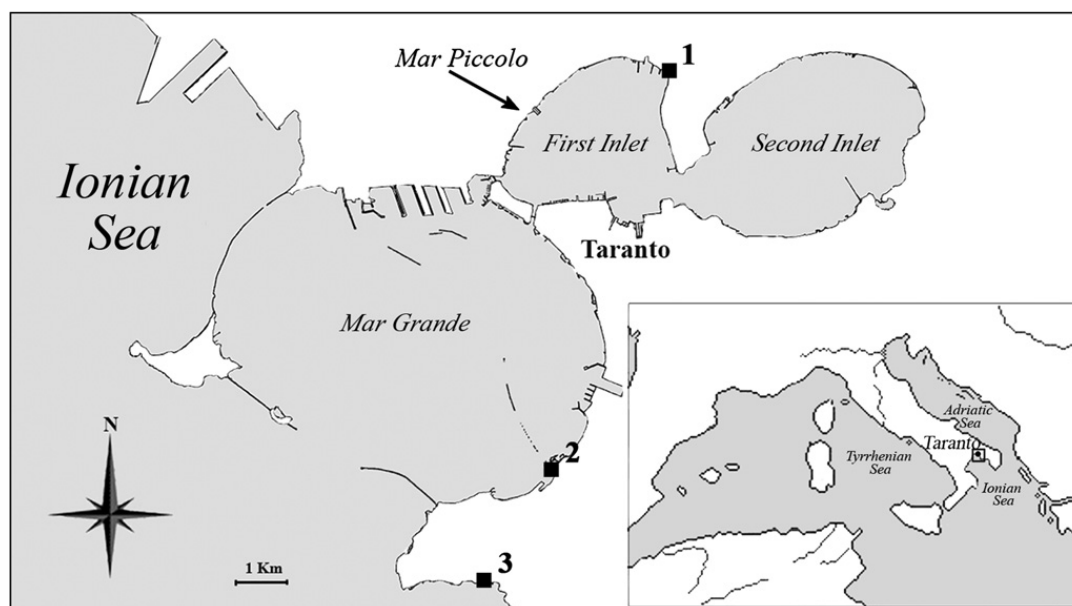
Fig. 1. Map of the sampling stations.

inverted microscope (Labovert FS Leitz equipped with phase contrast).

2.2. Isolation and cultivation of microalgae

Cultures were initially established by picking individual vegetative cells from both the rocks scraping and the seawater samples by using a micro-pipette and incubating them in f/2 medium (Guillard, 1983) at the salinity of 37‰. All cultures were uni-algal and clonal, but not axenic. Cultures were long term maintained in 250 mL Erlenmeyer flasks containing f/2 medium, under a 12:12 h light:dark cycle (100 µmol photons m^{-2} s^{-1}) at 24 ± 2 °C.

Before toxicological tests, algal cultures were counted, then diluted to obtain the required concentrations of the three strains.

2.3. Microscopy

Microalgae were examined under light microscopy. The thecal plate morphology of *C.* cfr. *monotis* and *O.* cfr. *ovata* was examined using phase contrast and UV epifluorescence microscopy after Calcofluor staining (Fritz and Triemer, 1985) under an Axioskop Zeiss microscope. Images of the thecated and the naked (*A. carterae*) species were acquired with a Canon Power Shot G5 digital camera.

2.4. Cell lysate

Amphidinium, Coolia and *Ostreopsis* cells from exponential growth phase culture were harvested by centrifugation (30 min at 3500 × g) freeze-dried and stored at −20 °C. Freeze-dried algal material was lyophilized, then suspended in distilled water (15 mg dry weight mL^{-1}) and maintained 1 h in the dark at room temperature. Fifteen milligrams of *Amphidinium, Coolia* and *Ostreopsis* dry

weight corresponded to solutions containing 9.03×10^6, 3.50×10^6, 0.31×10^6 cells mL^{-1}, respectively. Suspensions were then sonicated with an ultra-sound probe (Sonifer sonicator Model 250/240, Brain Ultrasonic Corporation) 5 times for 30 s on ice. Sonicated samples were checked under the microscope to ensure cell breakage. Samples were left on ice for 1 h in the dark before centrifugation at 20,000 × g for 30 min. The supernatants were recovered and stored at −20 °C until their use.

2.5. Paracentrotus lividus *embryo toxicity assay*

The procedure followed was adapted from the method already described (Pagliara and Caroppo, 2011). Briefly, a diluted sperm solution was added to an eggs suspension and *in vitro* fertilization was assured by the observation of the vitellin membrane under light microscope.

Fertilized eggs (zygotes) were immediately exposed to: 1) a culture containing the three harmful species at the concentration of 12×10^3 cells mL^{-1} (*Amphidinium*), 9×10^3 cells mL^{-1} (*Coolia*) and 0.67×10^3 cells mL^{-1} (*Ostreopsis*), respectively; 2) a culture containing a single algal species at a time with a two-fold serial dilutions over 6 wells; the starting concentrations were the same as previously given; 3) a two-fold serial dilutions over 10 wells of the single algae cell lysate at a time; the starting concentration of cell lysate was 15 mg mL^{-1} (dry weight mL^{-1}). A negative control was also performed by using a non-toxic alga (i.e. *Tetraselmis suecica*).

At appropriate intervals (1, 2, 3 h after fertilization) aliquots of 200 µL were fixed with the same volume of 10% formaldehyde. One hundred embryos from each well were counted to obtain the percentage of normal embryos. Their morphology was also observed under light microscope (Nikon Eclipse 600). Pictures were taken using a Nikon camera connected directly to the microscope. The *P. lividus* embryo bioassay was conducted in 24-well microplates.

Three replicates were performed for each treatment and control.

2.6. HOECHST stain

To reveal the nuclei, sea urchin embryos were washed in saline PBS, then incubated with Hoechst dye (10 mg mL^{-1}) for 15 min in the dark at room temperature. Unbound Hoechst dye was removed by washing three times in saline PBS and samples were examined under an epifluorescence microscope (Nikon Eclipse 600).

2.7. A. salina lethality assay

Dried cysts of *A. salina* (JBL Novotemia, Germany) were hatched in filtered seawater (1 g of cysts in 1 L of seawater) at 25 °C under continuous illumination and aeration. After 24 h of incubation, the *A. salina* nauplii were collected. Three replicates of the bioassay were conducted in 96-well microplates and a two-fold serial dilution of cell lysate over 7 wells were performed for each treatment. Ten individuals were transferred to each microplate well containing 100 μL of total volume. Cell lysate was prepared as described in paragraph 2.4 and the starting concentration was of 15 mg dry weight mL^{-1}. For a negative control the cell lysate of *T. suecica* (Prasinophyceae) was used.

After 24 h of exposure at 25 °C in darkness, the number of dead larvae in each well was counted.

2.8. Hemolysis assay

The hemolytic activity of the three algal lysate was evaluated by using human erythrocytes (RBCs), obtained from the peripheral blood (O positive) of a healthy volunteer. The blood was prepared as already described by Malagoli (2007).

For hemolysis assay, 100 μL of each cell lysate (prepared as described in paragraph 2.4) was mixed with 100 μL of 5% (v/v) erythrocytes suspension, and the mixture incubated for 60 min at 37 °C. The remaining unlyzed cells were centrifuged at 1500 g for 5 min. Hemoglobin content of supernatants was then evaluated spectrophotometrically at 540 nm. Negative and positive controls were performed using D-PBS and 1% Triton 100, respectively. The hemolytic activity of the sample was expressed as relative percentage against the positive control. Activity of at least 10% hemolysis has been considered and the average value from triplicate assays was calculated.

To evaluate the involvement of palytoxin-like molecules, the test was also performed by using a 5% (v/v) erythrocytes suspension pre-treated with 100 μM ouabain for 30 min at 37 °C.

2.9. Statistical analysis

Results are presented as mean (±SD) from at least three independent experiments.

Data were analyzed by a two-tailed paired Student's *t*-test and significant differences were considered at $p < 0.01$.

For *A. salina* test, results are presented as percentage of mortality ± SD and LC$_{50}$ values was estimated using the Probits statistical method (Finney, 1971).

3. Results

3.1. Occurrence and morphology of A. carterae, C. cfr. monotis and O. cfr. ovata

During the study period in the sampling sites water temperature ranged from 22.7 to 28.9 °C, salinity from 38.4 to 38.8 psu and pH from 8.05 to 8.10.

The three species were found at all the three considered stations of the northern Ionian Sea (Fig. 1, Table 1), throughout the sampling period. They were present in all the examined samples, both benthic (macroalgae) and planktonic (water) and were associated with the diatoms *Coscinodiscus* spp. and *Nitzschia* spp., the dinoflagellates *Prorocentrum* spp., and some filamentous cyanobacteria.

The highest *Coolia* and *Ostreopsis* abundances were observed at the St 3, whereas *A. carterae* was most abundant at the Mar Grande station.

3.1.1. A. carterae

A. carterae is athecate, oval in shape and flattened dorsoventrally, as light microscopy revealed (Fig. 2a). Its length vary between 12 and 20 μm and its width between 8 and 10 μm. The epicone was tongue shaped in ventral view and much smaller than the hypocone from which it was separated by a girdle encircling the epicone forming a V-shaped ending on the ventral side, below which lied the sulcus. A single large nucleus was located in the posterior part of the hypocone. Only one peripheral chloroplast, branched with a single prominent centrally placed pyrenoid, was present.

3.1.2. C. cfr. monotis

C. cfr. *monotis* cells were observed under the light microscope where cells appeared with an oblique axis when viewed from the side, and with a compressed spherical shape when viewed ventrally; a strongly lipped and defined cingulum and sulcus were present (Fig. 2b). Their epitheca was slightly smaller than the hypotheca. Cells size ranged from 28 to 40 μm in dorsoventral diameter (DV) and 22–35 μm in width. The thecal surface was smooth and covered with sparsely scattered pores.

Epifluorescence microscopy revealed the general thecal architecture of Po, 3′ (apical), 7″ (precingular), 5‴

Table 1

Abundance of the three harmful dinoflagellates. Values are reported as mean ± SD and expressed as 10^3 cells g^{-1} dw (dry weight) and as 10^3 cells L^{-1} ($n = 3$) if detected on macroalgae and water samples, respectively.

		Mar Piccolo	Mar Grande	Lido Bruno
Amphidinium carterae	Macroalgae	26.5 ± 12.4	43.6 ± 21.3	38.6 ± 9.8
	Water	1.7 ± 0.9	2.2 ± 1.4	2.6 ± 0.9
Coolia cfr. *monotis*	Macroalgae	12.8 ± 8.6	14.6 ± 7.5	27.5 ± 13.4
	Water	0.2 ± 0.1	0.4 ± 0.2	3.7 ± 2.1
Ostreopsis cfr. *ovata*	Macroalgae	171.3 ± 33.8	204.4 ± 14.8	422.3 ± 120.0
	Water	389.5 ± 85.3	175.1 ± 49.3	757.8 ± 114.3
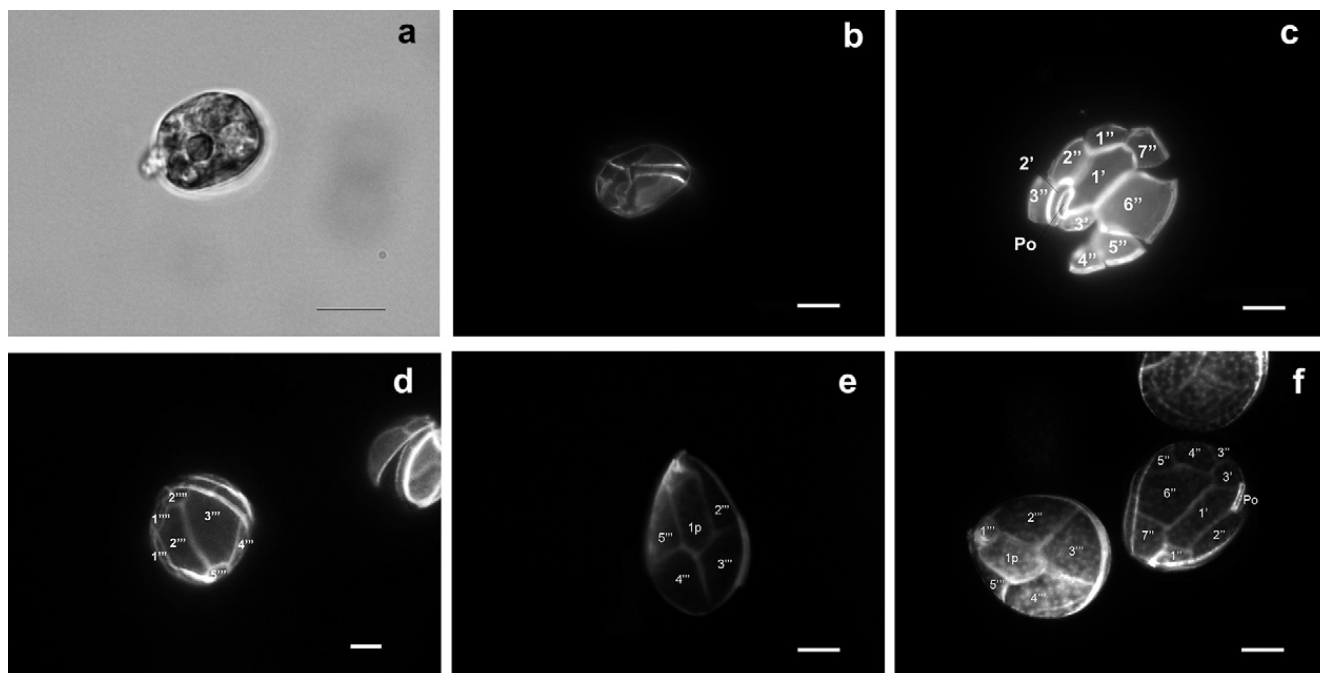
Fig. 2. a) *Amphidinium carterae* observed by light microscope; b-d) *Coolia* cfr. *monotis* observed by UV epifluorescence microscope: b) ventral view; c) epithecal view; d) hypothecal view; (e–f) *Ostreopsis* cfr. *ovata* observed by UV epifluorescence microscope: e) hypothecal view of a wild sample; f) hypothecal (left) and epithecal (right) views of a cultured sample. Scale bars, 10 μm. Po, pore plate.

(postcingular) and 2‴‴ (antapical) plates (Fig. 2c and d). The apical pore (9–10 μm long) was slit-like and surrounded by plates 1′, 2′ and 3′ (Fig. 2c). Postcingular plates were characterized by a fan-shaped arrangement (Fig. 2d). The existence of posterior intercalaries could not be confirmed. Six cingulum plates were recorded.

3.1.3. O. *cfr.* ovata

At light microscope, the cells appeared ovoid to oblong, with many golden chloroplasts. The dorsoventral diameter (DV) ranged from 34.3 to 56.7 μm, transdiameter (W) between 26.6 and 34.7 μm and anterioposterior (AP) diameter 16.8–32.2 μm.

Differences were found between cultured and wild cells especially in the W and in the AP diameter, being cells from culture less oblong and more rounded (DV/W = 1.35, $n = 50$; DV/AP = 1.86, $n = 30$) than field specimens (DV/W = 1.75, $n = 50$; DV/AP = 2.04, $n = 30$).

Furthermore in culture, large roundish forms, enclosed in a hyaline membrane and small cells were also found.

Epifluorescence microscopy showed a plate pattern Po, 3′, 7″, 6C, 6S?, 5‴, 1p, 2‴ (Fig. 2e and f) as identified for *Ostreopsis* species (Faust and Gulledge, 2002). In the epitheca, the plate 1′ was long and hexagonal, in contact with plates 2″, 3′, 1″, 2″, 6″ and 7″ (Fig. 2). In the hypotheca plate 1p was dorsoventrally elongated (Fig. 2f).

3.2. Effect of A. carterae, C. *cfr.* monotis and O. *cfr.* ovata on sea urchin embryonic development

The development of *P. lividus* embryos took place in glass tanks. In the control sample sea urchin embryos performed a normal cleavage reaching the two cells stage and the morula stage 1 h and 3 h after fertilization,

respectively. The gastrulation with blastula formation occurred within 4 h and the embryos became larvae with the typical shape of pluteus 24 h after fertilization.

The contemporary presence of *A. carterae*, *C.* cfr. *monotis* and *O.* cfr. *ovata* in the medium strongly affected the process. When the concentrations of the three dinoflagellates were similar to those of the aquatic environment, the sea urchin development was completely inhibited.

To understand the involvement of each microalga species in inhibiting this process, each single species was added to a sea urchin zygote suspension. No effect was observed when sea urchin embryos were reared in presence of *Amphidinium* or *Coolia*: segmentation and gastrulation took place regularly and development was observed for 24 h, until the pluteus stage was reached.

On the contrary the presence of *Ostreopsis* strongly affected the sea urchin embryonic development. One hour after fertilization control embryos were at the two cells stage (Fig. 3a) while all the embryos treated with the algal culture were still at the zygote stage, without sign of division (Fig. 3b). After 3 h, in the control, embryos developed reaching the morula stage (97.60 ± 0.87%) (Fig. 3c). The sea urchin embryos reared in presence of *Ostreopsis* remained at one cell stage or showed an anomalous cell division (Fig. 3d). The effect depended on the algal concentration being very toxic up to 338 cells mL^{-1} (Fig. 4). Indeed, when 338 cells mL^{-1} or more concentrated algal culture were added to sea urchin zygotes, these were unable to start segmentation. Fewer *Ostreopsis* cells did not prevent the segmentation, but this process was influenced, as the number of deformed embryos was consistent until the concentration of 42 cells mL^{-1}. At lower concentrations, the number of deformed embryos (3.90 ± 1.8) was not significantly ($p > 0.01$) different from the control (2.40 ± 0.87).
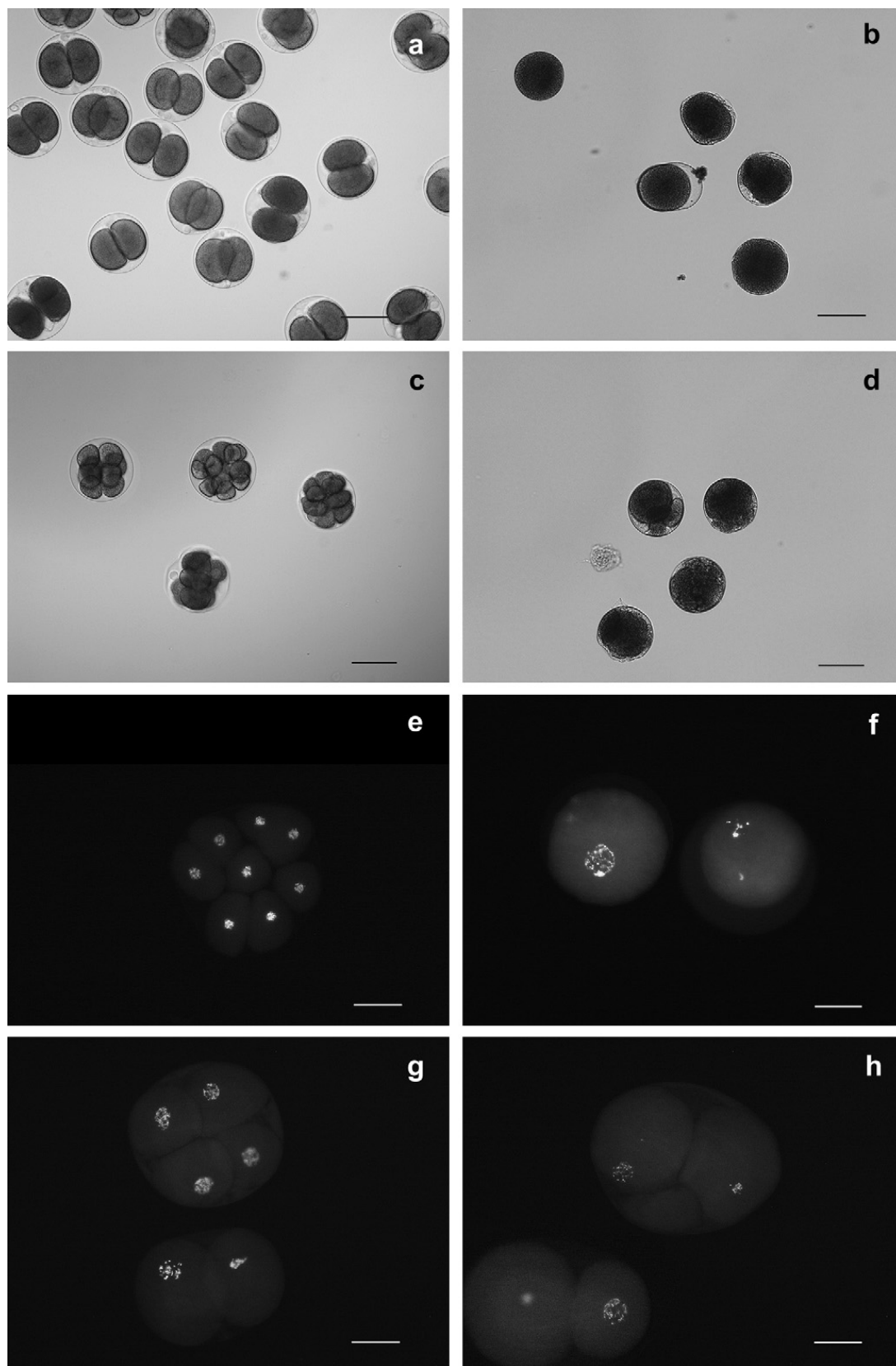
Fig. 3. Effect of *Ostreopsis* cfr. *ovata* on sea urchin embryos observed at optical (a–d) and UV epifluorescence (e–h) microscope; control (a) and treated (b) embryos 1 h after fertilization; control (c) and treated (d) embryos 3 h after fertilization. Bar = 100 μm. Control (e) and treated (f, g, h) sea urchin embryos stained by Hoechst 3 h after fertilization; f), g), h) are sea urchin embryos reared in presence of 338, 84 and 42 cell mL^{-1} of *Ostreopsis* respectively. Bar = 50 μm.
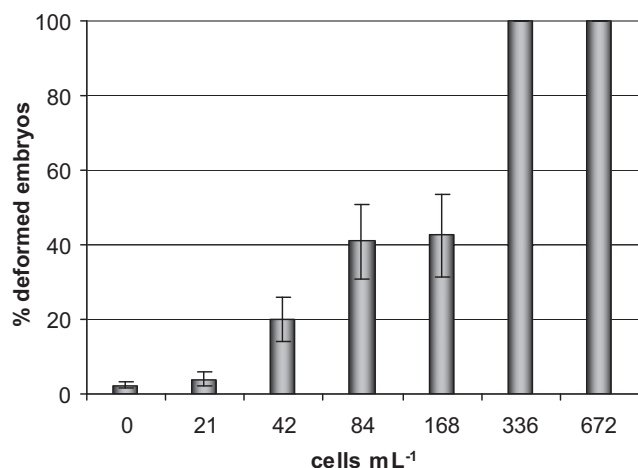
Fig. 4. Percentage of deformed sea urchin embryos reared in presence of different concentrations of *Ostreopsis* cfr. *ovata*. The results are reported as the mean of the percentage of deformed embryos ± SD.

Nuclear DNA specific staining, viewed with UV fluorescence, provided a more clear information about embryos damage evidencing a bright clumps and patchy distribution of chromatin. As shown in Fig. 3 the homogeneous distribution (Fig. 3e) of DNA into the nuclei of blastomere cells was not anymore detectable (Fig. 3f, g, h). In particular, when *Ostreopsis* concentration was high (338 or more cells mL^{-1}) the DNA remains localized at one pole of the cell, but it was strongly clumped and fragmented (Fig. 3f), while when the segmentation occurred, at the lower *Ostreopsis* concentration, the segmentation process was greatly delayed and an irregular mitosis was observed (Fig. 3g, h).

3.3. Effect of dinoflagellate cell lysate on sea urchin embryonic development

The embryos sensitivity to the three microalgae was different and related to cell lysate concentration. As shown in Fig. 5 the development of sea urchin embryos was completely inhibited in presence of 7.50 mg mL^{-1} of each algal cells lysate. At this concentration no zygotes was able to undergoing segmentation, while in presence of lower concentration of cell lysate (i.e. 3.75 mg mL^{-1}) a different
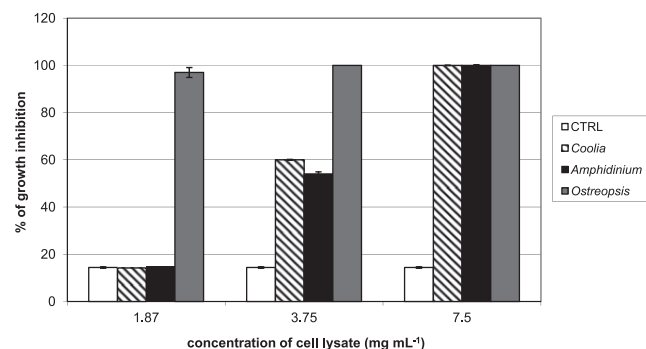
Fig. 5. Inhibitory effects of *Amphidinium carterae*, *Coolia* cfr. *monotis* and *Ostreopsis* cfr. *ovata* cell lysate on sea urchin embryos growth. Data are expressed as percentage of growth inhibition. Values are reported as the mean ± SD.

response pattern was observed if we referred to *Amphidinium*, *Coolia* or *Ostreopsis*. In fact, 58.00 ± 0.90% or 60.00 ± 0.20% of embryos growth inhibition was observed in presence of *Amphidinium* or *Coolia*, respectively. A further decrease in the percentage of development inhibition was observed at lower algal concentrations and in presence of 1.87 × 10^{-3} mg mL^{-1} of both the two algal species, the sea urchin embryos were very similar to the control.

A different response was observed when sea urchin embryos were reared in presence of *O.* cfr. *ovata* cell lysate that strongly inhibited the sea urchin development also at 3.75 mg mL^{-1} (Fig. 5). Three hours after fertilization, in the control sample, sea urchin embryos were at morula stage (Fig. 6a), while in sample treated with 1.87 × 10^{-2} mg mL^{-1} of *Ostreopsis* lysate, cleavage seemed to occur apparently without a segmentation inhibition (Fig. 6b). But a more careful observation of samples revealed that mitosis was not correctly performed. As better evidenced by nuclear staining, cell division deviated from the normal (Fig. 6c) and embryos presented the nuclear material not correctly distributed among blastomeres (Fig. 6d, e, f). A normal, but delayed segmentation was observed only from the concentration of 0.36 × 10^{-2} mg mL^{-1} cell lysate.

3.4. Effect of algal cell lysate on A. salina larvae

In the *A. salina* lethality bioassay, *A. carteri* cell lysate was lethal at the maximum concentration used (15 mg mL^{-1}). The LC$_{50}$ value obtained after 24 h of exposure was 3.67 mg mL^{-1} (95% Confidence Limits: 2.686–5.047 lower and upper respectively).

No effect was observed on *Artemia* vitality in presence of *C.* cfr. *monotis* cell lysate. On the contrary the lysate of *O.* cfr. *ovata* was very toxic. The LC$_{50}$ value obtained after 24 h of exposure was 0.03 mg mL^{-1} (95% Confidence Limits: 0.022–0.045 lower and upper respectively).

3.5. Hemolytic activity of algal cell lysate

The three algal lysates (at the concentration of 15 mg mL^{-1}) were able to induce hemolysis in human erythrocytes (Fig. 7), but the levels of the lysis were not comparable among them: low hemolytic activity was induced by *Amphidinium* (10.40 ± 0.03) and *Coolia* (12.30 ± 0.01) lysate, while those of *Ostreopsis* exhibited a very strong hemolytic activity (66.20 ± 0.08).

By using 100 μM ouabain a small difference of erythrolysis percentage has been observed ($p > 0.01$) in all the samples.

4. Discussion

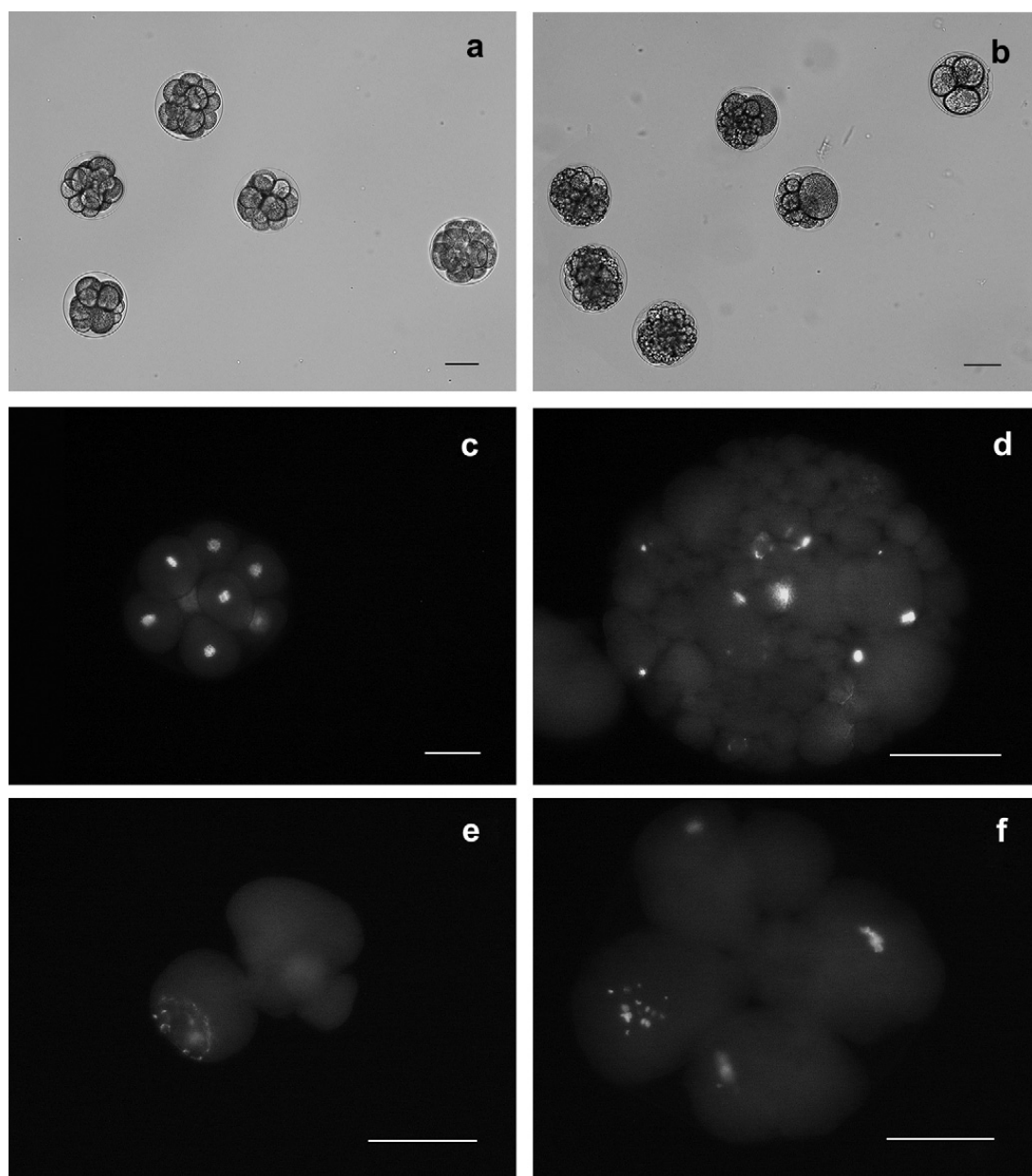### 4.1. Morphology and occurrence of A. carterae, C. cfr. monotis and O. cfr. ovata in the Ionian Sea

In this study for the first time we reported data on the morphology, abundances and toxicity of *A. carterae*, *C.* cfr. *monotis* and *O.* cfr. *ovata* in the Ionian Sea.

From a morphological point of view, the Ionian strain of *A. carterae* and *C.* cfr. *monotis* exibits features similar to the

Fig. 6. Sea urchin embryos observed at light (a, b) and fluorescence (c, d, e, f) microscope; a) control embryos 3 h after fertilization at morula stage; b) embryos 3 h after fertilization treated with algal cell lysate; c) control embryos 3 h after fertilization at morula stage stained by Hoechst; d), e) f) Hoechst stained embryos 3 h after fertilization treated with 1.47×10^{-2} mg mL^{-1} algal cell lysate. Bar a, b = 100 µm; c, d, e, f = 50 µm.

other Mediterranean strains (Penna et al., 2005; Tomas, 1997).

The identification of the genus *Ostreopsis* is actually unclear and needs to be reconstructed (Aligizaki et al., 2010; Penna et al., 2010). By considering the morphology of the species we evidenced that the Ionian strain was very similar to the other Mediterranean *O.* cfr. *ovata* strains. In fact, dimensions of the cells were similar to those observed by other authors, and the DV/AP ratio was lower than 2 (Aligizaki and Nikolaidis, 2006). This ratio has been indicated as the main distinctive tool between *O.* cfr. *ovata* and the other Mediterranean species *O.* cfr. *siamensis* (Penna et al., 2005).

Over the last decades, an increased incidence of the problems associated with the presence of harmful and toxic microalgae has been experienced all over the world.

While *A. carterae* and *C. monotis* are cosmopolitan dinoflagellates (Tomas, 1997), previously reported for Mediterranean (e.g. Barone, 2007; Dolapsakis et al., 2006; Riobò et al., 2004), *O. ovata* expanded its distribution. Particularly, in Italy the last five years have been marked by toxic outbreaks involving blooms of *Ostreopsis* species that had never been recorded in Italian coastal waters (Mangialajo et al., 2011). *Ostreopsis* blooms, in turn, have been originally recorded in the Adriatic coasts of Puglia and Tuscany (Aligizaki et al., 2011; Gallitelli et al., 2005; Sansoni et al., 2003; Simoni et al., 2003), but have moved northbound to the Adriatic Sea (Monti et al., 2007; Totti et al., 2010), and have spread in the Tyrrhenian Sea, from Sicily to Liguria (Barone, 2007; Ciminiello et al., 2006; Penna et al., 2005). The spreading reasons of this species are actually debated. It is unclear if its presence derives from
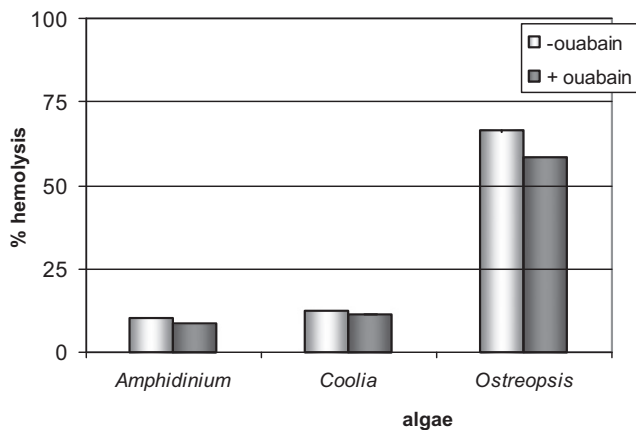
Fig. 7. Percentages of the hemolytic activity of *Amphidinium carterae*, *Coolia* cfr. *monotis* and *Ostreopsis* cfr. *ovata* cell lysates. Values are reported as the mean ± SD.

a new introduction supported by ballast waters or by the climate changes. It is also possible that *Ostreopsis* have already been present for a long time in temperate areas at low concentrations (Tognetto et al., 1995). Concerning the northern Ionian Sea, the presence of *Ostreopsis* could be linked to the increase in temperature observed during recent years (Giacomini and Alabiso, 2006). Also the possibility that *Ostreopsis* had been introduced by the ballast waters cannot be excluded, taking into account that the city of Taranto has an important commercial port and that exotic macroalgal species have been recently detected in this area (Cecere et al., 2004).

Our findings confirmed the association of the three dinoflagellates both in benthic and planktonic assemblages. As concerning their abundance, *Amphidinium* and *Coolia* did not reach high values during the sampling period both in water and in macroalgae samples. On the other hand, the *Ostreopsis* abundance, detected on macroalgae from the northern Ionian Sea, were lower than those reported for the north western Mediterranean Sea (Catalan Sea, Vila et al., 2001) and northern Adriatic Sea (Totti et al., 2010). As well, also in the water column we found abundances of *Ostreopsis* lower than those reported for the other Mediterranean sites (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008, 2011; Vila et al., 2001). However, our data suggest the necessity of further samplings not only to monitor the trend of these species but also to integrate their abundance with environmental variables which could affect their patterns.

4.2. Toxicity of A. carterae, C. cfr. monotis, and O. cfr. ovata

Dinoflagellates, as well as cyanobacteria, bacteria and diatoms, produce large amounts of toxins that affect the reproduction of aquatic organisms. These toxins may induce either a decrease in the amount or quality of gametes or an influence on embryonic development. Early stages are generally more sensitive than adults to toxins because they lack effective enzymatic systems to detoxify the toxins and are more exposed to the toxins due to a higher metabolic growth rate (Vasconcelos et al., 2010). In the present study we assessed the effects of *A. carterae*,

C. cfr. *monotis* and *O.* cfr. *ovata* on the *A. salina* vitality and on the sea urchin development, taking also into account the impact of marine toxins on invertebrate early life stages. The three considered dinoflagellates, isolated from the northern Ionian Sea (Mediterranean Sea), behaved differently showing various degree of toxicity to the sea urchin *P. lividus* embryos. Among the studied microalgae, *Ostreopsis* seem to be the most active on sea urchin embryos which development was inhibited by concentrations similar to those found in the Ionian Sea and lower than those detected in other Mediterranean sites or during their blooms. On the contrary, no effect was observed during sea urchin development in presence of *A. carterae* and *C.* cfr. *monotis*, also at concentrations higher than those we found in the marine environment. Sea urchin embryos exposed to *Ostreopsis* cells remained in the zygote stage and, as evidenced by UV-fluorescence microscopy, their nucleus showed a clumped chromatin that probably impedes the normal genome functioning. In a more general context chromatin organization is crucial for the correct occurrence of basic cellular processes such as DNA replication and gene expression (for a review see Cremer and Cremer, 2001). The marked toxicity of *Ostreopsis* produced deformed embryos also at the lower considered concentrations.

Sea urchins, as well as the most of aquatic invertebrates, exhibit an external fertilization, releasing a large number of sperms and eggs into the sea. In this environment, gametes and embryos are exposed to a number of substances and microorganisms which could affect their integrity and functionality. In particular, the presence of toxic species (i.e. *Ostreopsis*) can interfere with the gametes ability to give rise to a new organism and with their following development, affecting species survival and perturbing the equilibrium of the ecosystem where they live. Not much is known about the effects of the toxins produced by *Ostreopsis* (namely palytoxin and its analogues) on marine vertebrates and invertebrates (see a review of Munday, 2011), and particularly in respect to the early stages of the embryonic development (Ramos and Vasconcelos, 2010). The only documented effect of palytoxins on reproduction is the inhibition of sperm motility in sea urchins (Morton et al., 1982). Other information is available on the acute effects of palytoxins and its analogues on larval development of the brine shrimp *A. salina* and on bivalves (Ramos and Vasconcelos, 2010). In particular, *A. salina* resulted a very sensitive species, as demonstrated by Faimali et al. (2012), who compared the effects of different concentrations of *O. ovata* on crustaceans and fish larvae and juveniles. The toxicity of palytoxin has also been estimated by the FETAX assay, which results underlined that toxicity was concentration dependant (Franchini et al., 2008). Furthermore, a molecular biology approach demonstrated that the toxin acts by inducing modifications in the expression of genes involved in *Xenopus laevis* development (Franchini et al., 2009).

An important issue is related to the nature and availability of toxins, generally analyzed for their lipophilic nature and tested as isolated compounds. Here we present data on the toxic action evidenced, not only in presence of the whole alga, but also when the cells lysate of the three

microalgae was added to sea urchin embryos, to *A. salina* nauplii and to human erythrocytes.

In presence of *O. ovata* cell lysate an embryo fragmentation leading to degeneration occurred. Moreover, in these embryos nuclear material was not equally distributed into the cells, due to an uncorrected mitosis. So, we can hypothesize a direct action of toxic compounds on the DNA of embryo cells. No data are at now available on this aspect, while the specific action of some isolated toxins produced by *Ostreopsis* is well documented. As an example, palytoxins seem to have a specific action on Na/K pumps (Guennoun and Horisberger, 2000; Wang and Horisberger, 1997) and a possible close relation to H/K pump (Scheiner-Bobis et al., 2002). As a consequence an alteration on cellular pH derives. This parameter results a vital element for eggs development influencing those ionic signals that stimulate protein and DNA syntheses, the main events occurring during the first steps of embryonic development (Steinhardt and Epel, 1974; Whitaker and Steinhardt, 1981, 1982). But we may also hypothesize that this effect could be ascribed to one of the already identified toxins different from the palytoxins (Ciminiello et al., 2010) or to the synergic action of a number of bioactive molecules produced by *Ostreopsis*. In support to this hypothesis, we found that the Ionian strain of *Ostreopsis* cell lysate possess a strong hemolytic activity that was not inhibited by ouabain. All the available literature data report about the ouabain inhibition of hemolytic activity due palytoxin isolated from different strains of this dinoflagellate and from some invertebrates (Taniyama et al., 2001). At now no information are available on the bioactivity of other toxins produced by *Ostreopsis* which could interfere with ouabain inhibition.

Concerning *A. carterae*, here we present the first data on the toxicity of a Mediterranean strain. Its cell lysate, in fact, induced the *A. salina* death and interfere with the sea urchin embryo development. Some authors (Aligizaki et al., 2009; Mohammad-Noor et al., 2007) indicated *A. carterae* as a non toxic species, but some others (Echigoya et al., 2005; Tindall and Morton, 1998) recognized this dinoflagellate as a producer of powerful ichthyotoxins and hemolytic substances. Indeed, the genus *Amphidinium* is known to produce bioactive substances, such as cytotoxic macrolides, amphidinols and amphidinolides (Ishibashi and Kobayashi, 1997; Murata et al., 1999; Paul et al., 1995, 1996, 1997; Satake et al., 1991). The genotypic variability within the species, suggested by Murray et al. (2004), as well as an insufficient monitoring activity for potential harmful effects, suggests that there are both toxic and non toxic strains of this species.

Finally, *C.* cfr. *monotis* cell lysate, it did not exert any effect on *A. salina* vitality and a very low hemolytic activity towards human erythrocytes, but it was responsible for an evident delay in the sea urchin development. These results evidenced the capacity of this species to exert a toxic action when its cell content is released in the environmental medium, as during a stress condition. Previous findings assessed the non toxicity of Mediterranean strains of *C. monotis* (Penna et al., 2005) tested for hemolytic activity in methanolic extracts. Our investigation conducted with

microalgae cell lysate, proved the no toxicity by *A. salina* test and a low toxicity with hemolysis test, the latter being in contrast with previous findings reported by Penna et al. (2005) on another Mediterranean strain. From literature data, *C. monotis* appears to be toxic or non toxic, depending on its geographical origin (Penna et al., 2005; Rhodes et al., 2000; Riobò et al., 2004). Taking into account the results obtained in our experiments, we suppose that also the sensitivity of the used target (e.g. sea urchin embryos, brine shrimp and human erythrocytes) could be considered for evaluating the toxicity of the harmful species. Moreover, our toxicity tests suggest that the Ionian strain of *C.* cfr *monotis* should produce compounds whose nature need to be characterized.

It is important to emphasize that the toxic action of *Amphidinium* and *Coolia* was observed only with cell lysate. Cell lyses is an event that can occur in nature under certain environmental conditions. Temperature increase, changes in chemical–physical parameters and/or the end of a bloom represent stressors that could determine cells disruption. So, also the algal species which do not show toxicity during the vegetative stages of their life cycles, could become dangerous when their cell content with bioactive and/or toxic compounds are released into the marine environment.

5. Conclusions

The distribution and toxicity of the dinoflagellates *A. carterae, C.* cfr. *monotis* and *O.* cfr. *ovata* detected in the northern Ionian Sea is reported in this study. Among these species, *O.* cfr. *ovata* was able to exert a strong toxic effect on sea urchin embryos and on *A. salina*. Also *A. carterae* and *C.* cfr. *monotis*, non toxic when tested as cell cultures, resulted potentially dangerous when they loss their integrity. All the three species are able to produce water soluble compound(s) which nature and bioactivity need to be better characterized. Interesting, *O.* cfr. *ovata* seemed to affect processes such as DNA replication and gene expression.

Our data must be considered as preliminary, and more detailed studies will be carried out to enrich the data base which is necessary to forecast and manage the harmful algal blooms. We will take into consideration that: a) toxins produced in large amounts by dinoflagellates, cyanobacteria, bacteria and diatoms may accumulate in vectors that transfer the toxin along food chains and b) as also assessed by Vasconcelos et al. (2010), marine toxins have an impact on early life stages of invertebrate and vertebrate species, contributing to significant population changes. Finally, monitoring the presence and abundance of these microalgae is essential, considering both the influence that these dinoflagellates could have on the ecology of marine organisms and the economic negative effects on fisheries, aquaculture and touristic incomes.

Conflict of interest

The author declares that there are no conflicts of interest.

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