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The dual impact of *Ostreopsis* cf. *ovata* on *Mytilus galloprovincialis* and *Paracentrotus lividus*: Toxin accumulation and pathological aspects

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Abstract

Blooms of the toxic dinoflagellates *Ostreopsis* have become common along rocky shores of the Mediterranean Sea. In addition to health problems for beach-goers, *Ostreopsis* toxins may accumulate in benthic marine animals used for human consumption, which however at times have shown signals of stress and even mortality. In order to elucidate the actual relationships between *Ostreopsis* and benthic invertebrates, we exposed mussels *Mytilus galloprovincialis* and sea urchins *Paracentrotus lividus* from the Gulf of Naples to cultures and natural material of *O.* cf. *ovata* and assessed feeding and adverse effects on the animals, along with their acquired toxicity. Mussels exposed to *O.* cf. *ovata* for 24 hours filtered the microalgae at different rates, depending on both mussel size and microalgal density, and became weakly toxic in some cases. Under longer exposure most animals died and all survivors were toxic. Detoxification of a naturally toxic mussel populations from an area affected by *O*. cf. *ovata* blooms took more than two weeks. Sea urchins fed with the red alga *Asparagopsis taxiformis* epiphytised by *O.* cf. *ovata* did not show damages and became mildly toxic in some cases. However, the direct exposure of sea urchins to *O.* cf. *ovata* cultures caused the partial or total loss of the spines in a density-dependent way, with the death of the animals at the highest microalgal concentrations. Milder effects were registered with sonicated cultures or toxin extracts. Our results indicate that the balance between toxicity and animal health in these invertebrates depends on the mode and intensity of exposure to the toxic microalga, while the response varies between the two species but also within the same species. This scenario matches the variety of responses of benthic populations recorded in the natural environment in areas affected by *O.* cf. *ovata* blooms.

Keywords: *Ostreopsis*; *Mytilus galloprovincialis; Paracentrotus lividus*; mussels; sea urchins; toxicity.

Introduction

Ostreopsis spp. are benthic autotrophic dinoflagellates generally living as epiphytes on macroalgae or other substrates, such as submerged rocks, sediments and detritus aggregates (Totti *et al.,* 2010). These species were initially known to be restrained to tropical and subtropical seas, but since the late 1990s blooms were also observed along the coasts of many temperate areas of the west Pacific (Rhodes *et al.,* 2000; Pearce *et al.,* 2001; Shears & Ross, 2009; Sato *et al.,* 2011; Zhang *et al.,* 2018), west (Nascimento *et al.,* 2008; 2010; Tibiriçá *et al.,* 2019) and east Atlantic (David *et al.,* 2012; Santos *et al.,* 2019), as well as in the Mediterranean Sea (Tognetto *et al.,* 1995; Sansoni *et al.,* 2003; Turki *et al.,* 2006; Zingone *et al.,* 2006; Totti *et al.,* 2010; Illoul *et al.,* 2012; Abdennadher *et al.,* 2017; Gladan *et al.,* 2019; Zingone *et al.,* 2020). In the latter area, the most common and widespread *Ostreopsis* were initially classified as *O. ovata*, a species first described from Ryukyu Islands, French Polynesia and New Caledonia (Fukuyo, 1981). Presently, the taxon actually deserving the species name has not been defined, while the many taxa in this species-complex are referred to as *Ostreopsis* cf. *ovata*.

Among 12 *Ostreopsis* species described so far, seven are known to produce palytoxin analogues (Moestrup *et al.,* 2012); particularly, *O.* cf. *ovata* produces a variety of palytoxin-like molecules, the most abundant being ovatoxin-a (Ciminiello *et al.,* 2010; Rossi *et al.,* 2010; Scalco *et al.,* 2012; García-Altares *et al.,* 2015; Argyle *et al.,* 2016). *Ostreopsis* cf. *ovata* can cause damages to human health through exposure to marine aerosols or direct contact (Gallitelli *et al.,* 2005; Ciminiello *et al.,* 2006; Durando *et al*., 2007). In the Mediterranean Sea there are

no reports of intoxications by ingestion of contaminated seafood, which have been suspected, but not confirmed, in other areas where other toxic *Ostreopsis* species live (Tubaro *et al.,* 2011).

Regarding marine animals, clear impacts on the benthic fauna were first observed during *Ostreopsis* blooms in north Tyrrhenian waters in summers 1998, 2000 and 2001, when sea urchins and starfish showed loss of spines and anomalous position of arms, respectively (Sansoni *et al.,* 2003). Mass mortalities of sea urchins or other invertebrates were subsequently observed along Brazilian (Ferreira, 2006) and New Zealand coasts (Shears & Ross, 2009). In laboratory experiments, *O.* cf. *ovata* was shown to be detrimental to larval stages of invertebrates and fish (Simonini *et al.,* 2011; Faimali *et al.,* 2012), with sperm fertility reduction (Pagliara & Caroppo, 2012) and abnormal growth in embryos produced by fertilization of sea urchins collected during blooms (Migliaccio *et al.,* 2016; Neves *et al.,* 2018) and in jellyfish larvae (Giussani *et al*., 2016). At concentrations higher than 1.5 nM, palytoxin was highly cytotoxic to mussels (Louzao *et al*., 2010), causing a strong decrease of metabolic rates in mantle and hepatopancreas cells. In addition, palytoxin increased the percentage of mussel phagocytizing immunocytes (Malagoli *et al*., 2008). Mussel susceptibility to *Ostreopsis* was shown in the inhibition of the Na^+/K^+ ATPase and alteration of other enzymatic activities (Gorbi *et al.,* 2012), as well as in immunopathological signals (Gorbi *et al*., 2013). In fact, apparently healthy mussels exposed to these toxic microalgae activated an inflammatory response, mainly characterized by haemocyte aggregates and digestive tubule damages that could prevent further feeding and food assimilation (Carella *et al.,* 2015).

The observed impact of *Ostreopsis* spp. blooms on marine invertebrates should reduce the possibility that those animals accumulate toxins and transfer them to higher trophic levels and to humans. Nevertheless, palytoxin-like molecules have been detected in various marine organisms, like fish, crabs, mussels, sea anemones and sea urchins, all showing no apparent signs of damages (Briggs *et al.,* 1998; Gleibs & Mebs, 1999; Pearce *et al.,* 2001). In the Mediterranean Sea, palytoxin-like substances were detected in apparently healthy shellfish collected from the North Aegean Sea, French coasts and Gulf of Naples in concentrations up 97 μg kg^{-1} (Aligizaki *et al.,* 2008), 450 μg kg^{-1} (Amzil *et al.,* 2012) and 600 μg kg^{-1} (EF & PC, unpublished data), respectively. Relatively few laboratory studies have been performed to demonstrate how shellfish and other marine organisms accumulate palytoxin analogues. Previous experiments on three shellfish species demonstrated that those filter feeders were able to ingest *Ostreopsis siamensis* cells, but with no clear evidence of toxins in the animal tissues (Rhodes *et al.,* 2002).

In this study we aimed at shedding light on the feeding and toxicity response of the filter feeder mussel *Mytilus galloprovincialis* and the herbivore sea urchin *Paracentrotus lividus* exposed to cultures and natural samples of *Ostreopsis* cf. *ovata*. Both invertebrates are key species of the benthic community of the Mediterranean Sea and, due to their scarce motility, can be considered as sentinel species reflecting possible perturbations in the marine coastal environment. *Mytilus galloprovincialis* is also one of the most intensively cultivated marine species in the Mediterranean Sea and worldwide (Gazeau *et al.,* 2014). It is considered a target organism to detect bacterial and viral pollution (Popovic *et al.,* 2010; De Donno *et al.,* 2012; Lamon *et al.,* 2019), hydrocarbons (Balcioğlu *et al.,* 2017) and metals (Guendouzi *et al.,* 2020) in seawater. The sea urchin *P. lividus* is a basically herbivorous species greatly influencing macroalgal assemblages in benthic environments. It is also used for human consumption, being considered a delicacy in several regions. Its abundance is negatively affected by a plethora of anthropogenic impacts (Pagano *et al.,* 2017; Ruocco *et al.,* 2020), with consequent deleterious changes in biodiversity of seabeds. Our experiments were aimed at providing information about i) toxin accumulation in mussels and sea urchins fed with cultures and natural material of *O.* cf. *ovata*; ii) damages caused to mussels and sea urchins by the exposure to *O.* cf. *ovata*, and iii) time needed for detoxification of mussels contaminated by palytoxin-like molecules produced by *O.* cf. *ovata*.

Materials and Methods

Sampling and maintenance

Ostreopsis *cf.* ovata

Most experiments were performed with *Ostreopsis* cf. *ovata* strain D483, which was isolated from the red alga *Asparagopsis taxiformis* collected in September 2008 in the marine protected area (MPA) Gaiola (40.473323° N; 14.111879° W) on the Naples city coasts (Tyrrhenian Sea). The unialgal strain was obtained by single cell isolation with the capillary pipette method (Andersen & Kawaki, 2005) and cultivated in k/2 medium (Keller *et al.,* 1987) at 23.0±0.5 °C, under a 12:12 light: dark regime and at 100 μmol $m^{-2}s^{-1}$, refreshing the culture medium every 7–10 days.

For the experiments, cells were mass cultivated in 2-l glass bottles under the same conditions. Upon the death of strain D483, strain OOAPS0810-S1 was used for some of the experiments with sea urchins. The latter strain was isolated in August 2010 from the Passetto sampling site (Ancona, Adriatic Sea) and maintained under the same conditions as D483, except for slightly lower temperatures (21.5±0.5 °C).

For abundance estimation, 1-ml subsamples of formalin-fixed *Ostreopsis* cf. *ovata* cultures were counted in a Sedgewick-Rafter chamber, using an optical microscope (Zeiss Axiophot, Oberkochen, Germany) at 100x magnification. Cell numbers from 5 transects were averaged and concentrations were expressed as cells ml^{-1}. For epiphytic *O.* cf. *ovata*, macroalgal samples were placed in plastic bags and vigorously shaken in order to detach the microalgal cells. The macroalgae were removed, rinsed with tap water, gently dried with a tissue and weighted.

Total seawater sample volume was measured, and a subsample was collected in 250-ml plastic jars and fixed with neutral formalin at a final concentration of 5% of the standard solution. Cells were counted in the inverted microscope (Zeiss Axiophot, Oberkochen, Germany) following the Utermöhl method (Utermöhl, 1958). Results were expressed in cells g^{-1} of wet weight (WW) of macroalga.

Mussels

Wild mussels *Mytilus galloprovincialis* were collected between January and June 2009 from rocky shore beds of the Gulf of Naples and transported to the Stazione Zoologica Anton Dohrn of Naples (SZN). Mussel valve length increased from 4 cm in winter to 9 cm in late spring. Mussels were scrubbed free of all epiflora and -fauna, randomly distributed into three plastic open–meshed trays and suspended in a flow-through system with filtered (35-µm mesh size) seawater (25–27 °C, 36 salinity). The bottom of the tank was gently scraped every day to eliminate faecal pellets. Mussel subsamples were submitted to the mouse test (see below) although they were not expected to be toxic, as *Ostreopsis* is generally undetectable before mid-June in the area. For about two weeks mussels were fed with a mixture of *Isochrysis galbana* and *Tetraselmis suecica*, but one week before the experiment feeding was stopped to optimize *O.* cf. *ovata* ingestion.

For the detoxification experiment, wild mussels were collected from the rocks of the Rocce Verdi sampling site (Gulf of Naples, 40.478550° N; 14.120950° W) at 1 m depth (T~ 27 °C) on 20 July 2009, during a bloom of *O.* cf. *ovata* ($4.0 \cdot 10^5$ cells g^{-1} WW of *Asparagopsis taxiformis*, AZ, unpublished data).

Sea urchins

Sea urchins *Paracentrotus lividus* (5.0-6.0 cm test diameter, 45-60 g individual total weight) were collected from Gaiola and Rocce Verdi in June-August 2009, and maintained in 500-l tanks in suspended baskets for 10-30 days. A centralized Life Support System (cartridge filter 35µm, protein skimmer, ultraviolet sterilizer and refrigerator) maintained optimal sea water conditions (dissolved oxygen >90% saturation, pH 8.0±0.1, salinity 38.0±0.2 and temperature 22±1.0 °C). For each collection, a subsample of 15 animals was submitted to the mouse test. The other animals were nourished with *Ulva* sp. until one week before the experiments, during which they were starved in order to optimize *A. taxiformis* ingestion (see below). The tank bottom was siphoned daily to remove faeces and seaweed residuals.

Asparagopsis taxiformis

Macroalgae *A. taxiformis,* which host dense populations of *O.* cf. *ovata* as epiphytes in summer, were collected weekly at Gaiola and Rocce Verdi in July and August 2009 by scuba diving. The thalli were delicately enclosed in plastic bags and then cut at their base, closing the bag underwater. Samples were kept fresh until their arrival at the laboratory, where they were immediately used for the experiments and for the quantification of *O.* cf. *ovata* abundance.

Feeding experiments

Mussels

In each of the six experiments with *O.* cf. *ovata*, three replicates of ten individuals each of *M. galloprovincialis* of similar weight (88-215 g WW in the different experiments) were placed 24 h in 0.5-l borosilicate beakers containing cultures of *O.* cf. *ovata* (strain D483) at concentrations between 1.1 and $4.1 \cdot 10^3$ cells ml^{-1}. A gentle airstone aeration system was used to limit cell adhesion to the vessel walls and bottom. A mussel-free *O.* cf. *ovata* culture was used to evaluate feeding-independent changes in algal cell concentrations. The cell density of *O.* cf. *ovata* was estimated both in the control and in the experimental vessels at the beginning and at the end of each experiment. To assess possible effects of high microalgal concentrations and small container volume, mussels were preliminarily exposed in the same vessels to cultures of the non-toxic chlorophyte *Tetraselmis suecica*, at a tenfold series of concentrations up to 10^6 cells ml^{-1} for two days.

To investigate the effects of longer exposures to the toxic algae, animals (valve length: 5.5±0.5 cm) were exposed 72 h to *O.* cf. *ovata* cultures ($2.2 \pm 0.2 \cdot 10^3$ cells ml^{-1}) in the same conditions as above, refreshing the algal cultures every day in both control and experiment beakers.

At the end of the 24 h and 72 h experiments, mussel soft tissues were weighted, put in 50-ml falcon tubes, and frozen at -20 °C until toxicity analysis.

Sea urchins

Eight experiments were performed feeding sea urchins *P. lividus* with *O.* cf. *ovata* as epiphyte on the red alga *A. taxiformis* for 5 days. For each experiment, three replicates of 15 *P. lividus* specimens each, starved one week, were placed in borosilicate glass containers filled with 20 l of 35 µm filtered natural seawater at 22 °C. Thalli of *A. taxiformis* (60-130 g WW) were added to each container and a subsample (6-10 g) was collected for epiphytic *Ostreopsis* cell counts.

The tank was covered with a plastic lid and aerated with an airstone system gently, avoiding detachment of epiphytic cells and resuspension of faecal material. On the 3rd day, a refresh of 8 l of seawater was made. In the

fourth experiment, the same animals were submitted to two feeding cycles of 5 days each. At the end of the experiments, gonads and other soft parts of each animal were separated, weighted and immediately frozen at -20 °C until toxicity analyses.

Sea urchin exposure experiments

Exposure to whole O. *cf.* ovata *cells*

Prior to the experiments with *O*. cf. *ovata*, sea urchins were exposed to cultures of the non-toxic chlorophyte *Tetraselmis suecica* to assess possible generic damages not related to dinoflagellate toxicity. Cell density in the 2-l beakers ($1.4 \cdot 10^4$- $2.3 \cdot 10^4$ cells ml^{-1}) was more than 20-fold that of the dinoflagellate cultures used in the subsequent experiments ($1.6 \cdot 10^2$-$4.4 \cdot 10^3$ cells ml^{-1}) to compensate for the biovolume of *O.* cf. *ovata* cells ($1.7 \cdot 10^4$ μm^3) which is ca. 20-fold larger than that of *T. suecica* (ca. $9 \cdot 10^2$ μm^3).

To assess the direct effects of free-living *O.* cf. *ovata*, four sea urchins were exposed to either D483 or OOAPS0810-S1 strains at concentrations of 100, 200, 600, 1,200 and 4,800 cells ml^{-1} in 2-l beakers (22.5±0.5 °C, airstone aeration) for five days. Notes of any signs of stress were taken every day, including scarce adherence to the beaker walls and partial or total loss of the spines.

An arbitrary health index, similar to the index used by Shears & Ross (2010), was calculated by assigning scores between 5 and 0 to each of the four urchins in the batches: 5 = healthy, 4 = folded spines, 3 = loss of <50% spines, 2 = loss of >50% spines, 1 = death within 5 days, 0 = death within 4 days. The average of the scores of the four animals in each experiment was normalised dividing by 5, to obtain a value varying between 0 (all animals dead within 4 days) and 1 (all animals normal at the end of the experiment).

Exposure to filtered and sonicated cultures and toxin extracts

To detect possible effects of toxic substances released by the cells, sea urchins (n=4) were exposed to the medium of cultures of density of 100, 600, 740 and 1,000 cells ml^{-1}, obtained removing the cells by gravity filtration onto 0.22 µm filters.

Further, sea urchins (n=4) were exposed to lysed cultures of either *O.* cf. *ovata* strains obtained in the following way: 1 l of a culture of known concentration was centrifuged (4,500 rpm, 4 °C) for 10 min. The supernatant was stored at -20 °C, while the pellet was resuspended in about 2 ml in a 50-ml falcon tube by vortexing for about 5-10 s, and then ice-sonicated with a microtip sonicator for 10 min (pulsed sonication: duty cycles 30 s on/30 s off, 3 mm tip, power <10%, 60 amplitude). One droplet was used to assess the success of the lysis counting unbroken cells. After sonication, the sample was stored at -20 °C. Before the experiments, the supernatant and the pellet were thawed at room temperature, mixed and diluted with 0.22 µm-filtered seawater up to the concentration required.

Finally, sea urchins (n=4) were exposed to toxin extracts obtained from *O.* cf. *ovata* strain OOAPS0810-S1 at concentrations of 740, 1,000 and 1,900 cells ml^{-1}.

In each experiment set, sea urchins were observed daily and the health index was calculated as described above.

Mussel detoxification

Upon collection from the *O.* cf. *ovata* bloom area, mussels were immediately transferred to SZN and subsampled for initial toxicity assessment. The sample only included small specimens (< 3.0 cm long), as larger wild mussels are exhausted in summer. Mussels with valve length > 0.5 cm (ca. 6.5 kg total weight) were suspended in plastic baskets in a rectangular open system tank with maximum capacity of ca. 230 l, filled with 180 l of filtered (35 µm mesh-size) natural seawater flowing into the system. The average daily flow was of $4 \cdot 10^3$ l day^{-1} of filtered seawater (T= 25.6-26.6 °C) pumped in from the coastal waters off SZN. The tank walls and the baskets were scrubbed with a brush and the bottom cleaned daily with a siphon.

Over the whole maintenance period (3 weeks), the only food source for the mussels was natural plankton not retained by the filters, which was enumerated in the light microscope in the middle of the experiment. Since the day of collection, every 3-4 days 3 mussel replicates of 400 g (total weight) were collected and soft tissues were weighted and immediately frozen at -20 °C until toxicity analyses.

Toxicity analyses

Toxin extraction

Concentration values of ovatoxin-a in strain D483 considered in this study were those obtained by Scalco *et al.* (2012) from exponentially growing cultures maintained at 22 °C and 15:9 light:dark cycle. For strain OOAPS0810-S1, 100 ml of healthy exponentially growing cultures maintained as described above were centrifuged at 2,300 g for 10 minutes at 16 °C. The supernatant was discarded, while the pellets were frozen at -20 °C until analysis. Palytoxin-like molecules were extracted as described in Rossi *et al.* (2010). The palytoxin standard was purchased from Wako Chemicals GmbH (Neuss, Germany). Sample solutions of standard material (5, 2.5 and 1.25 ppm) were prepared in MeOH:water (1:1, v:v).

For animal tissues, the extraction was performed with a MeOH:W 60:40% solution. Extracts were dried under a stream of nitrogen and re-suspended in 4 ml of aqueous Tween 60 (1%).

Mouse bioassay

Because the chemical methods for detecting toxins were poorly sensitive at the time of our experiments and not all palytoxin-like molecules present in *O.* cf. *ovata* were known, the mouse test was used to determine toxicity in mussels and sea urchins. To this aim, 16 ml of palytoxin-like molecule extracts from animal samples dried and re-suspended in aqueous Tween 60 (1%) were used. For each sample, three Swiss mice (weight 18-20 g) were injected intraperitoneously with 1 ml of the Tween solution. Control mice were injected with an extract obtained from shellfish or sea urchin samples previously tested as negative. Mice were observed continuously for the first 30 min after the injection. Subsequent observations were performed at hour intervals during the next 6 h and hence after 22-24 h (Yasumoto *et al.,* 1978); for the purpose of our experiments, the death of any mice within the 24 h was considered as a sign of toxicity of the sample.

LC-MS

Algal pellets of strains OOAPS0810-S1 and samples of animal tissues resulting positive to the mouse bioassay were submitted to LC-MS analysis for toxin quantification.

For the algal strain, mass spectral analyses were performed using an Agilent LC/TOF/MS, a time-of-flight mass spectrometer equipped with an ElectroSpray interface coupled with an Agilent liquid chromatograph model 1100. Phenomenex Luna HILIC 3µ (150x2.00 mm) was used for chromatographic separation. Elution was accomplished with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 0.1% formic acid. The flow rate was 0.3 ml min^{-1}. The LC/TOF/MS analysis worked in positive ion mode, with mass range set at m/z 100– 3500 u at a resolving power of 10,000 (Rossi *et al.,* 2010). The conditions of ESI source were as follows: drying gas (N2) flow rate 11 ml min^{-1}, drying gas temperature 300 °C, nebulizer 45 psig, capillary voltage 4,000 V, fragmentor 250 V and skimmer voltage 60 V. All acquisitions and data analyses were controlled by Agilent LC/TOF/MS Software (Agilent, USA–Germany). Tuning mix (G1969-85003) was used for lock mass calibration in our assay. Final concentrations were determined by averaging the concentration values of two replicates.

For animal tissue analyses, MS experiments were carried out on an API-2000 triplequadrupole MS instrument (Applied Biosystems, Life Technologies, Foster City, CA, USA) equipped with a Turbo spray® (TSI) source coupled to an Agilent model 1100 LC (Palo Alto, CA, USA). A 3 µm Gemini C18 (150×2.00 mm) column (Phenomenex, Torrance, CA, USA) maintained at room temperature was used in all experiments; it was eluted at 0.2 ml min^{-1} with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. Analytical conditions were the same as in Ciminiello *et al.* (2011). Due to lack of standards for ovatoxin-a, quantitative analyses were carried out based on the tentative assumption that ovatoxin-a shows the same molar response as PLTX. All values displayed for toxin concentrations should hence be considered cautiously and are likely underestimated.

Results

Ostreopsis *toxin content*

Ovatoxin-a content in *O.* cf. *ovata* OOAPS0810-S1 (Adriatic Sea) analysed by LC/TOF/MS was 3.04 pg $cell^{-1}$, corresponding to less than half that of the Neapolitan strain D483 (8.05 pg $cell^{-1}$, Scalco *et al.*, 2012).

Mussel feeding with O. *cf.* ovata

In the 24 h experiments, mussel weight in each replicate varied between 14.5 and 46.6 g (WW) and initial *Ostreopsis* cf. *ovata* cell density between $1.1 \cdot 10^3$ and $4.1 \cdot 10^3$ cells ml^{-1} (Fig. 1A). Cell ingestion, estimated at the end of the experiment based on the cell density difference between the control (at times slightly lower than the initial one) and the beakers with the animals, showed a great variability (between $8.0 \cdot 10^3$ and $5.2 \cdot 10^4$ cells g^{-1} mussel WW in exp. 4 and 3, respectively; Fig. 1B). In the first three experiments (initial cell concentrations: $1.0 \cdot 10^3$-$2.5 \cdot 10^3$ cells ml^{-1}) animals were small (2.5-4.0 cm valve length) and filtered the culture effectively, ingesting, on an average, $3.3 \cdot 10^4$-$5.2 \cdot 10^4$ cells g^{-1} WW, with low or null residual algal concentrations at the end of the experiments. At higher concentrations ($3.1 \cdot 10^3$-$3.7 \cdot 10^3$ cells ml^{-1}, exp. 4 and exp. 5), small and medium-sized mussels (4.5-6.5 cm length) showed reduced ingestion ($8.2 \cdot 10^3$-$8.5 \cdot 10^3$ cells g^{-1} WW), while at still higher concentrations ($4.1 \cdot 10^3$ cells ml^{-1}, exp. 6) larger mussels (5.5-9.0 cm length) reduced the *O.* cf. *ovata* density in the batches to ca 20-30% of the initial values, ingesting $3.2 \cdot 10^4$ cells g^{-1} WW. Replicates generally gave similar results, with the exception of one batch of exp. 2, in which the ingested cell value was considerably higher.

At the end of the experiments the vessels contained broken cells often associated with faecal pellets, probably resulting from rejected food, not seen in the control. Mussels showed no evident adverse effects other than prolonged valve closures observed at times during the last hours of exposure. Mussels fed in parallel with cultures of non-toxic species showed no signs of stress and cleared the culture completely.

At the lowest cell concentration (exp. 1, $1.0 \cdot 10^3$ cells ml^{-1}) and with high weight-normalised ingested cell values (>$2.8 \cdot 10^4$ cells g^{-1} WW; exp. 2, batch 1, exp. 3, batches 1 and 2, exp. 6, all batches), mussel extracts caused the death of two or three mice in a time interval of 1-24 h. At higher concentrations (exp. 2-5), mussel extracts were negative to the mouse test or caused the death of a lower number of mice in a longer time (Supplementary Material, Table S1). Upon injection of toxin-containing samples, mice presented typical symptoms of palytoxin intoxication (Riobò *et al.,* 2008), with stretching of hind

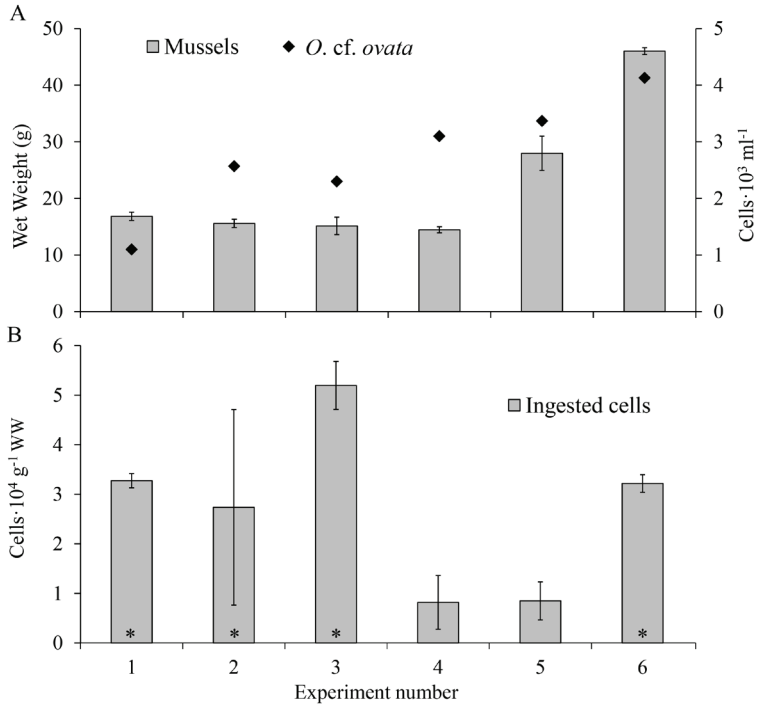

Fig. 1: Feeding of *Mytilus galloprovincialis* in six 24 h experiments with animals of different sizes exposed to different *Ostreopsis* cf. *ovata* cell concentrations. A) Mussel wet weight (WW) and *O.* cf. *ovata* cell density at the beginning of each experiment (avg±SDV). B) Weight-normalised ingested cells (avg±SDV). The asterisks indicate experiments in which some or all replicates were toxic to the mouse bioassay (Supplementary Material, Table S1).

limbs, gasping for breath, paralysis and finally death. Ovatoxin-a concentration in the extracts was below the detection limit of the LC-MS instrumentation.

During the 72 h experiment, mussels ingested averagely $4.3 \cdot 10^4$ cells g^{-1} WW during the first day of exposure (Fig. 2). In one replicate ingested cells were less than half those of the other two replicates ($2.5 \cdot 10^4$ and $5.8 \cdot 10^4$ cells g^{-1} WW, respectively). After 30-32 h, 7-8 animals per batch died, and the 2-3 surviving in each batch showed ingested cell values higher than on the first day (on average, $6.7 \cdot 10^4$ cells g^{-1} WW). On the third day, the animals stopped filtering and showed marked signs of stress, like frequent valve closures and reduction of byssus strength. Extracts of animal tissues collected at the end of the experiment caused a rapid death (1-2.5 h) of all specimens employed for the mouse-test, while ovatoxin-a concentration, assessed with LC-MS analysis, varied between 18 and 45 µg kg^{-1} of animal WW (Table 1).

Mussel detoxification

Wild mussels collected from rocks during the *Ostreopsis* cf. *ovata* bloom on 20 July 2009 caused mouse mortality in less than 30 minutes, while 107.14±12.50 µg kg^{-1} ovatoxin-a were found in their soft tissues. The animals did not show any signs of stress when they were collected, nor over the following weeks, when they were maintained in tanks with 35-µm filtered seawater flowing into the system from coastal waters. No additional food (algal cultures) was provided to the mussels. Cell counts in one sample of the tank water collected during the ex-

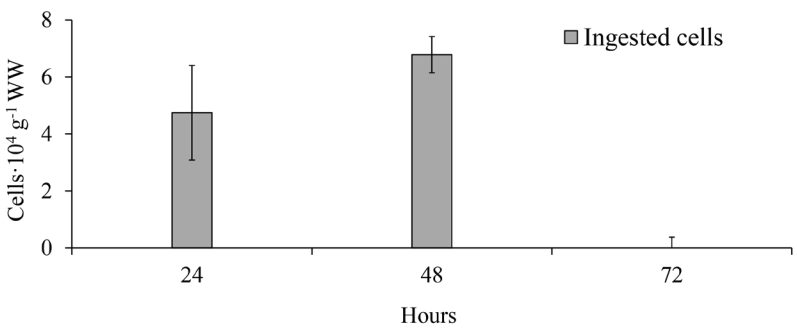

Fig. 2: Feeding of *Mytilus galloprovincialis* on *Ostreopsis* cf. *ovata* in a 72 h experiment. Weight-normalised ingested cells (avg±SDV) at different time intervals. Fresh microalgal cultures ($2.17 \pm 0.23 \cdot 10^3$ cells ml^{-1}) were provided every 24 h. Of the 10 animals of each replicate, 7-8 died at the beginning of the second day while the survivors were toxic (Table 1).

Table 1. Response of mussels exposed 72 h to *Ostreopsis* cf. *ovata* cultures (Fig. 2). Values of ingested cells and toxicity obtained at the end of the third day from the 2-3 survivors out of 10 initial individuals.

Replicate	Survived mussels	Ingested *O.* cf. *ovata* (cells·10^4 cells g^{-1} WW)	Mouse death times (h)	Ovatoxin-a (pg g^{-1})
1	2	4.28	1 - 2.5	45.19
2	3	7.54	1 - 2.5	18.36
3	3	8.05	1 - 2.5	29.07

periment showed phytoplankton cells at concentrations of $1.1 \cdot 10^5$ cells l^{-1}, with the most abundant species being diatoms (*Leptocylindrus danicus,* $6.0 \cdot 10^4$ cells l^{-1}, and an undetermined centric diatoms, $1.4 \cdot 10^4$ cells l^{-1}).

In the first 14 days from collection, differences in ovatoxin-a content were low, but the death time of the mice increased from less than 30 min to up to 3h (Table 2). A marked reduction of toxin levels occurred between the 15th and the 17th day, when ovatoxin-a was no longer detectable and a mild toxicity was revealed by the mouse test (Table 2).

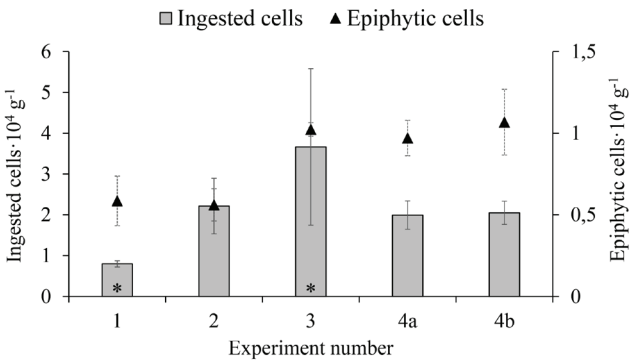

Fig. 3: Feeding of *Paracentrotus lividus* (avg±SDV, n = 15) on *Ostreopsis* cf. *ovata* epiphytic on the red alga *Asparagopsis taxiformis* in five experiments lasting five days each. Experiment 4b was performed with the same animals as 4a, which were given a second stock of seaweeds after a two- day interval. Macroalgae (55-131 g) were completely eaten in all cases. Four additional experiments at low epiphytic cell density (<$3.4 \cdot 10^3$ cells g^{-1}) are not represented. Asterisks indicate the experiments in which sea urchins were weakly toxic at the mouse bioassay (Supplementary Material, Table S2).

Sea urchin feeding on macroalgae epiphytised by O. *cf.* ovata

Sea urchins were fed with 60-130 g of the red macroalga *Asparagopsis taxiformis* for 5 days, with the exception of exp. 4, in which the same animals were fed again for 5 days after a two-day interval. Cell density of epiphytic *O.* cf. *ovata* in the natural samples varied over the season, ranging between $2.3 \cdot 10^4$ and $8 \cdot 10^4$ cells g^{-1} WW in late June-July, i.e., at the times of the first five experiments. In August, the epiphyte concentration decreased by two orders of magnitude compared to the maxima (≥ $2.2 \cdot 10^3$, exp. 7, not shown), in agreement with the normal course of the bloom in the sampling area. Despite all cares in handling the macroalgal samples, epiphyte cell densities in the tanks at the start of the experiments were considerably lower, varying between $1.6 \cdot 10^3$ and $5.7 \cdot 10^3$ cells g^{-1} of macroalga (Fig. 3), which corresponded to 5-36% of the initial densities with the exception of two cases, in which they were above 70% of those of the original samples.

Consumption rates varied between 1 and 2.6 g of seaweed ind. day^{-1}. Sea urchins ingested all the seaweed epiphytised by *O.* cf. *ovata* in all cases and apparently were not damaged. Assuming no further algal cell detachment during the five days of the experiments, the amount of ingested microalgae ranged between 0.2 and $4.0 \cdot 10^4$ cells g^{-1} of sea urchin WW (Fig. 3, Supplementary Material, Table S2).

Gonads were never positive to the mouse bioassay while other soft tissues were at times weakly toxic (Supplementary Material, Table S2). Ovatoxin-a concentration in all samples was below the instrumental detection limit.

Table 2. Toxicity decay of naturally toxic *Mytilus galloprovincialis* collected during a bloom of the toxic dinoflagellate *Ostreopsis* cf. *ovata* and maintained in 35-µm filtered natural seawater for 17 days. Na: no data available, Nd: not detectable.

Day	Mouse death times, h (n dead mice)	Ovatoxin-a (µg kg^{-1}; avg ± SDV)
0	<0.5 (9)	107.14 ± 12.50
3	0.5-2 (9)	98.81 ± 19.09
7	2-3 (9)	Na
10	2-3 (9)	89.67 ± 10.69
14	2.5-3 (9)	92.00 ± 21.93
17	≥ 20 (3)	Nd

Sea urchin exposure to O. *cf.* ovata

To assess the effects of *Ostreopsis* and its toxins in sea urchins, four animals in each experiment were exposed to *Ostreopsis* cultures or culture derivatives (culture filtrates, toxin extracts, and sonicated cultures) in batch systems.

The exposure of sea urchins to whole *O.* cf. *ovata* cultures did not affect the animals at low concentrations, whereas folded spines and different degrees of spine loss were observed at concentrations between 200 and 600 cells ml^{-1}. Lethal effects in some individuals occurred on the 5th day at 740-1,700 cells ml^{-1} concentrations, while at still higher concentrations all animals died in four days (Fig. 4). Residuals of the mucous net produced by *O.* cf. *ovata* were observed on sea urchin mouth and spines and at times on the bottom of the beakers, whereas *O.* cf. *ovata* cells were seen even inside the sea urchin pedicels. In the control, because of the high growth rate of *Isochrysis*, cell concentration and turbidity greatly increased during the experiment, but animals showed no adverse effects (data not shown).

Exposure to culture medium obtained by filtration of cultures (100, 600, 740 and 1,000 cells ml^{-1}) caused no adverse effects in sea urchins. Minor signs of stress were observed with sonicated cultures, with less serious damages than with cultures of comparable initial concentrations (Fig. 5). In sonicated cultures, lysis efficiency assessed by optical microscope showed several cells damaged (rough edges), but not completely broken. Usually, 3-4 over ten cells maintained their original shape, hence sonication operational efficiency was around 60-70%. However, unbroken cells appeared empty and strongly damaged, suggesting that toxins had been released also from partially lysed cells, thus leading to toxin concentration in the medium close to the toxin burden of the whole cells in the cultures of corresponding density.

Exposure to toxin extracts also caused a dose-dependent response in sea urchins, but with only sublethal effects (Fig. 6). The most serious response, observed at the highest toxin concentration, was the almost complete loss of spines in one over five urchins on the 5th day (health index = 2) with a toxin amount corresponding to that of a culture of 1,900 cells ml^{-1}.

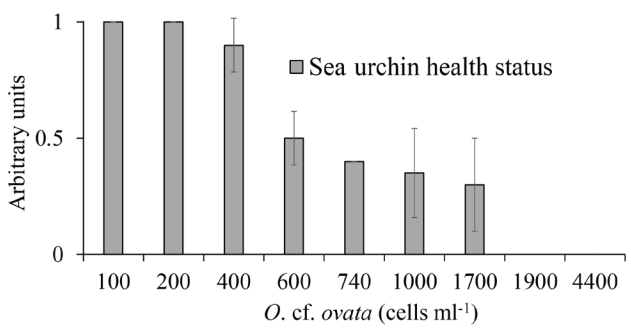

Fig. 4: Health status of sea urchins (avg±SDV, n = 4 animals) exposed to *Ostreopsis* cf. *ovata* (strain D483) for five days at different cell densities. Health index 1 corresponds to all four sea urchins alive after five days of exposure, 0 to all sea urchins dead in four days, intermediate values to different degrees of damage such as spine folded, partial and total spine loss and death in five days.

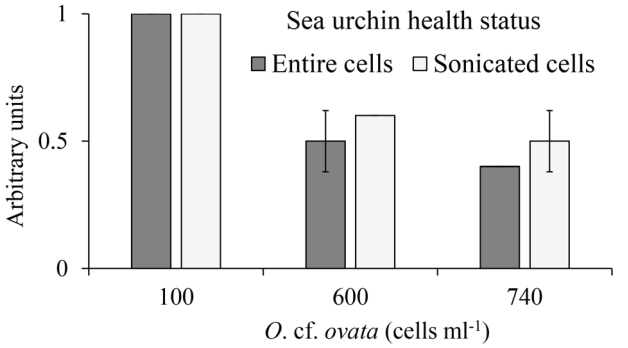

Fig. 5: Sea urchin health status (avg±SDV, n = 4) upon exposure to entire or sonicated *Ostreopsis* cf. *ovata* cultures (strain D483) of the same initial cell density.

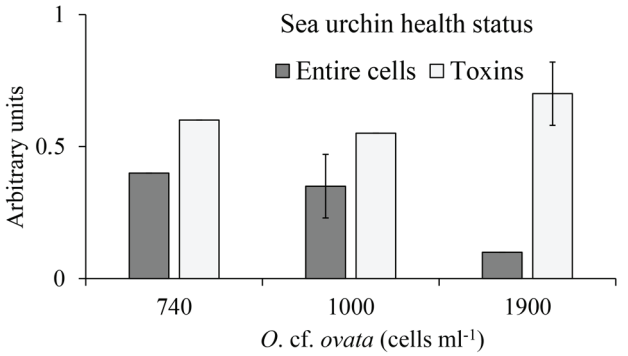

Fig. 6: Sea-urchin health status (avg±SDV, n = 4) after exposure to whole *Ostreopsis* cf. *ovata* cultures (strain 00APS0810-S1) or toxins extracted from cultures of the same cell density.

Discussion

The two key benthic Mediterranean species tested in this study, *Mytilus galloprovincialis* and *Paracentrotus lividus*, exhibited a variety of different responses to the toxic benthic dinoflagellate *O.* cf. *ovata*. The exposure to the microalgae at times caused evident adverse effects and even the death of some animals, whereas in other cases toxic cells were ingested without apparent damages to the animals, a result that mirrors the disparate responses observed in nature during blooms. The variability in the response depended on both the density of the toxic microalgae and the way the animals were exposed to them. Accumulation of toxic compounds at times occurred in mussels and less frequently in sea urchins.

Exposure of mussels to *O.* cf. *ovata* cultures for brief periods (24 h) rarely caused visible adverse effects in animals, in agreement with the observations of apparently healthy but toxic mussels during *Ostreopsis* blooms (e.g., Aligizaki *et al.,* 2008; this paper). The number of ingested cells greatly varied among experiments, and seemed to be dependent on both mussel size and culture density.

The modulation of filtration capacity is a behavioural response that is common in bivalve exposed, e.g., to species producing Paralytic Shellfish Toxins (PSTs, Bricelj & Shumway, 1998) and can provide indications on mussels' sensitivity to the toxic microalgae. In short term exposures, mussels appeared to filter actively the medium in which they were immersed, but the weight-normalised number of ingested cells was lower in small and medium-sized mussels exposed to high ($3.1 \cdot 10^3$-$3.4 \cdot 10^3$ cells ml^{-1}) cell densities. By contrast, small mussels in less concentrated cultures ($\leq 2.6 \cdot 10^3$ cells ml^{-1}), as well as larger individuals exposed to high cell densities ($4.1 \cdot 10^3$ cells ml^{-1}) exhibited the highest ingested cell values, indicating the relevance of the ratio between cell concentration and mussel size, rather than the mere cell densities, to the filtration capacity of the animals.

Based on ingested cell numbers and toxin content in *O.* cf. *ovata* strain D483, about 260 µg kg^{-1} of ovatoxin-a should have been accumulated by mussels. Although the mouse bioassay was positive in several experiments, the toxin content was much lower than expected and often undetectable to the chemical methods that were available at the time of the experiments. It should be noted that, in the natural environment, suspension-feeders such as bivalve mollusks are rarely exposed to such high *Ostreopsis* concentrations as those of our experiments, because these epiphytic microalgae are closely linked to their substrates and rarely attain concentrations higher than 10^4-10^5 cells l^{-1} in the water column (Mangialajo *et al.,* 2011; Amzil *et al.,* 2012). In analogy with other dinoflagellates, such as the PST producer *Alexandrium* (Bricelj *et al.,* 1990; Bricelj & Shumway, 1998), clearance rate could have been inhibited at high cell densities, under which the animals could enhance behavioural or physiological mechanisms that reduce the impact of toxic cells, such as reduced filtration or fast rejection. We did not investigate pseudofaeces, but the presence of damaged dinoflagellate cells in the beakers indicates the rejection of part of the filtered material, which could be the cause of the low toxicity of the animals. While mussel toxicity was hardly detectable in our 24 h experiments, a quite high amount of palytoxin-like molecules (130 µg kg^{-1}) were accumulated in 24 h by *Perna* mussels transplanted in an *O.* cf. *ovata* bloom area in Currais Arquipelago, Brazil (Tibiriçá, *et al.,* 2019). In our 72 h experiment, the few survived animals contained a detectable amount of ovatoxin-a (up to 45.19 µg kg^{-1}), with the highest toxin content in the replicate sample in which a lower filtration was observed, strengthening the hypothesis that part or all of the toxin burden could have been eliminated by cell rejection in the animals showing an apparently higher ingestion rate.

Overall, these results indicate a relatively high sensitivity of mussels to *O.* cf. *ovata*, enhanced by the high concentrations of the toxic algae used in our experiments, which stimulated defence mechanisms that prevented the accumulation of toxins at high rate. This explanation is supported by the death of the majority of the animals during the second day of the longer exposure experiment, which suggests that damages were simply not visible to the unaided eye in the shorter experiments. Enzymatic assays (Gorbi *et al.,* 2012) or histopathological analyses (Carella *et al.,* 2015) indeed revealed a clear impact of *Ostreopsis* on mussels at concentrations lower than those used in this study, as well as in apparently healthy mussels collected from areas affected by blooms.

An alternative explanation for low mussel toxicity in our feeding experiments could be that a large part of the toxins were biotransformed after their ingestion, as hypothesised in one of the few published experiments conducted on *Ostreopsis* fed to mussels over 27 and 84 h (Rhodes *et al.,* 2002). Such processes have been described for PSTs (Reis Costa *et al.,* 2018) and Diarrhoetic Shellfish Toxins (Blanco, 2019), where toxin profile in mussels were different from that of the producing dinoflagellate. Biotransformation of azaspiracid in mussels occurs within 6 hours, and ca. 50% of the toxins is converted in metabolites after 1-2 days (Jauffrais *et al.,* 2012). Unfortunately, since palytoxin metabolisms is still poorly known, it is not possible to establish the relative importance of cell rejection and biotransformation as the main mechanisms underlying the relatively low toxicity of the mussels in our experiments.

An interesting result of our study was the high variability in the response shown by mussels of different sizes to different concentrations of the toxic species in the 1-day experiments, but also by mussels of comparable sizes exposed to the same amount of toxic cells in the 3-days experiments. The variability in the latter case was particularly evident in the few animals that survived the others two whole days and exhibited a variable toxicity at the end of the experiment. Response variability among different bivalve species, and also within the same species, is well known in the case of PSTs (Bricelj & Shumway, 1998). Mussel response to toxic algae may also vary in relation to their previous history of exposure to toxins (Landsberg, 2002). For example, wild blue mussels from a region contaminated by PST-producing dinoflagellates accumulated twice as much toxins than cultured mussels from a pristine zone when subjected to identical bloom conditions (Chebib *et al.,* 1993). This should not be the case with our experiments, in which animals were all collected from the same area and at the same time.

Mussels in the Gulf of Naples can become toxic as a consequence of *O.* cf. *ovata* blooms in summer-early autumn (Carella *et al.,* 2015) and may remain toxic for several weeks after the bloom, thus requiring continued monitoring (ARPAC, 2008). In this study the period of detoxification of wild mussels collected during an *Ostreopsis* bloom was little longer than two weeks, with some changes in the first two weeks only detected by the mouse test, followed by a sudden toxicity decrease occurring between the 15th and 17th day. The persistence of quite high toxicity levels for two weeks could have been favoured by the maintenance of mussels in filtered seawater with relatively low algal concentrations, because increased ingestion rates in presence of higher cell concentrations would lead to a higher digestive activity and, thus, a faster decrease of toxin levels (Blanco *et al.,* 1999; Svensson, 2003). No toxin decrease in the first days of detoxification, typically occurring when recently ingested toxic al-

gae are eliminated through the faeces (Guéguen *et al.,* 2008), hints at a relatively long exposure of the mussels to toxic algae prior to collection, during which toxins could have been transferred to the body tissues of the animals. Lack of information on the toxification phase and maintenance of animals with no added food hamper the comparison between our results and those obtained with other toxic species. Yet, half-life of toxins in our experiments seems to be comparable to or even shorter than that of other toxins. In bivalves exposed to *Azadinium spinosum*, the amount of azaspiracids and their metabolites was above the regulatory limit after few hours of detoxification, but was still detectable after two weeks (Jauffrais *et al.,* 2012). Similar results were obtained with saxitoxins, still detectable in mussels previously fed with the toxic alga *Alexandrium catenella* (reported as *A. fundyense*) after a three-week exposure to the non-toxic diatom *Thalassiosira pseudonana* (Kwong *et al.,* 2006).

Differences between trends of mouse death times and ovatoxin-a content in our detoxification experiment suggest that palytoxin analogues other than ovatoxin-a that are present in *O.* cf. *ovata* may have a different fate and should be monitored during the detoxification period, along with their distribution in animal tissues and its change over time. The toxin profile analysis in the medium and in the faeces would also be needed in order to assess whether toxins are mainly eliminated (similar toxin profiles in seawater, faeces and animal tissues) or biotransformed (different toxin profiles). In fact, the detoxification time found in this study is comparable (Kwong *et al.,* 2006, Jauffrais *et al.,* 2012) or even lower (Mafra Jr. *et al.,* 2015) than that observed in other experiments in which toxin transfer among tissues or biotransformation have been demonstrated.

Adverse effects of *O.* cf. *ovata* through feeding were less evident in sea urchins. Little information about the effects of *Ostreopsis* and other toxic microalgae on adult sea urchins is available, as most of the experiments were performed on larvae or juveniles (Privitera *et al.,* 2012). Adult sea urchins fed with artificial (agarised) brevetoxin-contaminated substrata showed feeding rates similar to those fed with non-toxic food (Sotka *et al.,* 2009). In our experiments, the presence of the toxic microalga as epiphyte did not prevent macroalgae consumption, which occurred at the same rates as with other macroalgae (Boudouresque & Verlaque, 2013), nor animals showed any particular damages. Differently from our results, the sea urchin *Evechinus chloroticus* exposed to the brown alga *Carpophyllum plumosum* epiphytised by *O. siamensis* at bloom concentrations (in the order of 10^6 cell g^{-1} of macroalgal WW upon macroalgal collection) exhibited severe damages and death within 6 days (Shears & Ross, 2010). As the time of exposure to toxic algae was similar to that of our experiments, such a different response may have been caused by differences in microalgal density and/or toxicity, or in animal sensitivity to the toxins.

The absence of damages by epiphytic microalgae consumption matches the observations of ovatoxins often accumulated in apparently healthy sea urchins in the natural environment. Preferential feeding on *Ulva* and other green macroalgae (Boudouresque & Verlaque, 2013), generally less epiphytised than red and brown algae, should help avoiding excessive ingestion of toxic microalgae. Nonetheless, apparently healthy sea urchins collected during intense *O.* cf. *ovata* blooms (up to $1.6 \cdot 10^5$ cells g^{-1} macroalgal WW) in the Gulf of Naples had a relatively high toxin content (ca. 80 µg kg^{-1} ovatoxin-a, Migliaccio *et al.,* 2016), while animals collected from the southern French coast were even more toxic (up to 450 µg kg^{-1} ovatoxin-a and palytoxin) in presence of a bloom reaching up to $4 \cdot 10^5$ cells g^{-1} macroalgal WW (Amzil *et al.,* 2012). In our feeding experiments with *Ostreopsis*-epiphytised *Asparagopsis*, sea urchins were toxic only in a few cases, probably because of low epiphyte densities due to cell detachment before and during the experiments. Interestingly, the edible parts of the sea urchins, i.e. the gonads, were not toxic in the few cases of mild toxicity detected in the other sea urchin tissues, in agreement with studies showing the accumulation of toxins in the digestive tract (Amzil *et al.,* 2012; Brissard *et al.,* 2014). This information is particularly relevant as the gonads only are consumed in places where sea urchins are eaten.

While feeding on epiphytic *O.* cf. *ovata* apparently did not affect sea urchins, animals exposed to *Ostreopsis* cultures showed a clear density-dependent response to toxic cells, which matches cases of spine loss and mortality of sea urchins at times reported during blooms (Sansoni *et al.,* 2003; Shears & Ross, 2009; Amzil *et al.,* 2012; Accoroni & Totti, 2016). Our findings, along with the sporadic nature of those events compared to the recurrent seasonal pattern of blooms, suggest that sea urchins are affected macroscopically only when *Ostreopsis* spp. blooms are accompanied by a massive release of free cells and mucous net in the water column. Accordingly, in the above mentioned case of exposure of *Evechinus chloroticus* to epiphytic *O. siamensis* (Shears & Ross, 2010), mortality may have been caused by the contact with cells released by the macroalga rather than by ingested cells.

The most obvious culprit of the mortality and damages in sea urchins in the whole culture treatments would be the palytoxin-like molecules, which we attempted to demonstrate by enhancing the direct contact of the toxins with the animals. While culture filtrates were expected not to have a big impact as they do not contain high amounts of toxins (Simonini *et al.,* 2011; Guerrini *et al.,* 2010), surprisingly sonicated cultures caused less damages than whole cultures of the same initial concentration. Similar to our results, nauplii of the crustacean *Artemia salina* exposed to sonicated *Ostreopsis* cultures showed less serious signs of sufferance than those caused by undamaged cultures (Tartaglione *et al.,* 2016). A possible explanation is that the highest damage is caused by cell mucocysts and/or mucilaginous aggregates penetrating through the pedicels or being directly ingested. No information is available about the toxin content of the mucous produced by *Ostreopsis*, but the latter hypothesis would be supported by the even milder effects observed exposing sea urchins to toxin extracts, with no mortality and less serious damages compared to those caused by whole and sonicated cells.

Conclusions

The dual response of animals exposed to blooms of the toxic dinoflagellates *Ostreopsis* in nature was confirmed by our experimental results showing that, under short term exposures and at low cell concentrations, both mussels and sea urchins coexist with the microalga and feed on it with no apparent damages, while in other cases the animals are injured or hit to death by the toxic species. The apparently harmless coexistence between animals and toxic *Ostreopsis* is even more likely to occur in nature, where animal diets are more variable due to fluctuations in toxic cell abundance, animal movement (in sea urchins) and food selection. In these cases, the lack of adverse effects allows a more effective accumulation of toxins that make the animals unsafe as food.

One of the interesting results of our study was the lack of a linear and homogeneous mussel response to toxic cells, whereby the ratio between animal body weight and cell density seems to play a role. Differences in the modulation of filtration and actual ingestion rates may further contribute to the variability in the response among individual animals. The persistence of high toxicity levels for at least two weeks from exposure in mussels is also noteworthy, overall indicating a low level of defence mechanisms in the exposed animals.

Indeed, in both mussels and sea urchins, sublethal and even lethal effects are possible with exposures to higher cells concentrations, longer periods or different ways of exposure. Sea urchins seemed to be less damaged by the toxic dinoflagellate attached to the macroalgae, at least at the relatively low concentrations they experienced in our study. Conversely, exposure to free *O.* cf. *ovata* cells, either whole or broken, and to toxin extracts injured sea urchins in a dose-dependent manner. These results support the hypothesis that it is actually palytoxin-like compounds to hurt the animals, while the stronger impact in the presence of whole cells indicates that mucocysts and mucilaginous aggregates produced by live cells may act as an effective carrier for the toxins.

The results of our study corroborate those of other experiments that have shown subtle and sublethal effects in both mussels (Carella *et al.,* 2015; Gorbi *et al.,* 2012; 2013) and sea urchins, in their larval stages and even in the development of their progeny (Migliaccio *et al.,* 2016; Castellano *et al.,* 2018; Neves *et al.,* 2018). The clear negative impact of *O.* cf. *ovata* demonstrates that an adaptation of these animals to *Ostreopsis* toxins is far from being achieved. This would be in line with the hypothesis of a relatively recent introduction and expansion of *Ostreopsis* in the Mediterranean Sea, and points at serious damages caused by their toxic blooms in natural populations of these important key-species of the benthic marine fauna.

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Supplementary data

The following supplementary information is available on line for the article:

Table S1. Cell ingestion and mussel toxicity in the 24 h experiments in which *Mytilus galloprovincialis* were fed with *Ostreopsis* cf. *ovata* cultures. Toxins were always below the detection limit at the chemical analyses.

Table S2. Feeding of *Paracentrotus lividus* on the red macroalga *Asparagopsis taxiformis* with *O.* cf. *ovata* as epiphyte: sea-urchin and macroalga wet weight (WW) and total epiphytic cells in each experiment (avg±SDV). Macroalgae were completely eaten in all cases. Asterisks indicate samples in which sea urchin tissues were weakly positive to the mouse bioassay (1-2 mice dead in less than 24 hours in 1-2 replicate samples). Gonads analysed separately were not toxic.