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Highlights

A conceptual model of annual *Ostreopsis* cf. *ovata* blooms in the northern Adriatic Sea based on the synergic effects of hydrodynamics, temperature, and the N:P ratio of water column nutrients

Stefano Accoroni*, Patricia M. Glibert, Salvatore Pichierri, Tiziana Romagnoli, Mauro Marini, Cecilia Totti

- O. cf. ovata bloom is triggered by a combination of optimal temperature and N:P ratio.
- Synergic effects of hydrodynamics, temperature and nutrients affect the bloom onset.
- \bullet Water temperature threshold (25 °C) plays a key role on cyst germination.
- N:P ratio around Redfield value is a necessary condition to allow cell proliferation.
- \bullet After the onset, bloom development can be sustained at temperature values below 20 °C.

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A conceptual model of annual *Ostreopsis* cf. *ovata* blooms in the northern Adriatic Sea based on the synergic effects of hydrodynamics, temperature, and the N:P ratio of water column nutrients

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ABSTRACT

The ecology of Ostreopsis cf. ovata blooms in the Conero Riviera (N Adriatic Sea) was investigated based on sampling carried out from 2007 to 2012, in order to assess the role of environmental factors associated with blooms. Generally, the temporal trend of blooms showed the first cell appearance at the end of July/early August, the maximum abundances in late-summer at end of September/early October reaching up to 10^6 cells g⁻¹ fw on macrophyte samples, and the decline of the blooms at end October/ early November. Calm conditions appeared to be a prerequisite for blooms. When suitable hydrodynamic conditions exist, O. cf. ovata blooms appear to be triggered by a combination of optimal temperature and available nutrients. A water temperature threshold of 25 °C plays a key role on the germination of O. cf. ovata cysts and therefore on the bloom onset, and an N:P ratio around Redfield value is a necessary condition to allow cell proliferation. The synergy of higher temperatures and optimal N:P ratios resulted in a higher net growth rate of O. cf. ovata cells. After the onset, blooms can be maintained at temperature values even below 20 °C and at N:P ratios that are in excess of the Redfield ratio. Once the bloom is started it may be maintained not necessarily through high growth rates, but likely through other physiological mechanisms. Bloom decline occurred when temperatures dropped below 18 °C. The net effect of the synergy between local hydrodynamic conditions, temperature, and N and P availability may help to explain why blooms in the northern Adriatic Sea occur differently from those in other Mediterranean regions.

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1. Introduction

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Q4 Toxic benthic dinoflagellates belonging to the genus *Ostreopsis* are common components of tropical and subtropical marine environments and their presence has increased greatly in temperate waters in recent years (Rhodes, 2011; Parsons et al., 2012). In the last decade, massive *Ostreopsis* blooms, mostly *O*. cf. *ovata* (Battocchi et al., 2010; Perini et al., 2011), have occurred regularly during summer along Mediterranean Sea coasts (Vila et al., 2001; Turki, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011; Amzil

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et al., 2012; Illoul et al., 2012; Ismael and Halim, 2012; Pfannkuchen 20 et al., 2012). In these areas, blooms have been associated with both 21 noxious effects on human health (Gallitelli et al., 2005; Kermarec 22 et al., 2008; Tichadou et al., 2010; Del Favero et al., 2012) and mass 23 mortalities of benthic marine invertebrates and/or macroalgae 24 (Shears and Ross, 2009; Accoroni et al., 2011; Gorbi et al., 2012, 25 2013; Pagliara and Caroppo, 2012). Mortalities have been attributed 26 to the production of palytoxin-like compounds that include putative 27 palytoxin and ovatoxins-a, b, c, d, e and f (Ciminiello et al., 2011, 28 29 2012; Uchida et al., 2013), and mascarenotoxins-a and c (Rossi et al., 2010; Scalco et al., 2012). Furthermore, palytoxin-like compounds 30 produced by Ostreopsis spp. have been detected in tissues of both 31 filter-feeding bivalve molluscs and herbivorous echinoderms in 32 33 some Mediterranean areas (Aligizaki et al., 2008, 2011; Amzil et al., 2012; Brissard et al., 2014) constituting a further possible threat for 34 human health associated with seafood consumption. **Q5**35

36 Several environmental factors, including hydrodynamics, 37 water temperature, and nutrient concentrations may affect 38 bloom dynamics, growth rate, cell size and toxin production of Ostreopsis cf. ovata, as previously shown in both field and 39 40 experimental conditions (Aligizaki and Nikolaidis, 2006; Guerrini 41 et al., 2010; Granéli et al., 2011; Accoroni et al., 2012b; Pezzolesi 42 et al., 2012, 2014; Vanucci et al., 2012; Vidyarathna and Granéli, 43 2013). Several studies have shown hydrodynamics to be one of 44 the main factors affecting Ostreopsis blooms, highlighting that higher abundances are found in sheltered sites compared with 45 46 exposed ones (Chang et al., 2000; Shears and Ross, 2009; Totti 47 et al., 2010; Mabrouk et al., 2011; Selina et al., 2014). 48 Hydrodynamics may also have an important effect on the bloom 49 temporal variability as stormy events can lead a sudden decrease 50 of cell abundances on the benthic substrata (Totti et al., 2010; 51 Accoroni et al., 2011).

52 Several authors have proposed that temperature may represent 53 a key factor for bloom development, as Ostreopsis spp. need 54 relatively high temperatures to proliferate, and they have 55 suggested that regional warming may have influenced Ostreopsis 56 expansion in temperate areas such as the Mediterranean Sea 57 (Hallegraeff, 2010; Granéli et al., 2011). However, it is also 58 recognized that high temperatures may not be a consistent factor 59 for blooms throughout the Mediterranean area (Mangialajo et al., 60 2011); for example, in the northern Adriatic Sea, the bloom peak 61 occurs in the late summer when temperatures are decreasing 62 rather than when they are at their seasonal maximum (Accoroni 63 et al., 2012a). Nevertheless, a study on the life cycle of O. cf. ovata 64 conducted with northern Adriatic strains highlighted that temper-65 ature represents a key factor for bloom onset because it affects cyst 66 germination (Accoroni et al., 2014).

67 With regard to the role of nutrients, recent studies have 68 provided increasing evidence of a link between the nutrient 69 enrichment of coastal waters (anthropogenic eutrophication) 70 and harmful algal events (Anderson et al., 2002; Glibert and 71 Burkholder, 2006; Heisler et al., 2008; Glibert et al., 2010). 72 Furthermore, high input of N and P from the land and the 73 atmosphere not only increase their concentrations in the receiving 74 water bodies but may also result in an alteration of nutrient ratios 75 in relation to the optimum Redfield ratio (N:P = 16:1) for algal 76 growth (Redfield, 1934, 1958; Glibert et al., 2013, 2014). There are 77 some conflicting results regarding the relationships of nutrients 78 with Ostreopsis bloom trends in field observations. Several authors 79 did not observe any clear relationship between the abundance of 80 Ostreopsis cf. ovata during blooms and nutrient concentrations in the water column (Vila et al., 2001; Shears and Ross, 2009; 81 Accoroni et al., 2011; Cohu et al., 2011). Others have found positive 82 83 but variable nutrient relationships. For example, Parsons and 84 Preskitt (2007) found that Ostreopsis sp.1 abundance was 85 positively correlated with nutrient availability (nitrate (NO_3)), nitrite (NO_2^-) , phosphate (PO_4^{3-}) , and silicate $(Si(OH)_4)$ concentra-86 tions in the waters surrounding Hawaii, while Cohu et al. (2013) 87 reported that PO₄³⁻ concentrations, rather than those of nitrogen 88 89 (N) or Si(OH)₄, were positively associated with O. cf. ovata 90 abundances in northwestern Mediterranean Sea. Studies carried 91 out in experimental conditions have shown that the N:P ratio in the 92 growth medium affects growth rate, cell size, yield, and toxin 93 production of O. cf. ovata, suggesting that the Redfield proportion is 94 optimal for its growth (Vanucci et al., 2012; Vidyarathna and 95 Granéli, 2013). It has also been shown that diluted growth media 96 (L/4, f/4and f/10) seem to be most suitable for an optimal growth of 97 Ostreopsis cells (e.g. Guerrini et al., 2010; Mabrouk et al., 2011; 98 Nascimento et al., 2012; Accoroni et al., 2014) because the use of f/ 99 2 or GSe causes a decrease of growth rate and the appearance of 100 aberrant cell shape in Ostreopsis cultures (Rhodes et al., 2000; 101 Pearce et al., 2001; Nascimento et al., 2012).

Thus, despite the number of studies about the influence of 102 103 environmental parameters on Ostreopsis blooms, the mechanism for bloom development of this dinoflagellate is complex and far 104 from understood. In this study, we analyzed data from blooms in 105 the Conero Riviera, northern Adriatic Sea, from 2007 to 2012, in 106 order to compare the environmental factors associated with bloom 107 onset, maintenance and decline. These observations show how 108 environmental factors, including hydrodynamics, temperature and 109 nutrient availability, and their synergistic effects, may affect bloom 110 dynamics, explaining also the interannual variability of Ostreopsis 111 cf. ovata blooms in this area. These data permitted the develop-112 ment of a conceptual model of these factors on bloom dynamics in 113 the northern Adriatic Sea. 114

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2. Materials and methods

2.1. Sampling and sample treatment

Sampling was carried out from spring to fall of 2007–2012 at 117 the station of Passetto in the Conero Riviera, Ancona, N Adriatic Sea 118 (Fig. 1). This station is a sheltered site due to the presence of a 119 natural reef and is characterized by a rocky bottom, shallow depth, 120 and it is moderately affected by human impact during the summer 121 season. This site is also characterized by small caves derived from 122 human boring of the natural cliffs, with some wastewater 123 discharge facilities. The shore is a popular area for summer 124 125 holidays, when it is subjected to trampling by swimmers. 126

Sampling was conducted each year from April-June to November, fortnightly until the first appearance of *Ostreopsis* cells, and then about once a week until the bloom declined.

Surface temperature and salinity were measured with a CTD 129 (Model 30 handheld salinity, conductivity and temperature system, 130 YSI, Yellow Spring, OH USA) and wave height was recorded 131 according to the Douglas scale. Water samples for nutrient analysis 132 were collected in all years, except for 2007. Water samples were 133 collected in polyethylene bottles (50 ml) near the benthic substrata 134 (before the collection of biota or substratum, in order to avoid 135 resuspension), filtered through GF/F Whatman filters as soon as 136 possible thereafter (diameter 25 mm, nominal pore size 0.7μ m) and 137



Fig. 1. Map of the study area, showing the location of the sampling station along the Conero Riviera (N Adriatic Sea).

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138 stored in triplicate in 4 ml polyethylene bottles at $\sqrt{22}$ °C until 139 subsequent analysis.

140 Undisturbed benthic samples (macroalgae and pebbles) were 141 collected in triplicate at a depth of 0.5 m, following the protocol of 142 Totti et al. (2010). Several seaweed species were collected among 143 those always occurring in the study area during sampling period in 144 all investigated years: Ulva rigida (Ulvophyceae), Dictyota dichot-145 oma and Dictvopteris polypodioides (Phaeophyceae) and Hypnea 146 musciformis (Rhodophyceae). Surface seawater (one replicate) was 147 also sampled to analyze the abundance of benthic dinoflagellates 148 in the water column and preserved by adding 0.8% neutralized 149 formaldehyde (Throndsen, 1978).

150 2.2. Sample treatment

151 Macroalgae and pebbles were treated following the method 152 described in Totti et al. (2010). Seaweed samples with their 153 storage water were vigorously shaken into plastic jars to allow 154 the dislodgement of epiphytic cells, then thalli were rinsed 155 with filtered sea water (FSW) until the cells of Ostreopsis cf. 156 ovata were completely dislodged from their substratum. 157 Macrophyte thalli were weighed to determine fresh and dry 158 weight and their area were calculated (Accoroni et al., 2011). In 159 order to obtain a conversion factor useful for estimating the 160 thallus area only by measuring the wet or dry weight, the ratio 161 of 'fresh weight'/'dry weight'/'surface' was determined on 162 numerous samples.

Hard substrata, including pebbles, were accurately scraped
with a blade and rinsed with FSW, collecting the scraped material
with storage water. The area of the scraped surface was then
measured using a tape measure. Finally, the water sample
obtained, containing detached cells, rinsing and storage water,
was fixed with 0.8% neutralized formaldehyde (Throndsen, 1978)
and stored at 4 °C in the dark until analysis.

170 2.3. Microscope analysis

171 Abundances of Ostreopsis cf. ovata were estimated using an 172 inverted microscope (Zeiss Axiovert 135) equipped with phase 173 contrast, at $200 \times$ magnification. Subsamples (1–25 ml) were 174 settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method (Edler and Elbrachter, 175 176 2010). The identification of O. cf. ovata was carried by epifluor-177 escence microscopy, after staining with Calcofluor-White M2R. 178 Counting was performed on 10-30 random fields, 1-2 transects, or 179 the whole sedimentation chamber, in order to count a representa-180 tive cell number. Identifications were previously confirmed by 181 molecular analysis performed on the same samples (Accoroni et al., 182 2011; Perini et al., 2011).

183 *2.4. Nutrient analysis*

184 Analyses of NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} were conducted 185 following the colorimetric method by Strickland and Parsons 186 (1968), adapted for an Autoanalyzer QuAAtro Axflow. Limits of 187 detection were 0.02 μ mol l⁻¹ for NO₃⁻ and NO₂⁻, and 0.03 μ mol l⁻¹ 188 for PO₄³⁻.

189 2.5. Calculations and statistical analyses

190 Cell count data for *Ostreopsis* cf. *ovata* were expressed as 191 cells g^{-1} fw/dw and cells cm⁻² for macrophytes, cells cm⁻² for 192 pebbles and cells l^{-1} for water column. Each annual study period 193 was divided into three phases, identifying a pre-bloom condition 194 (no *O.* cf. *ovata* cells detected), a bloom onset (the first 195 appearance of *O.* cf. *ovata* cells), and a bloom maintenance period (the remaining period during which cells were detected 196 until their decline). 197

Estimates of the net growth rates (μday^{-1}) throughout the 198 bloom were calculated according to: 199

$$\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1) \tag{1}$$

where N_2 and N_1 are *Ostreopsis* cf. *ovata* abundances on each benthic substrata at respective sampling day, t_1 or t_2 . 202

Differences in environmental parameter values, i.e. water 203 temperature, salinity and concentrations of nutrients NO₃, NO₂, 204 NH_4^+ , and PO_4^{3-} and total dissolved inorganic N (as sum of NO_3^- , 205 NO₂, NH₄) and the ratio of inorganic N:P between the three bloom 206 phases (pre-bloom condition, bloom onset and bloom mainte-207 nance) were assessed through a one-way analysis of variance 208 (ANOVA). Differences in terms of Ostreopsis cf. ovata in situ growth 209 rates between (i) low (<20 °C) and high (>20 °C) water temperature and (ii) high (>24.5) and low (<24.5) N:P ratio, were also 210 211 assessed through an ANOVA test, while the effects of water 212 213 temperature and nutrient condition on *in situ* growth rate were tested using a two-way ANOVA. The abundances of Ostreopsis cf. 214 ovata were also tested for significant correlations (Pearson) with all 215 recorded environmental parameters and between abundances on 216 benthic substrata and those of water column. When significant 217 differences for the main effect were observed (p < 0.05), a Tukey's 218 pairwise comparison test was also performed. The statistical 219 analyses were conducted using Statistica (Statsoft) software. 220

3. Results

3.1. Temporal trend

Blooms of *Ostreopsis* cf. *ovata* were detected in all sampling 223 years at the Passetto station. Generally, the first cell appearance on 224 benthic substrata occurred at the end of July/early August, the 225 maximum abundances were recorded in early autumn (end of 226 September/early October), and the decline of the blooms occurred 227 at end October/early November (Fig. 2). 228

During summer 2007, the first cells appeared on 20th August, 229 maximum abundances were recorded on 1st October 230 $(1.7 \times 10^6 \text{ cells g}^{-1} \text{ fw, corresponding to } 62.0 \times 10^{'3} \text{ cells cm}^{-2})$ 231 and the bloom declined during the last days of October (Fig. 2A). 232 During summer 2009, the first cells appeared on 29th July, 233 maximum abundances were recorded on 9th September 234 $(1.3 \times 10^6 \text{ cells g}^{-1} \text{ fw, corresponding to } 63.8 \times 10 \text{ cells cm}^{-2})$ 235 and the bloom declined during the last days of October 236 (Fig. 2B). In contrast to these two years, in 2010 the first 237 appearance of Ostreopsis cf. ovata was recorded early, on 11th 238 June, but abundances remained very low and sporadically 239 observed until the end of July (Fig. 2C). The bloom started at the 240 beginning of August reaching the maximum value on 19th August 241 $(1.2 \times 10^6 \text{ cells g}^{-1} \text{ fw corresponding to } 47.8 \times 10^3 \text{ cells cm}^{-2})$, 242 and a second increase of abundances was observed on 16th 243 September, with lower values. The bloom decline occurred in the 244 first week of November (Fig. 2C). The pattern of the bloom recorded 245 in summer 2011 was the most different of the years studied: 246 although the first cells were recorded on 5th August, the bloom 247 developed very quickly, reaching the maximum abundances on 248 31st August $(1.6 \times 10^6 \text{ cells g}^{-1} \text{ fw corresponding})$ 249 to 25.6×10^3 cells cm⁻²), much earlier than in previous years 250 (Fig. 2D). Abundances rapidly decreased although the end of the 251 bloom occurred only in the last days of October (Fig. 2D). Lastly, in 252 summer 2012, the trend of the Ostreopsis bloom was similar to that 253 of the earlier years of the study, with the first cell appearance on 254 10th August, maximum abundances on 11th September 255 $(1.9 \times 10^6 \text{ cells g}^{-1} \text{ fw}, \text{ corresponding to } 33.3 \times 10^3 \text{ cells cm}^{-2})$, 256

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Fig. 2. Trend in *Ostreopsis* cf. *ovata* abundance on seaweeds expressed in cells cm⁻² (bold lines, 1st left *y*-axis), N:P ratio (bars, 2nd left *y*-axis) and water temperature (dotted lines, right *y*-axis), in years 2007 (A), 2009 (B), 2010 (C), 2011 (D) and 2012 (E). N:P ratio in 2007 is missing. Error bars indicate standard deviation. Bold lines under the *x*-axis mark dates of bloom onset in each year. The horizontal dotted line represents both (i) the lower temperature threshold permitting cyst germination and therefore the bloom onset and (ii) the upper N:P ratio threshold below which better nutrient conditions are supplied to 0. cf. *ovata* growth.

and the bloom decline occurred during the first part of November
(Fig. 2E). In this year, the bloom was the most intense in terms of
abundances recorded and duration (Fig. 2E).

260 In all years, Ostreopsis cf. ovata was also observed in the water 261 column, but with variable abundances (with maximum abundances of 14.7×10^3 cells l⁻¹ in 2007, 92.5×10^3 cells l⁻¹ in 2009, 262 263 $10.2 \times 10^4 \, cells \, l^{-1} in \ 2010, \ 39.5 \times 10^3 \, cells \, l^{-1}$ in 2011 and 77.9×10^3 cells l⁻¹ in 2012). The correlation between 0. cf. ovata 264 265 abundances on benthic substrata and those in water column was 266 positive and significant (n = 98; r = 0.44; p < 0.001; Fig. 3A). 267 Hydrodynamic events influenced this correlation as, excluding sampling days when sea state was not calm (Douglas scale 21), the 268 269 correlation improved (n = 32; r = 0.66; p < 0.001; Fig. 3B).

270 Among co-occurring benthic dinoflagellates, three species of 271 potentially toxic microalgae were most often found with *Ostreopsis* 272 cf. *ovata*, although with abundances 1–3 orders of magnitude lower: 273 *Prorocentrum lima* and *Amphidinum* cf. *carterae* were found on all 274 the analyzed substrata (6 ± 1 and 16 ± 10 cells cm⁻², respectively)



Fig. 3. Correlation between *Ostreopsis* cf. *ovata* abundances on benthic substrata (y-axis, cells cm⁻²) and in water column (y-axis, cells l⁻¹) for all the study years, on a logarithmic scale, considering all the hydrodynamic conditions (A) and excluding sampling days when sea state was not calm (Douglas scale values 1) (B).

and they did not display any clear seasonal pattern. Coolia monotis was275found as well $(104 \pm 20 \text{ cells cm}^{-2})$, but this species has been recently276removed from the list of harmful algae (www.marinespecies.org/hab/277index.php).278

3.2. Relationships between Ostreopsis cf. ovata bloom and environmental parameters

3.2.1. Temperature and salinity

Surface temperature ranged from 14.4 to 28.6 °C in all of these 282 5 years during the period when Ostreopsis cf. ovata was detected on 283 benthic substrata. The highest temperature values, between 284 25 and 28.6 °C, were generally observed from early July and were 285 maintained until middle August and generally corresponded with 286 bloom onset. Bloom onset was always observed on average about 287 30 days after a temperature of 25 °C was reached, with the only 288 exception observed in summer 2011 (Fig. 4). The bloom peak was 289 generally 70–100 days past this temperature threshold. 290

291 The bloom peak was mainly observed when surface temperature was decreasing from the seasonal maximum (18.8-24 °C), 292 with values in the middle of the two previously described 293 extremes. However, in summer 2011 the early bloom peak was 294 recorded close to the seasonal maximum (27 °C) with water 295 temperature of 25.9 °C. Water temperature was significantly lower 296 in bloom maintenance phase $(22.7 \pm 3.3 \text{ °C})$ than in the onset 297 298 (26.5 \pm 1.1 °C, Tukey HSD test, *p* < 0.05). Lowest temperature values were recorded at the end of the bloom, with values ranging from 299 14.4 to 17.5 °C (Fig. 5). 300

During the period when *Ostreopsis* cf. *ovata* was detected on 301 benthic substrata, the salinity ranged from 31.3 to 39.1 °C (not shown). 303

3.2.2. Nutrients

Concentrations of DIN were highly variable between and within 305 the <u>Ostreopsis</u> cf. ovata blooms, ranging from 0.55 to 306 19.40 μ mol l⁻¹ (Fig. 6). In 2009, 2010 and 2011, NH₄⁺ was the 307 major contributor to DIN from June to early-October (Fig. 6A–C), 308 while in 2012 NO₃⁻ represented the dominant N source for the 309 entire sampling period (Fig. 6D). 310

Concentrations of PO_4^{3-} ranged from 0.02 to 0.49 μ mol l^{-1} 311 during the blooms (Fig. 6). PO_4^{3-} concentrations were significantly 312 higher in the bloom onset periods $(0.35 \pm 0.27 \ \mu mol \ l^{-1})$ than in 313 pre-bloom $(0.10 \pm 0.09 \,\mu\text{mol}\,l^{-1})$, Tukey HSD test, p < 0.001) or 314 bloom maintenance periods ($0.18 \pm 0.10 \mu$ mol l⁻¹, Tukey HSD test, 315 p < 0.01). A recognizable PO₄³⁻ seasonal trend was observed in 2009, 316 when an increase in concentrations from the end of May to mid-317 August was followed by a decrease until early October and a new 318 increase at the end of October when the bloom declined (Fig. 6A), 319 while during the other studied years, a clear seasonal trend was not 320 observed (Fig. 6B, C and D). 321

Considering the ratios of inorganic N:P, they were generally far 322 above the Redfield ratio with values >1000. Ratios of N:P far below 323



Fig. 4. Timing of the bloom onset of *Ostreopsis* cf. *ovata* blooms in terms of days past temperature threshold of 25 °C, in each sampling year.

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Fig. 5. Water temperature recorded each year at bloom onset, peak and end. Each radius represents the temperature values axis (°C) of year indicated at the corner.

324 Redfield ratio were a more rare event, observed only in some 325 sampling days in 2011 and 2012 (Fig. 2). Noteworthy, the summer 326 of 2011 was characterized by frequent and lasting periods of low 327 N:P, around Redfield ratio during both pre-bloom conditions and 328 bloom development. For all years of study for which nutrient data 329 are available, the N:P ratio was significantly lower in the bloom 330 onset (24.5 \pm 7.6) than in pre-bloom conditions (139.2 \pm 40.8, Tukey HSD test, p < 0.05). Moreover, the N:P ratio during the bloom 331 332 maintenance phase (68.2 ± 13.7) was higher than on the bloom onset 333 and lower than in pre-bloom conditions, although not significantly in 334 both cases.

335 3.2.3. Relationship between environmental parameters and the in situ336 growth rate

The net *in situ* growth rate of *Ostreopsis* cf. *ovata* ranged from 337 5.77 to 1.36 day⁻¹. The maximum growth rates for each year 338 339 $(1.36, 0.62, 0.80, 1.16 \text{ and } 0.80 \text{ day}^{-1})$ were recorded on 13th 340 September 2007, 4th August 2009, 5th August 2010, 17th August 2011, and 29th August 2012, respectively. For those years for 341 342 which nutrient data were available, these values corresponded 343 with inorganic N:P ratio values ranging from 16.06 to 45.85 (Fig. 7) 344 as well as temperature values ranging from 24.5 to 26.3 °C. In 345 2009 and 2012, all the positive values of growth rate were detected 346 at inorganic N:P values below 100, while in 2010 and 2011 a small 347 percentage of them (12% and 7%, respectively) were recorded also 348 at inorganic N:P ratios >100 (Fig. 7). On the contrary, the lowest 349 values, in fact negative net growth rates, were recorded on 29th 350 September 2009, 15th October 2010, 29th September 2011, and 351 17th October 2012, when inorganic N:P ratios generally exceeded 352 50 (Fig. 7) and when temperatures were ≤ 25 °C.

353 Water temperatures affected the Ostreopsis cf. ovata in situ growth rates: at temperatures ≤20 °C, O. cf. ovata average net 354 growth rates were significantly lower $(-0.23 \pm 0.05 \text{ day}^{-1})$ than at temperatures $\geq 20 \degree \text{C} (0.03 \pm 0.03 \text{ day}^{-1})$ Tukey HSD test, p < 0.01). 355 356 357 Assuming the average N:P ratio recorded during bloom onset (24.5) as 358 the threshold below which better nutrient conditions are supplied to 359 O. cf. ovata growth, O. cf. ovata showed growth rate higher in optimal nutrient conditions $(0.06 \pm 0.04 \text{ day}^{-1})$ than in higher N:P ratio 360 values ($-0.02\pm0.03~day^{-1}$), although not significantly. However, 361 analyzing these data considering also the effect of the temperature, 362

O. cf. *ovata* show a significantly higher growth rate in optimal nutrient conditions and temperature $\gtrsim 20 \degree \text{C} (0.09 \pm 0.04 \text{ day}^{-1})$ than at higher N:P ratios and temperatures $_{\sim} < 20 \degree \text{C} (-0.31 \pm 0.10 \text{ day}^{-1})$, Tukey HSD test, p < 0.05).

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No significant correlations were found between salinity values and both benthic *Ostreopsis* cf. *ovata* abundances and *in situ* growth rate.

4. Discussion

The Passetto station, Conero Riviera, northern Adriatic Sea, has been defined a hot-spot area for Ostreopsis cf. ovata blooms by the Italian Regional Agencies for the Protection of the Environments (ISPRA, 2012). In this area, O. cf. ovata blooms are among the most intense of the entire Mediterranean coasts (Mangialajo et al., 2011), with maximum abundances reaching the magnitude of 10^6 cells g⁻¹ fw (corresponding to 10^7 cells g⁻¹ dw and 10⁴ cells cm⁻²; Accoroni et al., 2012a). Given the human health and ecological health problems associated with these blooms (Shears and Ross, 2009; Del Favero et al., 2012), there is a great need to understand the factors contributing to their proliferation. Although several studies have highlighted the influence of environmental factors on bloom dynamics of O. cf. ovata in field conditions (Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Cohu et al., 2011, 2013), the complexity of conditions leading to blooms of this dinoflagellate are still far from understood.

A clear and important role of the hydrodynamic condition on the trend of Ostreopsis blooms has been previously recognized and investigated in the same area (Totti et al., 2010; Accoroni et al., 2011, 2012a). Although blooms occur even in exposed sites, higher abundances are observed under sheltered conditions (Chang et al., 2000; Shears and Ross, 2009; Totti et al., 2010; Mabrouk et al., 2011; Selina et al., 2014). Moreover, the removing of the artificial breakwater reefs from a documented Ostreopsis cf. ovata bloom hot-spot area in Conero Riviera (i.e. Numana, see Totti et al., 2010), led to a marked decrease of Ostreopsis abundances in the following years (unpublished data). The data herein further highlight how hydrodynamic events may influence the correlation between the abundances of O. cf. ovata on benthic substrata and those in water column. In fact, a relatively good correlation between epiphytic and planktonic cell abundances was found only if the hydrodynamic effect was excluded. The O. cf. ovata mat is only loosely attached to the substrata and is easily resuspended in the water column; thus, hydrodynamic events can either increase planktonic cell abundance by resuspension or decrease them by dilution if a hydrodynamic event is very strong.

On the contrary, there are some conflicting results regarding the 407 408 relationships between Ostreopsis bloom dynamics and both water temperature and nutrient concentrations. Ostreopsis blooms are 409 summer events in temperate areas and maximum abundances 410 have been associated with high temperatures (Aligizaki and 411 Nikolaidis, 2006; Mangialajo et al., 2008), except for the northern 412 Adriatic Sea (Monti et al., 2007; Accoroni et al., 2012a) and the Sea 413 of Japan (Selina et al., 2014). Our data from the Conero Riviera show 414 that the bloom onset was always observed at high temperature 415 416 (25–28.6 °C). This seems to suggest that Ostreopsis needs to reach a temperature threshold to start its bloom, probably linked to cyst 417 germination that generally occurs around values of 25 °C (Accoroni 418 et al., 2014). Once Ostreopsis cysts are germinated, its vegetative 419 forms seem to actively proliferate even if temperature values 420 decrease. In this area, the peak in bloom abundance was found to 421 occur in the late summer when temperatures were decreasing 422 from the seasonal maximum (18.8–24 °C) and water temperatures 423 were significantly lower in the bloom maintenance phase 424 $(22.7 \pm 3.3 \text{ °C})$ than in the bloom onset period $(26.5 \pm 1.1 \text{ °C})$. In 425 426 this area, Ostreopsis cf. ovata blooms can persist on benthic substrata

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Fig. 6. Temporal variability of nutrient concentration (μ mol l⁻¹) in years 2009 (A), 2010 (B), 2011 (C) and 2012 (D): dissolved inorganic nitrogen (DIN) with the detail of NO₃⁻ (crossed), NO₂⁻ (black) and NH₄⁺ (gray) contribution (left *y*-axis) and PO₄³⁻ (bold line, right *y*-axis). Vertical bars indicate the period of *Ostreopsis* cf. *ovata* blooms. (A): modified from Accoroni et al., 2011; (B) modified from Accoroni et al. (2012a).

427 until temperature values decrease to values ranging from 14.4 to 428 17.5 °C. These observations completely agree with what observed in 429 experimental conditions about the ecophysiology of O. cf. ovata from 430 the northern Adriatic Sea: although Ostreopsis strains can growth at 20, 25, and 30 °C, the highest growth rates were recorded at 20 °C 431 432 (Pezzolesi et al., 2012) and O. cf. ovata resting cysts were able to 433 germinate after a 5-month-dormancy period only at 25 °C and not at 434 21 °C (Accoroni et al., 2014). Binder and Anderson (1987) also found 435 that in experimental conditions the optimal temperature range for 436 the germination of the dinoflagellate Scrippsiella trochoidea does not 437 coincide exactly with the optimal range for growth.

In all the studied years except 2011, the bloom onset was
observed on average about 30 days after reaching a temperature
threshold of 25 °C, suggesting that other environmental factors
beside temperature may affect the development of *Ostreopsis* cf. *ovata* blooms. Recent studies have provided increasing evidence of

a link between the nutrient enrichment of coastal waters 443 (anthropogenic eutrophication) and harmful algal events (Glibert 444 and Burkholder, 2006; Heisler et al., 2008; Glibert et al., 2010). 445 However, there is very limited information on relationships 446 between nutrient concentrations and trends in development of 447 benthic dinoflagellate blooms. Vila et al. (2001) and Cohu et al. 448 (2011) in the NW Mediterranean Sea and Shears and Ross (2009) in 449 NE New Zealand, did not find any relation between epiphytic O. cf. 450 ovata and nutrients, while Parsons and Preskitt (2007) found that 451 Ostreopsis sp.1 abundance was positively correlated with all 452 inorganic nutrients concentrations in the waters surrounding 453 Hawaii. Cohu et al. (2013) reported that PO₄³⁻ concentration, rather 454 than N or Si(OH)₄, was also positively associated with O. cf. ovata 455 abundances and that N:P ratio was significantly and negatively 456 related to Ostreopsis abundances in the northwestern Mediterra-457 nean Sea. In fact, if the bloom cells are consuming nutrients, one 458

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would not necessarily expect a positive and direct relationshipbetween the abundance of cells and the nutrient availability.

Although no clear relationship was found between nutrient 461 462 concentrations and *Ostreopsis* cf. ovata abundances in this study, it 463 was found that in the bloom onset period PO_4^{3-} concentrations 464 were significantly higher than in either the bloom maintenance 465 phase or the pre-bloom conditions, resulting in an N:P ratio that 466 was significantly lower in the bloom onset period (24.5 ± 7.6) than 467 in pre-bloom conditions (139.2 \pm 40.8). Both Vanucci et al. (2012) 468 and Pezzolesi et al. (2014) found, in experimental studies of different 469 Adriatic O. cf. ovata strains, that the depletion of P was proportion-470 ately more rapid than that of N, highlighting the strong P demand of 471 this dinoflagellate. A decrease in N:P ratio has previously been 472 associated with the onset of a number of planktonic dinoflagellate 473 blooms; comparatively fewer reports are available of this relation-474 ships for benthic dinoflagellates. For example, planktonic Prorocen-475 trum species generally achieve maximum growth rates at N:P ratios 476 just below Redfield proportions (Zhang and Hu, 2011, Glibert et al., 477 2012); furthermore, Hodgkiss and Ho (1997), in their study of 478 dinoflagellate blooms in Hong Kong water found that annual 479 frequency of blooms increased with declines in the N:P ratio well below Redfield proportions. Bloom onset is the result of both cyst 480 481 germination and survival/proliferation of germinating cells. Although 482 water temperature has been identified as the most important factor in 483 cyst germination of dinoflagellates (Anderson and Morel, 1979; 484 Anderson, 1980), nutrient availability may modulate the cyst 485 germination, strongly influencing the timing and the rate of 486 excystment or the growth of cells following excystment (Anderson 487 and Wall, 1978: Anderson and Morel, 1979: Dale, 1983: Anderson and 488 Keafer, 1987: Binder and Anderson, 1987: An et al., 1992: Hallegraeff et al., 1998; Rengefors and Anderson, 1998; Figueroa et al., 2005). To 489 date the only information about the nutritional demand of Ostreopsis 490 cysts during germination is based on studies in which an N:P ratio of 491 492 24 was used to perform germination experiments on resting cysts 493 (Accoroni et al., 2014). Assuming the N:P ratio value recorded during 494 bloom onset (24.5) as the threshold below which better nutrient 495 conditions are supplied to O. cf. ovata growth, the growth rate is higher when proportionately more PO_4^{3-} is available and when the 496 N:P is ≤ 24.5 . The synergy of higher temperatures and lower N:P ratio 497 498 resulted in a higher net growth rate.

499Interestingly, blooms were maintained in a condition of500elevated N:P ratios, well above Redfield proportions, a condition501normally taken to be indicative of P limitation. However, PO_4^3 was502never depleted from the water column, and thus true P limitation503likely never developed physiologically. However, net growth rates504were slower than during the initial phase of blooms development.505Such a progression of bloom growth in relation to N:P ratios would

be consistent with the progression of Prorocentrum blooms 506 previously described by Glibert et al. (2012). They suggested that 507 508 such blooms may be initiated at low N:P levels (even less than Redfield ratio, but not in severely N limiting conditions), often 509 stimulated by a "flush" of nutrients or organic materials that may 510 allow a latent resident population (in our case, the newly 511 germinating cells) to increase growth rate, as observed in blooms 512 in Tolo Harbor, Chesapeake Bay, and Neuse River Estuary, After that 513 growth rate increase, bloom biomass is able to increase often 514 accompanied by N:P values greater than Redfield. At this point, 515 these blooms are able to sustain high biomass levels, not 516 necessarily by high growth rates, but also through other 517 mechanisms, including (i) metabolic dissipatory strategies that 518 allow the maintenance of cellular nutrient and energy balance in 519 an environment where energy flow and nutrients are provided at 520 unbalanced proportions and (ii) allelopathic and mixotrophic 521 interactions with other species. Thus, as suggested by our 522 observations on Ostreopsis blooms as well, while high growth 523 rates (stimulated by an injection of P-rich water) may allow 524 blooms to initiate, adaptive physiology would enable the mainte-525 nance of blooms at less than maximal growth rates and at non-526 527 optimal N:P ratios.

Therefore, a synergic action of optimal water temperature and 528 optimal nutrient conditions may explain why in the N Adriatic Sea 529 there is a delay between the maximum temperature values and the 530 peak Ostreopsis cf. ovata bloom. Generally, these simultaneous 531 conditions happen between the second part of July and early 532 August. An exception to this trend was observed in 2011, as after 533 water temperature reached the threshold of 25 °C, optimal 534 nutrient conditions occurred, allowing the immediate bloom 535 development. Noteworthy, in 2010 nutrient conditions remained 536 unfavorable for rapid bloom development, and only in three 537 sampling days N:P ratios ≤24.5 were recorded, leading to a 538 proportionately smaller bloom and very low abundances com-539 pared to what observed during the other years. 540

However, other factors both abiotic and biotic, such as ability to 541 use organic forms of nutrients and interactions with other 542 organisms, should be investigated to further clarify the 543 Ostreopsis cf. ovata natural bloom mechanism. Actually, many 544 HAB genera can use organic (dissolved or particulate) forms of 545 nutrients for their nutritional demands (Cucchiari et al., 2008; 546 Heisler et al., 2008) and mixotrophy has been hypothesized in O. cf. 547 ovata that showed a particular defensive strategy where small 548 organisms remain trapped in its mucilaginous web (Barone, 2007). 549 Moreover, allelopathic interactions between Ostreopsis and other 550 organisms should be considered as well: although a weak 551 inhibitory effect of O. cf. ovata was observed towards other 552



Fig. 8. Conceptual model of the effect of N:P ratio, water temperature and hydrodynamic conditions on both bloom onset and maintenance of Ostreopsis cf. ovata. Green and red arrows symbolize positive and negative effect, respectively. Dotted line represents a slight effect relative to solid line.

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Fig. 9. Conceptual diagram of the effect of N:P ratio and water temperature on *Ostreopsis* cf. *ovata* bloom. Panel A represents the typical pre-bloom condition, showing low temperature and very high N:P ratio. Panel B represents the pre-bloom condition just before the bloom onset: although the temperature (>25 °C) is optimal for cyst germination, the high N:P ratio does not allow the start of the bloom. Bloom onset (panel C) occurs at *T* >25 °C and at N:P ratio (around 24) balanced by an injection of P-rich water, allowing both cyst germination and cell proliferation. Panel D shows the optimal environmental conditions for the bloom maintenance: temperature is not limiting, as bloom peak occurs at *T* around 20 °C, and the slight increase of N:P ratio accompanied by a general decrease of N and P could be attributable to *O*. cf. *ovata* growth.

553 benthic dinoflagellates (Monti and Cecchin, 2012), studies about 554 the interactions between *O.* cf. *ovata* and the co-occurring 555 microphytobentic community have not been developed yet, and 556 they may further clarify the *O.* cf. *ovata* natural bloom mechanism.

557 In conclusion, our data suggest that in the northern Adriatic Sea 558 Ostreopsis cf. ovata blooms are triggered by a combination of calm 559 hydrodynamic conditions, optimal temperature and favorable 560 nutrients (Figs. 8 and 9). Calm conditions are a prerequisite for 561 blooms, and only when this condition exists do temperature and 562 nutrient start to have a decisive effect. The water temperature 563 threshold of 25 °C plays a key role in the germination of O. cf. ovata 564 cysts and therefore in bloom onset, and an N:P ratio around 565 Redfield value is a necessary condition to allow cell proliferation. 566 After the bloom onset, the decrease of water temperature to values 567 around 20 °C and slight increase of N:P ratio (likely attributable to 568 O. cf. ovata uptake) allow the bloom maintenance, as indicated by 569 the values of in situ growth rate. Hydrodynamic conditions 570 enhance these effects both directly acting on the benthic 571 dinoflagellate abundances and indirectly influencing the values of both temperature and nutrient concentration in sea water. This 572 573 synergic effect on the bloom might explain both (i) the interannual 574 variability of O. cf. ovata blooms along Conero Riviera and (ii) why 575 in the northern Adriatic Sea the O. cf. ovata blooms occur in the late 576 summer when temperature values are decreasing, differently from 577 other Mediterranean areas.

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