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Allelopathic interactions between the HAB dinoflagellate *Ostreopsis* cf. *ovata* and macroalgae

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ABSTRACT

Intense blooms of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* have been a recurrent phenomenon along several Mediterranean coasts during summer in the last few years. These blooms are often associated with noxious effects on humans and deaths of benthic invertebrates. Previous studies carried out on the Conero Riviera (northern Adriatic Sea) highlighted that *Ostreopsis* abundances recorded on rocks were significantly higher than on the surface of seaweeds, suggesting that some allelopathic interactions might occur between *Ostreopsis* and macroalgal substrates. In this study we investigated under experimental conditions the interactions between *O.* cf. *ovata* and three of the most common macroalgae in this area: *Dictyota dichotoma* (brown alga), *Rhodymenia pseudopalmata* (red alga) and *Ulva rigida* (green alga). Three different experiments were set up: *O.* cf. *ovata* was grown (i) together with fresh macroalgal tissues, (ii) in media in which macroalgae were previously cultured, and (iii) in media with the addition of dry macroalgal powder at different concentrations. The results indicated that all the investigated seaweeds exerted negative effects toward the benthic dinoflagellate *O.* cf. *ovata. D. dichotoma* inhibited the growth of *O.* cf. *ovata* in all tested experimental conditions; *U. rigida* had inhibitory effect both in form of fresh thalli and dry powder but not as growth medium filtrate, suggesting that either *Ulva* does not release any allelopathic compound in the medium in absence of *O.* cf. *ovata* or the alleged released allelochemicals are rapidly degradable. Neither the fresh thalli of *R. pseudopalmata* or the filtrate of its culture medium showed any inhibitory effects, while a negative effect was only observed at high concentrations of dry thallus powder. With the exception of *D. dichotoma* co-culture experiment, a complete algicidal effect was never observed partly because *O.* cf. *ovata* produced a large amount of resting stages, which permitted its survival.

1. Introduction

Q2 In the last decade, blooms of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* Fukuyo have been a recurrent phenomenon along several Mediterranean coastal areas during summer (Vila et al., 2001; Turki, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008, 2011; Totti et al., 2010; Amzil et al., 2012; Illoul et al., 2012; Ismael and Halim, 2012; Pfannkuchen et al., 2012; Casabianca et al., 2014). In these areas, *O.* cf. *ovata* is well-known for its toxin production, including both palytoxin-like compounds (isobaric palytoxin and ovatoxin-a, b, c, d, e, f and g) and mascarenotoxin-a and c (Rossi et al., 2010; Ciminiello et al., 2011, 2012; Scalco et al., 2012; Uchida et al., 2013; García-Altares et al., 2015) that cause both mortality of benthic marine organisms (Shears and Ross, 2009; Accoroni et al., 2011; Gorbi et al., 2012, 2013; Pagliara and Caroppo, 2012) and noxious effects on human health (Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 2010; Del Favero et al., 2012).

Several studies have been conducted to assess the role of abiotic factors (mainly hydrodynamics, water temperature and nutrients) on the blooms dynamics (Chang et al., 2000; Vila et al., 2001; Monti et al., 2007; Shears and Ross, 2009; Totti et al., 2010; Accoroni et al.,

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2012a,b, 2015; Mabrouk et al., 2011; Mangialajo et al., 2011; Selina et al., 2014), while biotic factors such as allelopathic interactions between *Ostreopsis* and other organisms have only been partially addressed. The allelopathic interactions have been well documented among toxic dinoflagellates and co-occurring microalgae under unfavorable environmental conditions (Fistarol et al., 2003, 2004; Granéli and Johansson, 2003; Granéli and Hansen, 2006; Prince et al., 2008), and Monti and Cecchin (2012) showed that *O.* cf. *ovata* had a weak allelopathic activity toward other benthic dinoflagellates as well. However, to the best of our knowledge, allelopathic interactions between *Ostreopsis* and macroalgae have never been considered. *O.* cf. *ovata* commonly grows over all benthic substrata (rocks, pebbles, seaweed thalli, mollusc shells, etc.), and during periods of intense proliferation produces a conspicuous brownish mat which is only loosely attached to the substrata. Previous studies on the Conero Riviera, where *O.* cf. *ovata* blooms reach abundances among the highest of the entire Mediterranean coasts (Mangialajo et al., 2011), highlighted that *Ostreopsis* abundances on rocks were significantly higher than those recorded on seaweeds suggesting that some allelopathic interactions might occur between *Ostreopsis* and its macroalgal hosts (Totti et al., 2010).

Algal-bloom control is an important issue for the protection of the water environment due to the negative impacts on human economy and health, especially when the involved bloom-forming species are toxic. Moreover, the development of environment-friendly and cost-effective strategies for controlling algal blooms, such as using the allelopathy of aquatic macrophytes has gained great interest and has been suggested by several authors (Jeong et al., 2000; Nan et al., 2004; Jin et al., 2005; Wang et al., 2007a,b; Hu and Hong, 2008; Tang and Gobler, 2011). The interactions between microalgae and macroalgae have been investigated between phytoplankton species that form blooms and a number of both freshwater (Gross, 2003; Hu and Hong, 2008) and seawater macrophytes (Gross, 2003; Jin and Dong, 2003; Nan et al., 2004, 2008; Jin et al., 2005; Wang et al., 2007a,b; Ye and Zhang, 2013) but no information is available about macroalgae and benthic dinoflagellate interactions.

In this study, we investigated the interactions between *Ostreopsis* cf. *ovata* and three macroalgal species under laboratory conditions. The macroalgae were chosen among the most common species on the Conero Riviera during the bloom period of *Ostreopsis*: *Dictyota dichotoma* (Hudson) J.V. Lamouroux (brown alga), *Rhodymenia pseudopalmata* (J.V. Lamouroux) P.C. Silva (red alga) and *Ulva rigida* C. Agardh (green alga). Three different experiments were carried out: *O.* cf. *ovata* was grown (i) together with fresh macroalgal tissues, (ii) in filtered culture media in which macroalgae were previously grown, and (iii) in media with addition of dry macroalgal powder at different concentrations.

2. Materials and methods

2.1. Ostreopsis cf. ovata cultures

A strain of *Ostreopsis* cf. *ovata* was isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from seawater samples collected from the bloom that occurred on the Conero Riviera (N Adriatic Sea) in summer 2007 (strain OoAPn0807/E). After initial growth in microplates, cells were cultured at 21 ± 0.1 °C under a 12:12 h L:D cycle and an irradiance of 90–100 μmol m^{-2} s^{-1}, in modified f/4 medium prepared by adding macronutrients at a f/4 medium concentration without silica (Guillard and Ryther, 1962) and selenium to filtered and autoclaved natural seawater (salinity 35). Trace metals, iron, vitamins (H, B1 and B12) and HEPES pH 7.1 were added at levels corresponding to f/2 medium. The same physico-chemical conditions were used in all experiments described below, with the addition of germanium dioxide (6 mg l^{-1}), when specified, to prevent the growth of diatoms.

Microalgae were cultured to the exponential phase before inoculation in the following experiments. All the experiments were carried out in three replicates.

2.2. Macroalgae sampling and pre-treatment

Thalli of three macroalgal species, i.e., *Dictyota dichotoma*, *Rhodymenia pseudopalmata* and *Ulva rigida*, were collected on the Conero Riviera in summer 2009 and treated to remove the epiphytes from their surface as follows: they were washed carefully in filtered sea water (FSW) containing 1% of surfactant, rinsed in FSW and then observed at a stereo-microscope in order to mechanically remove the epiphytes with scalpels and tweezers; afterwards, a 3-min dip in tap water followed by a washing with chloramphenicol (50 ppm in FSW) led to the complete removal of residual epiphytic cells and bacteria respectively. After rinsing in FSW, macroalgal thalli were cut into fragments of approximately 9 cm^2 and acclimated for one week in FSW (containing 6 mg l^{-1} germanium dioxide).

2.3. Co-cultures of Ostreopsis cf. ovata and fresh macroalgal thalli

The co-cultures were set up with 1 g of thallus segments of each tested seaweed in 500 ml of medium containing germanium dioxide. For each species, 3 flasks were inoculated with 100 cells ml^{-1} of *O.* cf. *ovata*, and 3 flasks containing 100 cells ml^{-1} 1 of *Ostreopsis* cf. *ovata* without macroalgal thalli were used as control. The flasks were incubated for 20 days in a culture chamber at the conditions previously described. Every 2 days, 3 aliquots (2 ml) were taken from each flask (after gentle shaking of each thallus with a pair of tweezers and a homogenization of the medium) and preserved with Lugol's solution in the dark to assess the cell densities, and 1 aliquot (4 ml) was filtered (GF/F Whatman, diameter 25 mm, nominal pore size 0.7 μm) and stored in polyethylene bottles at −22 °C for nutrient analysis. Every 7 days, pH was checked and adjusted to maintain it to value of 8 until the end of the experiment.

2.4. Cultures of Ostreopsis cf. ovata in macroalgal culture medium filtrate

For each macroalgal species, 24 g of thallus segments pre-treated as above were maintained in 1 l FSW for one week. Afterwards, macroalgal thalli were removed and the culture medium was filtered (0.22 μm pore size) and used to prepare the medium for the experiment: the pH was adjusted to 8 and the nutrients were added to obtain a modified f/4 medium as described previously. Culture flasks containing 250 ml of medium were inoculated with *Ostreopsis* cf. *ovata* cells to obtain a final concentration of 500 cells ml^{-1}. As a control, 500 cells ml^{-1} of *O.* cf. *ovata* were inoculated in fresh modified f/4 medium. Subsamples (2 ml) were sampled every 2–3 days for 23 days and fixed with Lugol's solution.

2.5. Cultures with dry powder of macroalgae

Fresh thalli of each macroalga were dried at room temperature and pulverized using a mortar and a pestle. Different amounts of dry powder (0.4, 0.8, 1.6 g l^{-1}) were added to flasks containing *Ostreopsis* cultures (500 cells ml^{-1}) in 300 ml modified f/4 medium. Microalgal cultures without addition of dry macroalgal powder were used as controls. Cultures were maintained for 18 days during which subsamples (2 ml) were taken every 2 days and fixed with Lugol's solution.

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2.6. Microscope counting

Densities of *Ostreopsis* cf. *ovata* were estimated after homogenization using either Utermöhl (Edler and Elbrachter, 2010) or Sedgewick-Rafter (Guillard, 1978) chambers, through an inverted light microscope (Zeiss Axiovert 135) and a light microscope (Zeiss Axioskop) respectively, both equipped with phase contrast, at 200× magnification. Counting was performed on 10–30 random fields, 1–2 transects, or the whole chamber, in order to count a representative cell number. Total densities (vegetative cells and cysts) were expressed as cells ml^{-1}.

2.7. Cyst isolation for germination tests

Cysts of *Ostreopsis* cf. *ovata* were isolated at the end of the experiments and used to perform germination tests. Single cysts were isolated by the capillary pipette method according to the classical methodology (Hoshaw and Rosowski, 1973) and put separately in 24-well culture plates filled with fresh growth medium. Plates were placed in the culture chamber in the conditions previously described and were observed every day for 8 weeks.

2.8. Nutrient analysis

Nutrient variation during the co-cultures of *Ostreopsis* cf. *ovata* with fresh thalli was monitored throughout the experiment. Analyses of NO_3^-, NO_2^-, NH_4^+, and PO_4^{3-} were conducted following the colorimetric method by Strickland and Parsons (1968), adapted for an Autoanalyzer QuAAtro Axflow. Limits of detection were 0.02 μmol l^{-1} for NO_3^- and NO_2, and 0.03 μmol l^{-1} for PO_4^{3-}.

2.9. Calculations and statistical analyses

The microalgal growth was expressed as specific growth rate (μ) using the equation

$$\mu = \frac{(\ln N_1 - \ln N_0)}{T}$$

where N_1 is the final density, N_0 is the initial density, and T is the number of days between two measurements.

Differences in final cell densities of *Ostreopsis* cf. *ovata* in each experiment (i.e., co-cultures, macroalgal filtrate and dry macroalgal powder) between the three different macroalgal species were assessed through a one-way analysis of variance (ANOVA). Moreover, in the co-culture experiment, differences in nutrient concentrations between the three different macroalgae were assessed through an ANOVA test as well. When significant differences for the main effect were observed ($p < 0.05$), a Tukey's pairwise comparison test was also performed. The statistical analyses were conducted using Statistica (StatSoft Inc., Tulsa, OK, USA) software.

3. Results

3.1. Co-cultures of Ostreopsis cf. ovata with fresh macroalgal thalli

The first general observation regarding the pattern of colonization in each flask is that *Ostreopsis* cf. *ovata* cells colonized the bottom of the flask rather than macroalgal thalli. Our results showed that *O.* cf. *ovata* growth was markedly affected only by the presence of two macroalgal species, i.e., *Dictyota dichotoma* and *Ulva rigida*: at the end of the experiment (day 20) *Ostreopsis* cell abundance in co-culture with *D. dichotoma* and *U. rigida* was reduced by 100% and 94% respectively, compared to the day of the inoculum (Fig. 1). The density of *O.* cf. *ovata* was significantly lower with *D. dichotoma* (0.08 ± 0.06 cells ml^{-1}) and *U. rigida* (31.6 ± 4.6 cells ml^{-1}) than with *Rhodymenia pseudopalmata* (823.2 ± 59.8 cells ml^{-1}, $p < 0.001$) and in the control (735.4 ± 125.1 cells ml^{-1}, $p < 0.001$). The average lethal times (LT50), which represents the time at which 50% of the *Ostreopsis* cells were dead, in co-cultures with *U. rigida* and *D. dichotoma* were 12 and 8 days, respectively. On the contrary, the growth of *O.* cf. *ovata* was not inhibited by the presence of *R. pseudopalmata*, as no significant difference was observed between the maximum yield of *O.* cf. *ovata* in *R. pseudopalmata* co-culture and the control. The maximum yield was reached at the day 18 with 1535 ± 538 cells ml^{-1} in the control and 1016 ± 210 cells ml^{-1} in the *R. pseudopalmata* co-culture. The growth rate of *Ostreopsis* in *R. pseudopalmata* co-culture was comparable with that of the control (0.17 and 0.19 day^{-1} respectively).

Regarding the nutrient concentrations during the entire experiment (Fig. 2), we considered the phosphate (PO_4^{3-}) and the dissolved inorganic nitrogen (DIN) as a sum of nitrates, nitrites and ammonia. A marked decrease of DIN throughout the experiment was observed in *Rhodymenia pseudopalmata* and *Ulva rigida* co-cultures, while PO_4^{3-} concentration did not show appreciable variation.

Comparing nutrient content in different co-cultures, significantly higher values of both DIN and PO_4^{3-} were observed in *Dictyota dichotoma* co-culture than in *Rhodymenia pseudopalmata* and *Ulva rigida* ($p < 0.001$, Table 1). Comparing *U. rigida* and *R. pseudopalmata* co-cultures, PO_4^{3-} was significantly higher in the former than in the latter ($p < 0.05$, Table 1), while no significant differences were observed in DIN concentrations.

3.2. Cultures of Ostreopsis cf. ovata in macroalgal culture medium filtrate

Among the macroalgae tested, only *Dictyota dichotoma* culture filtrate exhibited inhibiting effects on *Ostreopsis* cf. *ovata* growth: *Ostreopsis* cell numbers immediately decreased (LT50 = 2) (Fig. 3). At the end of the experiment (day 23), the cell density of *O.* cf. *ovata* was significantly lower in the culture filtrate of *D. dichotoma* than in those of *Rhodymenia pseudopalmata* ($p < 0.001$), *Ulva rigida* ($p < 0.001$) and control ($p < 0.001$, Table 2). It is noteworthy that since the second sampling day in *D. dichotoma* treatment we observed the appearance of cysts (both thin and double-walled cysts, Fig. 4B and C). The percent abundances of double-walled cysts increased toward the end of the experiment, becoming the predominant morphotype observed in the final day of the experiment.

On the contrary, in filtrates of *Ulva rigida* and *Rhodymenia pseudopalmata* the *Ostreopsis* maximum yield was significantly

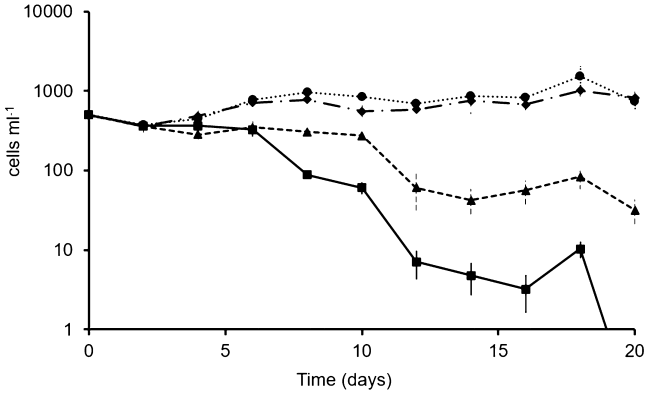

Fig. 1. Growth pattern of *Ostreopsis* cf. *ovata* cells growing in co-culture with fresh thalli of (■) *Dictyota dichotoma*, (◆) *Rhodymenia pseudopalmata*, (▲) *Ulva rigida* and (●) control. Bars indicate standard deviation.

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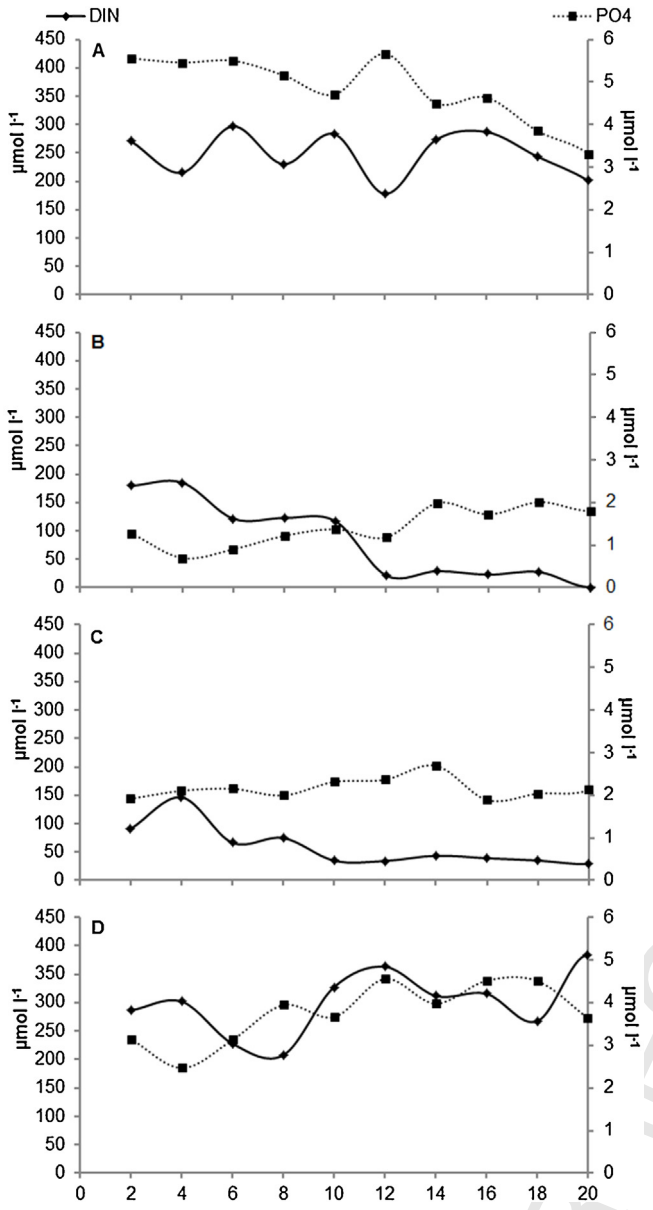

Fig. 2. Temporal variability of nutrient concentration (µmol l^{-1}) in cultures of *Ostreopsis* cf. *ovata* (A) without macroalgal thalli (control) and in co-culture with fresh thalli of (B) *Rhodymenia pseudopalmata*, (C) *Ulva rigida* and (D) *Dictyota dichotoma*. Dissolved inorganic nitrogen (DIN, left *y*-axis) and phosphate (PO_4^{3-}, right *y*-axis).

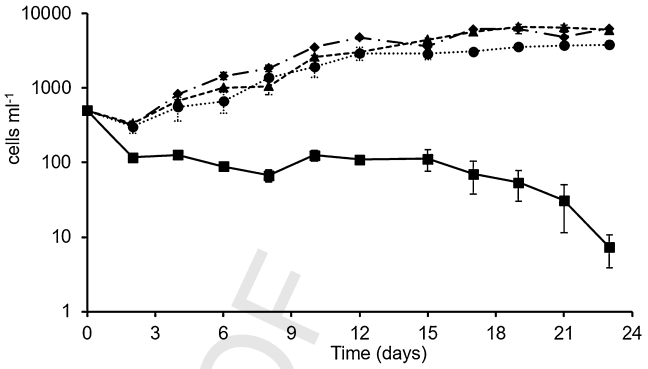

Fig. 3. Growth pattern of *Ostreopsis* cf. *ovata* cells growing with macroalga culture medium filtrates of (■) *Dictyota dichotoma*, (◆) *Rhodymenia pseudopalmata* and (▲) *Ulva rigida* and (●) control. Bars indicate standard deviation.

higher than in the control ($p < 0.001$, Table 2) and the growth rates, 0.28 and 0.38 day^{-1} respectively, were higher than for the control (0.25 day^{-1}). In the filtrate of *R. pseudopalmata*, maximum value was recorded in the final sampling day, while in the filtrate of *U. rigida* was recorded on day 19 (6597 ± 515 cells ml^{-1}).

3.3. Cultures with dry powder of macroalgae

The results of this experiment showed that all the macroalgal species exhibited an inhibitory effect on cultures of *Ostreopsis* cf. *ovata* (Fig. 5) as all the powder concentrations tested led to significantly lower values of cell densities at the end of the experiment compared to the control ($p < 0.001$, Table 3), except for the powder of *Rhodymenia pseudopalmata* at the lowest concentration (0.4 g l^{-1}) which allowed the growth of *O.* cf. *ovata* (growth rate = 0.19 day^{-1}).

Generally, the trend of total densities throughout the experiment showed a gradual decrease. At the end of the experiment (day 18), the abundance of *Ostreopsis* was reduced to values between 59 and 79% respect to the inoculum for powder of *Dictyota dichotoma* (LT50 of 10, 16 and 4 for 0.4, 0.8 and 1.6 g l^{-1} respectively), of 17–37% for powder of *Ulva rigida* (at all the powder concentrations tested), while for powder of *Rhodymenia pseudopalmata* such decrease was observed only at concentrations higher than 0.4 g l^{-1} (i.e., 19% for 0.8 g l^{-1} and 52%, for 1.6 g l^{-1}, LT50 = 18, Fig. 5). In all experiments (except for powder of *R. pseudopalmata* at concentration 0.4 g l^{-1}) vegetative cells were replaced by cysts: both thin and double-walled cysts were observed with an increase of percent abundances of the latter toward the end of the experiment.

3.4. Germination tests

Thin and double-walled cysts isolated at the end of the experiments (see above) were put in favorable conditions (fresh replete medium, 21 °C) to stimulate the germination. Among them, only thin-walled cysts germinated producing vegetative cells in a short time interval (3 h–2 d), while double-walled cysts did not germinated. The estimated percentage of germination of thin walled cysts was around 60%.

4. Discussion

The results obtained in this study highlighted negative interactions that occurred between the benthic dinoflagellate *Ostreopsis* cf. *ovata* and the macroalgae *Dictyota dichotoma* (brown alga), *Rhodymenia pseudopalmata* (red alga) and *Ulva rigida* (green alga): *D. dichotoma* and *U. rigida* showed an evident inhibitory effect, while such effect was not observed for *R. pseudopalmata* except that at high concentrations of dry macroalgal powder. The inhibitory effect of macroalgae toward microalgae has been investigated previously and could be interpreted as due to various factors including nutrient and light competition, pH changes and secondary metabolites (allelochemicals) production. Microalgae and macroalgae are known to have an antagonistic relationship in both natural and experimental aquatic ecosystems (Lee and Olsen, 1985; Fong et al., 1993). Firstly, they compete for the nutrients. Pedersen and Borum (1996) analyzed the nitrogen storage and nitrogen-dependent growth rates of microalgae and macroalgae and suggested that microalgae would be superior at high nutrient availability, while at low nutrient availability slow-growing macroalgae would be more successful. Nevertheless, it has been observed in field studies that microalgae are sometimes suppressed by macroalgae at high nutrient availability (Smith and

Table 1
Results of ANOVA and Tukey's test about the concentrations of nutrient levels in the co-cultures of *Ostreopsis* cf. *ovata* with fresh thalli. Mean concentration ($\mu mol\,l^{-1}$) ± standard error (SE) of dissolved inorganic nitrogen (DIN) and PO_4^{3-} in the entire experiment and in the control are reported.

	Dictyota dichotoma ($\mu mol\,l^{-1}$) Avg ± SE	*Ulva rigida* ($\mu mol\,l^{-1}$) Avg ± SE	*Rhodymenia pseudopalmata* ($\mu mol\,l^{-1}$) Avg ± SE	Control ($\mu mol\,l^{-1}$) Avg ± SE	*p*-level	Tukey test
DIN	299.23 ± 17.37	59.45 ± 11.65	82.80 ± 22.15	248.13 ± 12.83	***	Control > *Rhodymenia pseudopalmata*
					***	Control > *Ulva rigida*
					***	*Dictyota dichotoma* > *Rhodymenia pseudopalmata*
					***	*Dictyota dichotoma* > *Ulva rigida*
PO_4^{3-}	3.7522 ± 0.2191	2.16080 ± 0.0769	1.4193 ± 0.1424	4.838 ± 0.2495	**	Control > *Dictyota dichotoma*
					***	Control > *Rhodymenia pseudopalmata*
					***	Control > *Ulva rigida*
					***	*Dictyota dichotoma* > *Rhodymenia pseudopalmata*
					***	*Dictyota dichotoma* > *Ulva rigida*
					*	*Ulva rigida* > *Rhodymenia pseudopalmata*

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Horne, 1988; Sfriso et al., 1989; Sfriso and Pavoni, 1994). Although a high rate of nutrient uptake has been reported particularly for *Ulva* species (see e.g., Zertuche-González et al., 2009; Luo et al., 2012), we excluded the hypothesis that the inhibitory effect observed in the co-culture experiments was due to nutrient competition, as nutrient levels in both *D. dichotoma* and *U. rigida* co-cultures showed values comparable to those of the control and even higher than those in co-cultures of *R. pseudopalmata* where no inhibition was observed. Changing in pH in surrounding medium due to macroalgae growth is a common event, as high rates of photosynthesis may drawdown CO_2, increasing pH levels and making the environment unsuitable for microalgal growth (Goldman et al., 1982; Taraldsvik and Myklestad, 2000). In this regard, during our experiments the pH was maintained at values of 8, in order to prevent that the effect of an increased pH masking possible allelopathic interactions (Keating, 1977; Schmidt and Hansen, 2001; Granéli et al., 2008). Finally, macroalgae and microalgae strongly interact through release of allelopathic substances and these chemical interactions may suggest interesting opportunities to obtain algicidal products which may be used in the bloom control (Nan et al., 2004; Hu and Hong, 2008; Tang and Gobler, 2011). Although in this study chemical analyses were unfortunately not performed, we can hypothesize that such interactions likely occurred. Among the macroalgae tested, *D. dichotoma* inhibited the growth of *O.* cf. *ovata* in all tested conditions. In particular, the experiment with macroalgal powder and macroalgal filtrate medium suggests that *D. dichotoma* could contain/release, irrespective of the presence of microalgae, some molecules able to exert an inhibitory effect on *Ostreopsis* growth. Species belonging to the genus *Dictyota* are known to produce secondary metabolites, i.e., hundreds of terpenes (Cronin et al., 1997; Vallim et al., 2005), with ecological functions including defense against feeding by generalist marine herbivores (Paul et al., 2001; Paul and Ritson-Williams, 2008). In addition, brown algae are well-known for the production of other secondary metabolites, the phlorotannins, which are considered to be an important chemical defence against marine herbivores, as well as epiphytes (Jennings and Steinberg, 1997; Amsler and Fairhead, 2005; Iken et al., 2009) and have been shown to exert negative effects also on several red tide microalgae (Nagayama et al., 2003; Wang et al., 2007a).

Ulva rigida showed a moderate inhibitory effect on *Ostreopsis* cf. *ovata*, particularly as both fresh thalli and dry powder, while no inhibition occurred with *U. rigida* growth medium filtrate. This suggests that either *Ulva* does not release any compound in the medium in absence of stimulating factors such as microalgae could represent, or if allelochemicals are released they are rapidly degraded. Similar results have been obtained by other authors who hypothesized that the continuous release of small quantities of rapidly degradable allelochemicals from the fresh tissue of *Ulva* spp. was essential to inhibit the growth of several microalgae (Jin and Dong, 2003; Nan et al., 2004, 2008; Jin et al., 2005; Wang et al., 2007a). Tang and Gobler (2011) suggested that the polyunsaturated fatty acids produced by *Ulva* species (Alamsjah et al., 2005, 2008) probably act as allelochemicals, explaining the inhibitory effect of *Ulva* on several planktonic HAB species. Other authors (e.g., Wang et al., 2007b) observed that the culture filtrate of *Ulva* can exert contrasting species-specific allelopathic interactions on different bloom-forming dinoflagellates, i.e., both inhibitory (on *Prorocentrum donghaiense*, *Alexandrium tamarense* and *Scrippsiella trochoidea*) and stimulatory (on *Amphidinium carterae*) effects. Our results show a stimulatory effect of culture filtrates of both *U. rigida* and *Rhodymenia pseudopalmata* on *Ostreopsis* growth as well, suggesting that in absence of *O.* cf. *ovata* they could release some

Table 2
Results of ANOVA and Tukey's test about the mean cell abundances (cells ml^{-1}) ± standard error (SE) of *Ostreopsis* cf. *ovata* at the day 23 in the experiment with macroalga culture medium filtrate and in the control.

Dictyota dichotoma cells ml^{-1} Avg ± SE	*Ulva rigida* cells ml^{-1} Avg ± SE	*Rhodymenia pseudopalmata* cells ml^{-1} Avg ± SE	Control cells ml^{-1} Avg ± SE	*p*-level	Tukey test
7.3 ± 3.6	6009.9 ± 305.06	6283.1 ± 450.2	3823.4 ± 343.8	***	*Dictyota dichotoma* < *Rhodymenia pseudopalmata*
				***	*Dictyota dichotoma* < *Ulva rigida*
				***	*Dictyota dichotoma* < Control
				***	*Rhodymenia pseudopalmata* > Control
				***	*Ulva rigida* > Control

*** $p < 0.001$.

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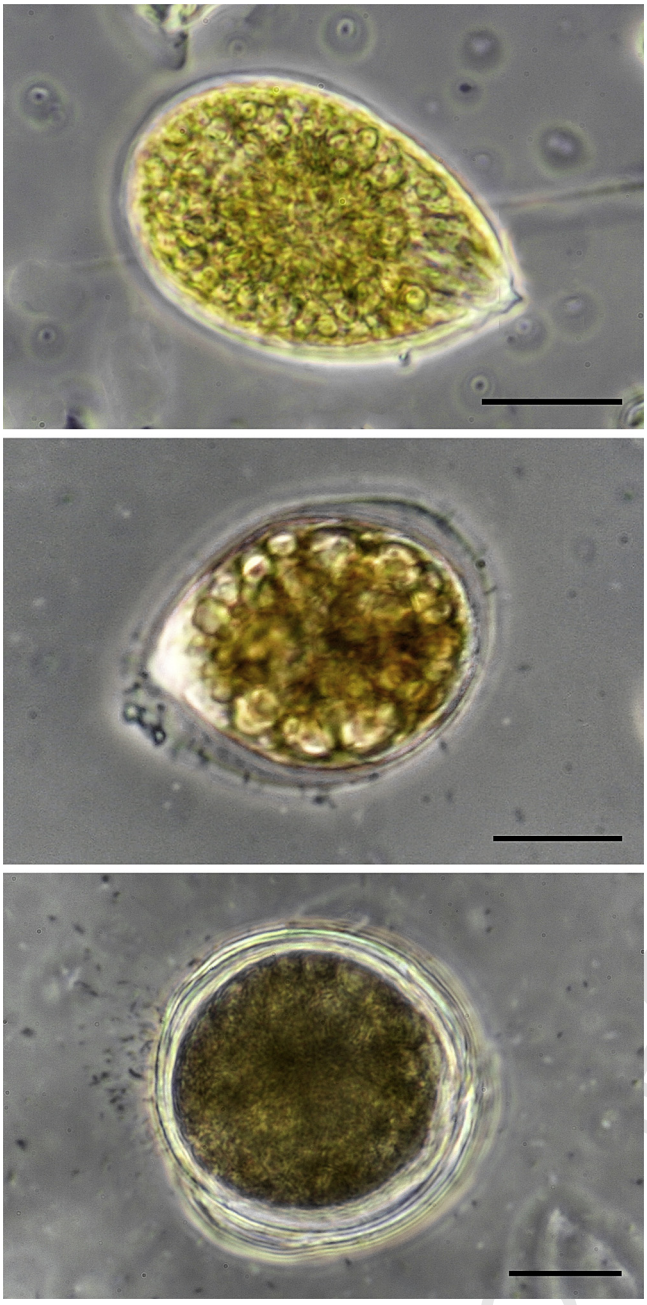

Fig. 4. Different morphotypes of *Ostreopsis* cf. *ovata* cells observed during the experiments: (A) vegetative cell, (B) thin- and (C) double-walled cysts. Scale bars = 20 μm.

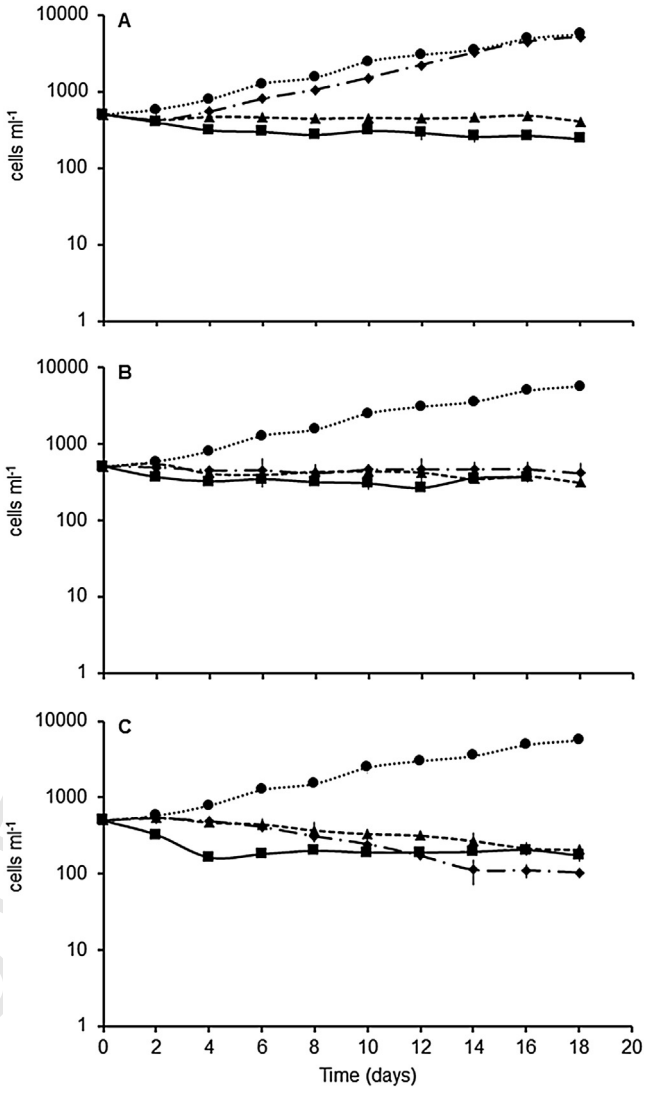

Fig. 5. Growth pattern of *Ostreopsis* cf. *ovata* cells growing with different concentrations (◆ 0.4, ▲ 0.8, ■ 1.6 g l^{-1} and ● control) of dry macroalgal powder of (A) *Rhodymenia pseudopalmata*, (B) *Ulva rigida* and (C) *Dictyota dichotoma*. Bars indicate standard deviation.

compounds which may operate as growth enhancers, also considering that the filtrate experiment has been carried out with high thallus concentrations (12 times higher than in co-culture experiment).

On the contrary, *Rhodymenia pseudopalmata* showed inhibitory effects only beyond a threshold level of thallus powder concentration. Red algae are reported as a source of secondary metabolites including terpenes, which exhibit a range of activities such as feeding inhibition for marine herbivores (Sakata et al., 1991; Kurata et al., 1998) and antialgal effect (König et al., 1999). Our results might suggest that *Rhodymenia* is not a strong producer of chemical-deterrents, unlike other red algal species: *Corallina pilulifera*, *Gracilaria lemaneiformis* and *Gracilaria tenuistipitata* displayed algicidal activity against several harmful algae (Jeong et al., 2000; Wang et al., 2007a,b; Ye and Zhang, 2013). The lower inhibitory effect observed in *Rhodymenia* in this study could partly explain results reported in field studies in both northern Adriatic Sea and North Aegean Sea, where the maximum abundances of

Table 3
Mean cell abundances (cells ml^{-1}) ± standard error (SE) of *Ostreopsis* cf. *ovata* at the day 18 and growth rate (day^{-1}) in the experiment with dry macroalgal powder at different concentrations (0.4, 0.8 and 1.6 g l^{-1}) and in the control.

Macroalga	Powder concentration g l^{-1}	AVG ± SE cells ml^{-1}	Growth rate day^{-1}
Dictyota dichotoma	0.4	103.7 ± 2.9	/
Dictyota dichotoma	0.8	206.0 ± 7.1	/
Dictyota dichotoma	1.6	172.8 ± 9.9	/
Ulva rigida	0.4	417.3 ± 43.8	/
Ulva rigida	0.8	313.2 ± 16.0	/
Ulva rigida	1.6	369.3 ± 10.2	/
Rhodymenia pseudopalmata	0.4	5184.3 ± 149.8	0.19
Rhodymenia pseudopalmata	0.8	407.1 ± 11.8	/
Rhodymenia pseudopalmata	1.6	241.6 ± 5.2	/
Control		5651.2 ± 152.0	0.25

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O. cf. *ovata* were generally recorded on Rhodophyceae (e.g., *Corallina* sp., *Ceramium* spp., *Hypnea musciformis*) rather than on other macroalgal groups (Aligizaki and Nikolaidis, 2006; Totti et al., 2010). So far, this has been explained considering that tridimensional articulate thalli morphotypes (as are the above species) could support higher abundances of epibiontic dinoflagellates than simple filamentous thalli (Vila et al., 2001; Parsons and Preskitt, 2007; Totti et al., 2010), but the results of this study suggest that other factors such as chemical interactions might affect the growth of epiphytic dinoflagellates.

Although the powder of *Ulva rigida*, *Dictyota dichotoma* and *Rhodymenia pseudopalmata* as well as the *D. dichotoma* culture filtrate strongly inhibited the *Ostreopsis* growth, in such conditions a complete algicidal effect was not observed, as the dinoflagellate population suddenly produced a lot of resting stages, and in the last days of the experiment the majority of the population was represented by cysts (thin and double-walled cysts), maintaining the total densities at a stable level and comparable to values of the initial inoculum throughout the entire experiment. Thin-walled and double-walled cysts have been previously observed in *O.* cf. *ovata* cultures and interpreted as short-term and resting cysts respectively; while thin-walled cysts have been demonstrated to germinate already at 21 °C, the double-walled ones germinated at 25 and not at 21 °C (Accoroni et al., 2014). The cyst formation in presence of macroalgae (both powder and filtrate conditions) indicates that the co-existence of macroalgae represented a stress for *Ostreopsis*, suggesting that some allelochemicals might be produced, although unfortunately in this study we did not made a chemical analysis. The cyst production allowed *Ostreopsis* populations to persist, contrarily to results reported by Nagayama et al. (2003) who found that phlorotannins of the brown macroalgae *Ecklonia kurome* killed the cells of *Karenia mikimotoi* and *Cochlodinium polykrikoides*, firstly causing a loss of motility in the cells, then making the cells become round, expand and burst, suggesting an interaction of phlorotannins with microalgal proteins disturbing the control of osmotic pressure.

In conclusion, our results highlighted that the investigated seaweed species exert negative effects toward the benthic dinoflagellate *Ostreopsis* cf. *ovata*, inhibiting growth and inducing cyst formation, suggesting that these inhibiting effects could be of allelopathic nature. In order to control and/or mitigate HABs, different physical approaches and chemical ways have been developed (Sugawara et al., 2003; Sun and Choi, 2004; Lee et al., 2008; Chen et al., 2009). Considering our results added to the substantial experimental evidence demonstrating the inhibitory activity of several macroalgae on the growth of HAB species, we think that the use of macroalgae in the prevention, control, and mitigation of HABs represents a potential option which could be taken in account in the coastal management.

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