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Allelopathic interactions between the HAB dinoflagellate Ostreopsis cf. ovata and macroalgae / Accoroni, Stefano; Percopo, Isabella; Cerino, Federica; Romagnoli, Tiziana; Pichierri, Salvatore; Cesira, Perrone; Totti, Cecilia Maria. - In: HARMFUL ALGAE. - ISSN 1568-9883. - STAMPA. - 49:(2015), pp. 147-155. [10.1016/j.hal.2015.08.007]

Availability:

This version is available at: 11566/227858 since: 2022-06-01T12:57:45Z

Publisher:

Published DOI:10.1016/j.hal.2015.08.007

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## Harmful Algae



journal homepage: www.elsevier.com/locate/hal

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

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### ARTICLE INFO

Article history: Received 15 April 2015 Received in revised form 31 August 2015 Accepted 31 August 2015

Keywords: Ostreopsis Macroalgae Allelopathy Harmful algae Growth inhibition Cysts

#### 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 coculture experiment, a complete algicidal effect was never observed partly because O. cf. ovata produced a large amount of resting stages, which permitted its survival.

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### 1. Introduction

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**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.,

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http://dx.doi.org/10.1016/j.hal.2015.08.007 1568-9883/© 2015 Elsevier B.V. All rights reserved. 2012; Ismael and Halim, 2012; Pfannkuchen et al., 2012; 15 Casabianca et al., 2014). In these areas, O. cf. ovata is well-known 16 for its toxin production, including both palytoxin-like compounds 17 (isobaric palytoxin and ovatoxin-a, b, c, d, e, f and g) and 18 19 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., 20 2015) that cause both mortality of benthic marine organisms 21 (Shears and Ross, 2009; Accoroni et al., 2011; Gorbi et al., 2012, 22 2013; Pagliara and Caroppo, 2012) and noxious effects on human 23 health (Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 24 2010: Del Favero et al., 2012). 25

Several studies have been conducted to assess the role of abiotic 26 factors (mainly hydrodynamics, water temperature and nutrients) 27 on the blooms dynamics (Chang et al., 2000; Vila et al., 2001; Monti 28 et al., 2007; Shears and Ross, 2009; Totti et al., 2010; Accoroni et al., 29

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30 2012a,b, 2015; Mabrouk et al., 2011; Mangialajo et al., 2011; Selina 31 et al., 2014), while biotic factors such as allelopathic interactions 32 between Ostreopsis and other organisms have only been partially 33 addressed. The allelopathic interactions have been well docu-34 mented among toxic dinoflagellates and co-occurring microalgae 35 under unfavorable environmental conditions (Fistarol et al., 2003, 36 2004; Granéli and Johansson, 2003; Granéli and Hansen, 2006; 37 Prince et al., 2008), and Monti and Cecchin (2012) showed that 38 O. cf. ovata had a weak allelopathic activity toward other benthic 39 dinoflagellates as well. However, to the best of our knowledge, 40 allelopathic interactions between Ostreopsis and macroalgae have 41 never been considered. O. cf. ovata commonly grows over all 42 benthic substrata (rocks, pebbles, seaweed thalli, mollusc shells, 43 etc.), and during periods of intense proliferation produces a 44 conspicuous brownish mat which is only loosely attached to the 45 substrata. Previous studies on the Conero Riviera, where O. cf. ovata 46 blooms reach abundances among the highest of the entire Mediterranean coasts (Mangialajo et al., 2011), highlighted that 47 48 Ostreopsis abundances on rocks were significantly higher than 49 those recorded on seaweeds suggesting that some allelopathic 50 interactions might occur between Ostreopsis and its macroalgal 51 hosts (Totti et al., 2010).

52 Algal-bloom control is an important issue for the protection of 53 the water environment due to the negative impacts on human 54 economy and health, especially when the involved bloom-forming species are toxic. Moreover, the development of environment-55 56 friendly and cost-effective strategies for controlling algal blooms, 57 such as using the allelopathy of aquatic macrophytes has gained 58 great interest and has been suggested by several authors (Jeong 59 et al., 2000; Nan et al., 2004; Jin et al., 2005; Wang et al., 2007a,b; 60 Hu and Hong, 2008; Tang and Gobler, 2011). The interactions 61 between microalgae and macroalgae have been investigated 62 between phytoplankton species that form blooms and a number 63 of both freshwater (Gross, 2003; Hu and Hong, 2008) and seawater 64 macrophytes (Gross, 2003; Jin and Dong, 2003; Nan et al., 2004, 65 2008; Jin et al., 2005; Wang et al., 2007a,b; Ye and Zhang, 2013) but 66 no information is available about macroalgae and benthic 67 dinoflagellate interactions.

68 In this study, we investigated the interactions between 69 Ostreopsis cf. ovata and three macroalgal species under laboratory 70 conditions. The macroalgae were chosen among the most common 71 species on the Conero Riviera during the bloom period of 72 Ostreopsis: Dictyota dichotoma (Hudson) J.V. Lamouroux (brown 73 alga), Rhodymenia pseudopalmata (J.V. Lamouroux) P.C. Silva (red 74 alga) and Ulva rigida C. Agardh (green alga). Three different 75 experiments were carried out: O. cf. ovata was grown (i) together 76 with fresh macroalgal tissues, (ii) in filtered culture media in which 77 macroalgae were previously grown, and (iii) in media with 78 addition of dry macroalgal powder at different concentrations.

## 79 2. Materials and methods

## 80 2.1. Ostreopsis cf. ovata cultures

A strain of Ostreopsis cf. ovata was isolated by the capillary 81 82 pipette method (Hoshaw and Rosowski, 1973) from seawater 83 samples collected from the bloom that occurred on the Conero 84 Riviera (N Adriatic Sea) in summer 2007 (strain OoAPn0807/E). 85 After initial growth in microplates, cells were cultured at 86  $21 \pm 0.1$  °C under a 12:12 h L:D cycle and an irradiance of 90– 100  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, in modified f/4 medium prepared by adding 87 88 macronutrients at a f/4 medium concentration without silica 89 (Guillard and Ryther, 1962) and selenium to filtered and autoclaved 90 natural seawater (salinity 35). Trace metals, iron, vitamins (H, B1 and 91 B12) and HEPES pH 7.1 were added at levels corresponding to f/2 92 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 before95inoculation in the following experiments. All the experiments were96carried out in three replicates.97

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## 2.2. Macroalgae sampling and pre-treatment

99 Thalli of three macroalgal species, i.e., Dictyota dichotoma, Rhodymenia pseudopalmata and Ulva rigida, were collected on the 100 Conero Riviera in summer 2009 and treated to remove the 101 epiphytes from their surface as follows: they were washed 102 carefully in filtered sea water (FSW) containing 1% of surfactant, 103 rinsed in FSW and then observed at a stereo-microscope in order to 104 mechanically remove the epiphytes with scalpels and tweezers; 105 afterwards, a 3-min dip in tap water followed by a washing with 106 chloramphenicol (50 ppm in FSW) led to the complete removal of 107 residual epiphytic cells and bacteria respectively. After rinsing in 108 FSW, macroalgal thalli were cut into fragments of approximately 109  $9\,\text{cm}^2$  and acclimated for one week in FSW (containing  $6\,\text{mg}\,l^{-1}$ 110 germanium dioxide). 111

## 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 113 tested seaweed in 500 ml of medium containing germanium 114 dioxide. For each species, 3 flasks were inoculated with 115 100 cells ml<sup>-1</sup> of O. cf. ovata, and 3 flasks containing 100 cells ml<sup>-1</sup> 116 1 of Ostreopsis cf. ovata without macroalgal thalli were used as 117 control. The flasks were incubated for 20 days in a culture chamber 118 at the conditions previously described. Every 2 days, 3 aliquots 119 (2 ml) were taken from each flask (after gentle shaking of each 120 thallus with a pair of tweezers and a homogenization of the 121 medium) and preserved with Lugol's solution in the dark to assess 122 the cell densities, and 1 aliquot (4 ml) was filtered (GF/F Whatman, 123 diameter 25 mm, nominal pore size 0.7 µm) and stored in 124 polyethylene bottles at -22 °C for nutrient analysis. Every 7 days, 125 pH was checked and adjusted to maintain it to value of 8 until the 126 end of the experiment. 127

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

For each macroalgal species, 24 g of thallus segments pre-130 treated as above were maintained in 1 l FSW for one week. 131 Afterwards, macroalgal thalli were removed and the culture 132 133 medium was filtered (0.22  $\mu$ m pore size) and used to prepare the medium for the experiment: the pH was adjusted to 8 and the 134 nutrients were added to obtain a modified f/4 medium as described 135 previously. Culture flasks containing 250 ml of medium were 136 inoculated with Ostreopsis cf. ovata cells to obtain a final 137 concentration of 500 cells  $ml^{-1}$ . As a control, 500 cells  $ml^{-1}$  of 0. 138 cf. ovata were inoculated in fresh modified f/4 medium. 139 Subsamples (2 ml) were sampled every 2-3 days for 23 days 140 and fixed with Lugol's solution. 141

## 2.5. Cultures with dry powder of macroalgae

Fresh thalli of each macroalga were dried at room temperature 143 and pulverized using a mortar and a pestle. Different amounts of 144 dry powder (0.4, 0.8,  $1.6 \text{ g l}^{-1}$ ) were added to flasks containing 145 Ostreopsis cultures (500 cells ml<sup>-1</sup>) in 300 ml modified f/4 medi-146 um. Microalgal cultures without addition of dry macroalgal 147 powder were used as controls. Cultures were maintained for 148 18 days during which subsamples (2 ml) were taken every 2 days 149 150 and fixed with Lugol's solution.

Please cite this article in press as: Accoroni, S., et al., Allelopathic interactions between the HAB dinoflagellate *Ostreopsis* cf. *ovata* and macroalgae. Harmful Algae (2015), http://dx.doi.org/10.1016/j.hal.2015.08.007

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## 151 2.6. Microscope counting

Densities of Ostreopsis cf. ovata were estimated after homoge-152 153 nization using either Utermöhl (Edler and Elbrachter, 2010) or 154 Sedgewick-Rafter (Guillard, 1978) chambers, through an inverted 155 light microscope (Zeiss Axiovert 135) and a light microscope (Zeiss 156 Axioskop) respectively, both equipped with phase contrast, at 157  $200 \times$  magnification. Counting was performed on 10–30 random fields. 1-2 transects, or the whole chamber, in order to count a 158 159 representative cell number. Total densities (vegetative cells and 160 cysts) were expressed as cells  $ml^{-1}$ .

161 2.7. Cyst isolation for germination tests

Cysts of Ostreopsis cf. ovata were isolated at the end of the 162 163 experiments and used to perform germination tests. Single cysts were isolated by the capillary pipette method according to the 164 classical methodology (Hoshaw and Rosowski, 1973) and put 165 166 separately in 24-well culture plates filled with fresh growth 167 medium. Plates were placed in the culture chamber in the 168 conditions previously described and were observed every day for 8 weeks. 169

### 170 2.8. Nutrient analysis

171Nutrient variation during the co-cultures of Ostreopsis cf. ovata172with fresh thalli was monitored throughout the experiment.173Analyses of  $NO_3^-$ ,  $NO_2^-$ ,  $NH_4^+$ , and  $PO_4^{3-}$  were conducted174following the colorimetric method by Strickland and Parsons175(1968), adapted for an Autoanalyzer QuAAtro Axflow. Limits of176detection were 0.02  $\mu$ mol l<sup>-1</sup> for NO\_3<sup>-</sup> and NO<sub>2</sub>, and 0.03  $\mu$ mol l<sup>-1</sup>177for PO<sub>4</sub><sup>3-</sup>.

## 178 2.9. Calculations and statistical analyses

179 The microalgal growth was expressed as specific growth rate 180  $(\mu)$  using the equation 181  $(\ln N_1 - \ln N_2)$ 

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

182where  $N_1$  is the final density,  $N_0$  is the initial density, and T is the184number of days between two measurements.

185 Differences in final cell densities of Ostreopsis cf. ovata in each experiment (i.e., co-cultures, macroalgal filtrate and dry macro-186 187 algal powder) between the three different macroalgal species were assessed through a one-way analysis of variance (ANOVA). 188 189 Moreover, in the co-culture experiment, differences in nutrient 190 concentrations between the three different macroalgae were 191 assessed through an ANOVA test as well. When significant 192 differences for the main effect were observed (p < 0.05), a Tukev's 193 pairwise comparison test was also performed. The statistical analyses were conducted using Statistica (StatSoft Inc., Tulsa, OK, 194 195 USA) software.

## 196 **3. Results**

## 197 3.1. Co-cultures of Ostreopsis cf. ovata with fresh macroalgal thalli

198 The first general observation regarding the pattern of coloniza-199 tion in each flask is that Ostreopsis cf. ovata cells colonized the 200 bottom of the flask rather than macroalgal thalli. Our results 201 showed that O. cf. ovata growth was markedly affected only by the 202 presence of two macroalgal species, i.e., Dictyota dichotoma and 203 Ulva rigida: at the end of the experiment (day 20) Ostreopsis cell 204 abundance in co-culture with D. dichotoma and U. rigida was 205 reduced by 100% and 94% respectively, compared to the day of the inoculum (Fig. 1). The density of O. cf. ovata was significantly lower 206 with *D. dichotoma*  $(0.08 \pm 0.06 \text{ cells ml}^{-1})$  and *U. rigida*  $(31.6 \pm 4.6)$ 207 cells ml<sup>-1</sup>) than with *Rhodymenia* pseudopalmata ( $823.2 \pm 59.8$ 208 cells ml<sup>-1</sup>, p < 0.001) and in the control (735.4 ± 125.1 cells ml<sup>-1</sup>, 209 p < 0.001). The average lethal times (LT50), which represents the time 210 at which 50% of the Ostreopsis cells were dead, in co-cultures with U. 211 rigida and D. dichotoma were 12 and 8 days, respectively. On the 212 contrary, the growth of O. cf. ovata was not inhibited by the presence 213 of *R. pseudopalmata*, as no significant difference was observed 214 between the maximum yield of O. cf. ovata in R. pseudopalmata co-215 culture and the control. The maximum yield was reached at the day 216 18 with  $1535\pm538\ cells\ ml^{-1}$  in the control and  $1016\pm210$ 217 cells ml<sup>-1</sup> in the *R. pseudopalmata* co-culture. The growth rate of 218 Ostreopsis in R. pseudopalmata co-culture was comparable with that of 219 the control (0.17 and 0.19  $day^{-1}$  respectively). 220

Regarding the nutrient concentrations during the entire 221 experiment (Fig. 2), we considered the phosphate  $(PO_4^{3-})$  and 222 the dissolved inorganic nitrogen (DIN) as a sum of nitrates, nitrites 223 and ammonia. A marked decrease of DIN throughout the 224 experiment was observed in *Rhodymenia pseudopalmata* and 225 *Ulva rigida* co-cultures, while  $PO_4^{3-}$  concentration did not show 226 appreciable variation. 227

Comparing nutrient content in different co-cultures, significantly higher values of both DIN and  $PO_4^{3-}$  were observed in 229 *Dictyota dichotoma* co-culture than in *Rhodymenia pseudopalmata* 230 and *Ulva rigida* (p < 0.001, Table 1). Comparing *U. rigida* and *R*. 231 *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. 234

3.2. Cultures of Ostreopsis cf. ovata in macroalgal culture medium 235 filtrate 236

Among the macroalgae tested, only Dictyota dichotoma culture 237 filtrate exhibited inhibiting effects on Ostreopsis cf. ovata growth: 238 Ostreopsis cell numbers immediately decreased (LT50 = 2) (Fig. 3). 239 At the end of the experiment (day 23), the cell density of O. cf. ovata 240 was significantly lower in the culture filtrate of *D. dichotoma* than 241 in those of Rhodymenia pseudopalmata (p < 0.001), Ulva rigida 242 (p < 0.001) and control (p < 0.001, Table 2). It is noteworthy that 243 since the second sampling day in D. dichotoma treatment we 244 observed the appearance of cysts (both thin and double-walled 245 cysts, Fig. 4B and C). The percent abundances of double-walled 246 cysts increased toward the end of the experiment, becoming the 247 predominant morphotype observed in the final day of the 248 249 experiment.

On the contrary, in filtrates of *Ulva rigida* and *Rhodymenia* 250 pseudopalmata the Ostreopsis maximum yield was significantly 251



**Fig. 1.** Growth pattern of *Ostreopsis* cf. *ovata* cells growing in co-culture with fresh thalli of  $(\blacksquare)$  *Dictyota dichotoma*.  $(\spadesuit)$  *Rhodymenia pseudopalmata*.  $(\blacktriangle)$  *Ulva rigida* and  $(\spadesuit)$  control. Bars indicate standard deviation.

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**Fig. 2.** Temporal variability of nutrient concentration ( $\mu$ mol l<sup>-1</sup>) 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<sub>4</sub><sup>3-</sup>, right *y*-axis).

higher than in the control (p < 0.001, Table 2) and the growth rates, 0.28 and 0.38 day<sup>-1</sup> respectively, were higher than for the control (0.25 day<sup>-1</sup>). 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<sup>-1</sup>).

## 257 3.3. Cultures with dry powder of macroalgae

258 The results of this experiment showed that all the macroalgal 259 species exhibited an inhibitory effect on cultures of Ostreopsis cf. 260 ovata (Fig. 5) as all the powder concentrations tested led to 261 significantly lower values of cell densities at the end of the 262 experiment compared to the control (p < 0.001, Table 3), except for 263 the powder of Rhodymenia pseudopalmata at the lowest concentration (0.4 g  $l^{-1}$ ) which allowed the growth of O. cf. ovata (growth 264 rate =  $0.19 \text{ day}^{-1}$ ). 265



**Fig. 3.** Growth pattern of *Ostreopsis* cf. *ovata* cells growing with macroalga culture medium filtrates of ( $\blacksquare$ ) *Dictyota dichotoma*, ( $\blacklozenge$ ) *Rhodymenia pseudopalmata* and ( $\blacktriangle$ ) *Ulva rigida* and ( $\blacklozenge$ ) control. Bars indicate standard deviation.

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<sup>-1</sup> 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<sup>-1</sup> (i.e., 19% for 0.8 g l<sup>-1</sup> and 52%, for 1.6 g l<sup>-1</sup>, LT50 = 18, Fig. 5). In all experiments (except for powder of *R. pseudopalmata* at concentration 0.4 g l<sup>-1</sup>) 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.

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## 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%. 281

## 4. Discussion

The results obtained in this study highlighted negative 289 interactions that occurred between the benthic dinoflagellate 290 Ostreopsis cf. ovata and the macroalgae Dictyota dichotoma (brown 291 alga), Rhodymenia pseudopalmata (red alga) and Ulva rigida (green 292 alga): D. dichotoma and U. rigida showed an evident inhibitory 293 effect, while such effect was not observed for *R. pseudopalmata* 294 except that at high concentrations of dry macroalgal powder. The 295 inhibitory effect of macroalgae toward microalgae has been 296 investigated previously and could be interpreted as due to various 297 factors including nutrient and light competition, pH changes and 298 secondary metabolites (allelochemicals) production. Microalgae 299 and macroalgae are known to have an antagonistic relationship in 300 both natural and experimental aquatic ecosystems (Lee and Olsen, 301 1985; Fong et al., 1993). Firstly, they compete for the nutrients. 302 Pedersen and Borum (1996) analyzed the nitrogen storage and 303 nitrogen-dependent growth rates of microalgae and macroalgae 304 305 and suggested that microalgae would be superior at high nutrient availability, while at low nutrient availability slow-growing 306 macroalgae would be more successful. Nevertheless, it has been 307 308 observed in field studies that microalgae are sometimes sup-309 pressed by macroalgae at high nutrient availability (Smith and

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#### 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  $(\mu mol l^{-1}) \pm$  standard error (SE) of dissolved inorganic nitrogen (DIN) and PO<sub>4</sub><sup>3-</sup> in the entire experiment and in the control are reported.

	Dictyota dichotoma (µmol l <sup>-1</sup> ) Avg ± SE	Ulva rigida (µmol l <sup>-1</sup> ) Avg ± SE	Rhodymenia pseudopalmata (µmol1 <sup>-1</sup> ) Avg±SE	Control (µmol l <sup>-1</sup> ) Avg ± SE	p-level	Tukey test
DIN	$299.23 \pm 17.37$	$59.45 \pm 11.65$	82.80±22.15	$248.13 \pm 12.83$	•••	Control > Rhodymenia pseudopalmata Control > Ulva rigida Dictyota dichotoma > Rhodymenia pseudopalmata Dictyota dichotoma > Ulva rigida
PO4 <sup>3-</sup>	$3.7522 \pm 0.2191$	$2.16080 \pm 0.0769$	$1.4193 \pm 0.1424$	$4.838 \pm 0.2495$	··· ··· ···	Control > Dictyota dichotoma Control > Rhodymenia pseudopalmata Control > Ulva rigida Dictyota dichotoma > Rhodymenia pseudopalmata Dictyota dichotoma > Ulva rigida Ulva rigida > Rhodymenia pseudopalmata

*p* < 0.01.

p < 0.001.

310 Horne, 1988; Sfriso et al., 1989; Sfriso and Pavoni, 1994). Although a high rate of nutrient uptake has been reported particularly for 311 312 Ulva species (see e.g., Zertuche-González et al., 2009; Luo et al., 313 2012), we excluded the hypothesis that the inhibitory effect 314 observed in the co-culture experiments was due to nutrient 315 competition, as nutrient levels in both D. dichotoma and U. rigida 316 co-cultures showed values comparable to those of the control and 317 even higher than those in co-cultures of *R. pseudopalmata* where no 318 inhibition was observed. Changing in pH in surrounding medium 319 due to macroalgae growth is a common event, as high rates of photosynthesis may drawdown CO<sub>2</sub>, increasing pH levels and 320 making the environment unsuitable for microalgal growth (Gold-321 322 man et al., 1982; Taraldsvik and Myklestad, 2000). In this regard, 323 during our experiments the pH was maintained at values of 8, in 324 order to prevent that the effect of an increased pH masking 325 possible allelopathic interactions (Keating, 1977; Schmidt and 326 Hansen, 2001; Granéli et al., 2008). Finally, macroalgae and 327 microalgae strongly interact through release of allelopathic 328 substances and these chemical interactions may suggest interest-329 ing opportunities to obtain algicidal products which may be used 330 in the bloom control (Nan et al., 2004; Hu and Hong, 2008; Tang and Gobler, 2011). Although in this study chemical analyses were 331 332 unfortunately not performed, we can hypothesize that such interactions likely occurred. Among the macroalgae tested, D. 333 334 dichotoma inhibited the growth of O. cf. ovata in all tested conditions. In particular, the experiment with macroalgal powder 335 336 and macroalgal filtrate medium suggests that D. dichotoma could 337 contain/release, irrespective of the presence of microalgae, some 338 molecules able to exert an inhibitory effect on Ostreopsis growth. 339 Species belonging to the genus Dictyota are known to produce 340 secondary metabolites, i.e., hundreds of terpenes (Cronin et al., 1997; Vallim et al., 2005), with ecological functions including 341

defense against feeding by generalist marine herbivores (Paul et al., 342 2001; Paul and Ritson-Williams, 2008). In addition, brown algae 343 are well-known for the production of other secondary metabolites, 344 the phlorotannins, which are considered to be an important 345 chemical defence against marine herbivores, as well as epiphytes 346 (Jennings and Steinberg, 1997; Amsler and Fairhead, 2005; Iken 347 et al., 2009) and have been shown to exert negative effects also on 348 several red tide microalgae (Nagayama et al., 2003; Wang et al., 349 2007a). 350

Ulva rigida showed a moderate inhibitory effect on Ostreopsis cf. 351 ovata, particularly as both fresh thalli and dry powder, while no 352 inhibition occurred with U. rigida growth medium filtrate. This 353 suggests that either Ulva does not release any compound in the 354 medium in absence of stimulating factors such as microalgae could 355 represent, or if allelochemicals are released they are rapidly 356 degraded. Similar results have been obtained by other authors who 357 hypothesized that the continuous release of small quantities of 358 rapidly degradable allelochemicals from the fresh tissue of Ulva 359 spp. was essential to inhibit the growth of several microalgae (Jin 360 and Dong, 2003; Nan et al., 2004, 2008; Jin et al., 2005; Wang et al., 361 2007a). Tang and Gobler (2011) suggested that the polyunsaturat-362 ed fatty acids produced by Ulva species (Alamsjah et al., 2005, 363 2008) probably act as allelochemicals, explaining the inhibitory 364 effect of Ulva on several planktonic HAB species. Other authors 365 (e.g., Wang et al., 2007b) observed that the culture filtrate of Ulva 366 can exert contrasting species-specific allelopathic interactions on 367 different bloom-forming dinoflagellates, i.e., both inhibitory (on 368 Prorocentrum donghaiense, Alexandrium tamarense and Scrippsiella 369 trochoidea) and stimulatory (on Amphidinium carterae) effects. Our 370 results show a stimulatory effect of culture filtrates of both U. rigida 371 and Rhodymenia pseudopalmata on Ostreopsis growth as well, 372 suggesting that in absence of O. cf. ovata they could release some 373

#### Table 2

Results of ANOVA and Tukey's test about the mean cell abundances (cells ml<sup>-1</sup>)± 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 $\pm$ SE	Ulva rigida cells ml $^{-1}$ Avg $\pm$ SE	Rhodymenia pseudopalmata cells ml <sup>-1</sup> Avg±SE	Control cells $ml^{-1}$ Avg $\pm$ SE	p-level	Tukey test
7.3 ± 3.6	$6009.9 \pm 305.06$	$6283.1 \pm 450.2$	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 =  $20 \ \mu$ m.

374 compounds which may operate as growth enhancers, also
375 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 378 379 effects only beyond a threshold level of thallus powder concentra-380 tion. Red algae are reported as a source of secondary metabolites 381 including terpenes, which exhibit a range of activities such as 382 feeding inhibition for marine herbivores (Sakata et al., 1991; 383 Kurata et al., 1998) and antialgal effect (König et al., 1999). Our 384 results might suggest that Rhodymenia is not a strong producer of 385 chemical-deterrents, unlike other red algal species: Corallina 386 pilulifera, Gracilaria lemaneiformis and Gracilaria tenuistipitata 387 displayed algicidal activity against several harmful algae (Jeong



**Fig. 5.** Growth pattern of *Ostreopsis* cf. *ovata* cells growing with different concentrations ( $\blacklozenge$  0.4,  $\blacktriangle$  0.8,  $\blacksquare$  1.6 g l<sup>-1</sup> and  $\blacklozenge$  control) of dry macroalgal powder of (A) *Rhodymenia pseudopalmata*, (B) *Ulva rigida* and (C) *Dictyota dichotoma*. Bars indicate standard deviation.

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 388

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### Table 3

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

Macroalga	Powder concentration	$AVG\pm SE$	Growth rate
	$g l^{-1}$	cells ml <sup>-1</sup>	day <sup>-1</sup>
Dictyota dichotoma	0.4	$103.7\pm2.9$	1
Dictyota dichotoma	0.8	$206.0\pm7.1$	/
Dictyota dichotoma	1.6	$172.8\pm9.9$	/
Ulva rigida	0.4	$417.3\pm43.8$	/
Ulva rigida	0.8	$313.2\pm16.0$	1
Ulva rigida	1.6	$\textbf{369.3} \pm \textbf{10.2}$	1
Rhodymenia pseudopalmata	0.4	$5184.3 \pm 149.8$	0.19
Rhodymenia pseudopalmata	0.8	$407.1 \pm 11.8$	/
Rhodymenia pseudopalmata	1.6	$241.6\pm5.2$	1
Control		$5651.2 \pm 152.0$	0.25

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392 O. cf. ovata were generally recorded on Rhodophyceae (e.g., 393 Corallina sp., Ceramium spp., Hypnea musciformis) rather than on 394 other macroalgal groups (Aligizaki and Nikolaidis, 2006; Totti et al., 395 2010). So far, this has been explained considering that tridimen-396 sional articulate thalli morphotypes (as are the above species) 397 could support higher abundances of epibiontic dinoflagellates than 398 simple filamentous thalli (Vila et al., 2001; Parsons and Preskitt, 399 2007: Totti et al., 2010), but the results of this study suggest that 400 other factors such as chemical interactions might affect the growth 401 of epiphytic dinoflagellates.

402 Although the powder of Ulva rigida, Dictyota dichotoma and 403 Rhodymenia pseudopalmata as well as the D. dichotoma culture 404 filtrate strongly inhibited the Ostreopsis growth, in such condi-405 tions a complete algicidal effect was not observed, as the 406 dinoflagellate population suddenly produced a lot of resting 407 stages, and in the last days of the experiment the majority of the 408 population was represented by cysts (thin and double-walled 409 cysts), maintaining the total densities at a stable level and 410 comparable to values of the initial inoculum throughout the entire 411 experiment. Thin-walled and double-walled cysts have been 412 previously observed in O. cf. ovata cultures and interpreted as 413 short-term and resting cysts respectively; while thin-walled cysts 414 have been demonstrated to germinate already at 21 °C, the 415 double-walled ones germinated at 25 and not at 21 °C (Accoroni et al., 2014). The cyst formation in presence of macroalgae (both 416 417 powder and filtrate conditions) indicates that the co-existence of 418 macroalgae represented a stress for Ostreopsis, suggesting that 419 some allelochemicals might be produced, although unfortunately 420 in this study we did not made a chemical analysis. The cyst 421 production allowed Ostreopsis populations to persist, contrarily to 422 results reported by Nagayama et al. (2003) who found that 423 phlorotannins of the brown macroalgae Ecklonia kurome killed the 424 cells of Karenia mikimotoi and Cochlodinium polykrikoides, firstly 425 causing a loss of motility in the cells, then making the cells become 426 round, expand and burst, suggesting an interaction of phlor-427 otannins with microalgal proteins disturbing the control of 428 osmotic pressure.

In conclusion, our results highlighted that the investigated 429 430 seaweed species exert negative effects toward the benthic 431 dinoflagellate Ostreopsis cf. ovata, inhibiting growth and induc-432 ing cyst formation, suggesting that these inhibiting effects could be of allelopathic nature. In order to control and/or mitigate 433 434 HABs, different physical approaches and chemical ways have 435 been developed (Sugawara et al., 2003; Sun and Choi, 2004; Lee 436 et al., 2008; Chen et al., 2009). Considering our results added to 437 the substantial experimental evidence demonstrating the 438 inhibitory activity of several macroalgae on the growth of 439 HAB species, we think that the use of macroalgae in the prevention, control, and mitigation of HABs represents a 440 441 potential option which could be taken in account in the coastal 442 management.

#### 443 Acknowledgements

This research was partially funded by ISPRA - Italian Ministry of 444 03 445 the Environment. The authors wish to gratefully thank Rossella 446 Pistocchi and Franca Guerrini for their useful indications about the experimental design and Mauro Marini for the nutrient analysis. A 447 special thanks to Fabio Rindi for the English revision.[SS] 448

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Please cite this article in press as: Accoroni, S., et al., Allelopathic interactions between the HAB dinoflagellate Ostreopsis cf. ovata and macroalgae. Harmful Algae (2015), http://dx.doi.org/10.1016/j.hal.2015.08.007

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