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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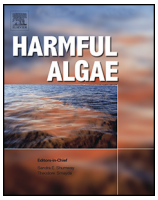

Highlights

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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

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.
- 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.
- 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.

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Sampling and sample treatment

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

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

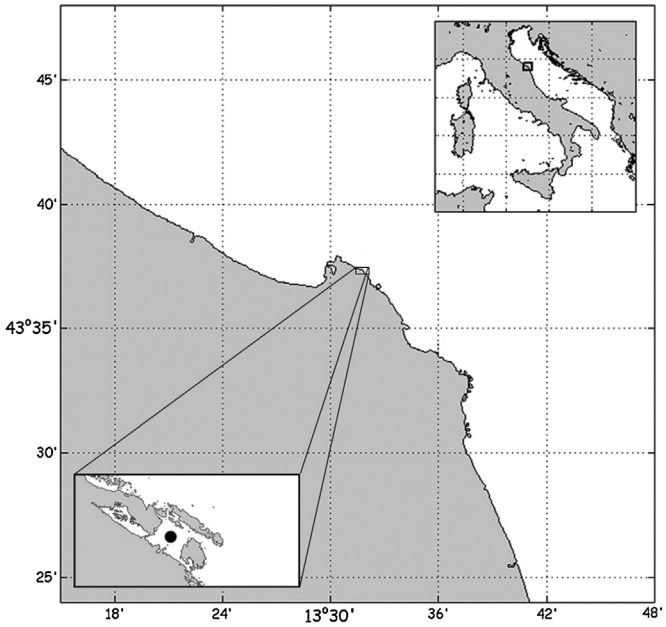

Fig. 1. Map of the study area, showing the location of the sampling station along the Conero Riviera (N Adriatic Sea).

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stored in triplicate in 4 ml polyethylene bottles at −22 °C until subsequent analysis.

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

2.2. Sample treatment

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

2.3. Microscope analysis

Abundances of *Ostreopsis* cf. *ovata* were estimated using an inverted microscope (Zeiss Axiovert 135) equipped with phase contrast, at 200× magnification. Subsamples (1–25 ml) were settled in counting chambers after homogenization, according to the Utermöhl's sedimentation method (Edler and Elbrachter, 2010). The identification of *O.* cf. *ovata* was carried by epifluorescence microscopy, after staining with Calcofluor-White M2R. Counting was performed on 10–30 random fields, 1–2 transects, or the whole sedimentation chamber, in order to count a representative cell number. Identifications were previously confirmed by molecular analysis performed on the same samples (Accoroni et al., 2011; Perini et al., 2011).

2.4. Nutrient analysis

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-}.

2.5. Calculations and statistical analyses

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

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

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1) \quad (1)$$

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

Differences in environmental parameter values, i.e. water temperature, salinity and concentrations of nutrients NO_3^-, NO_2^-, NH_4^+, and PO_4^{3-} and total dissolved inorganic N (as sum of NO_3^-, NO_2^-, NH_4^+) and the ratio of inorganic N:P between the three bloom phases (pre-bloom condition, bloom onset and bloom maintenance) were assessed through a one-way analysis of variance (ANOVA). Differences in terms of *Ostreopsis* cf. *ovata in situ* growth 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 assessed through an ANOVA test, while the effects of water temperature and nutrient condition on *in situ* growth rate were tested using a two-way ANOVA. The abundances of *Ostreopsis* cf. *ovata* were also tested for significant correlations (Pearson) with all recorded environmental parameters and between abundances on benthic substrata and those of water column. 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) software.

3. Results

3.1. Temporal trend

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

During summer 2007, the first cells appeared on 20th August, maximum abundances were recorded on 1st October (1.7×10^6 cells g^{-1} fw, corresponding to 62.0×10^3 cells cm^{-2}) and the bloom declined during the last days of October (Fig. 2A). During summer 2009, the first cells appeared on 29th July, maximum abundances were recorded on 9th September (1.3×10^6 cells g^{-1} fw, corresponding to 63.8×10^3 cells cm^{-2}) and the bloom declined during the last days of October (Fig. 2B). In contrast to these two years, in 2010 the first appearance of *Ostreopsis* cf. *ovata* was recorded early, on 11th June, but abundances remained very low and sporadically observed until the end of July (Fig. 2C). The bloom started at the beginning of August reaching the maximum value on 19th August (1.2×10^6 cells g^{-1} fw corresponding to 47.8×10^3 cells cm^{-2}), and a second increase of abundances was observed on 16th September, with lower values. The bloom decline occurred in the first week of November (Fig. 2C). The pattern of the bloom recorded in summer 2011 was the most different of the years studied: although the first cells were recorded on 5th August, the bloom developed very quickly, reaching the maximum abundances on 31st August (1.6×10^6 cells g^{-1} fw corresponding to 25.6×10^3 cells cm^{-2}), much earlier than in previous years (Fig. 2D). Abundances rapidly decreased although the end of the bloom occurred only in the last days of October (Fig. 2D). Lastly, in summer 2012, the trend of the *Ostreopsis* bloom was similar to that of the earlier years of the study, with the first cell appearance on 10th August, maximum abundances on 11th September (1.9×10^6 cells g^{-1} fw, corresponding to 33.3×10^3 cells cm^{-2}),

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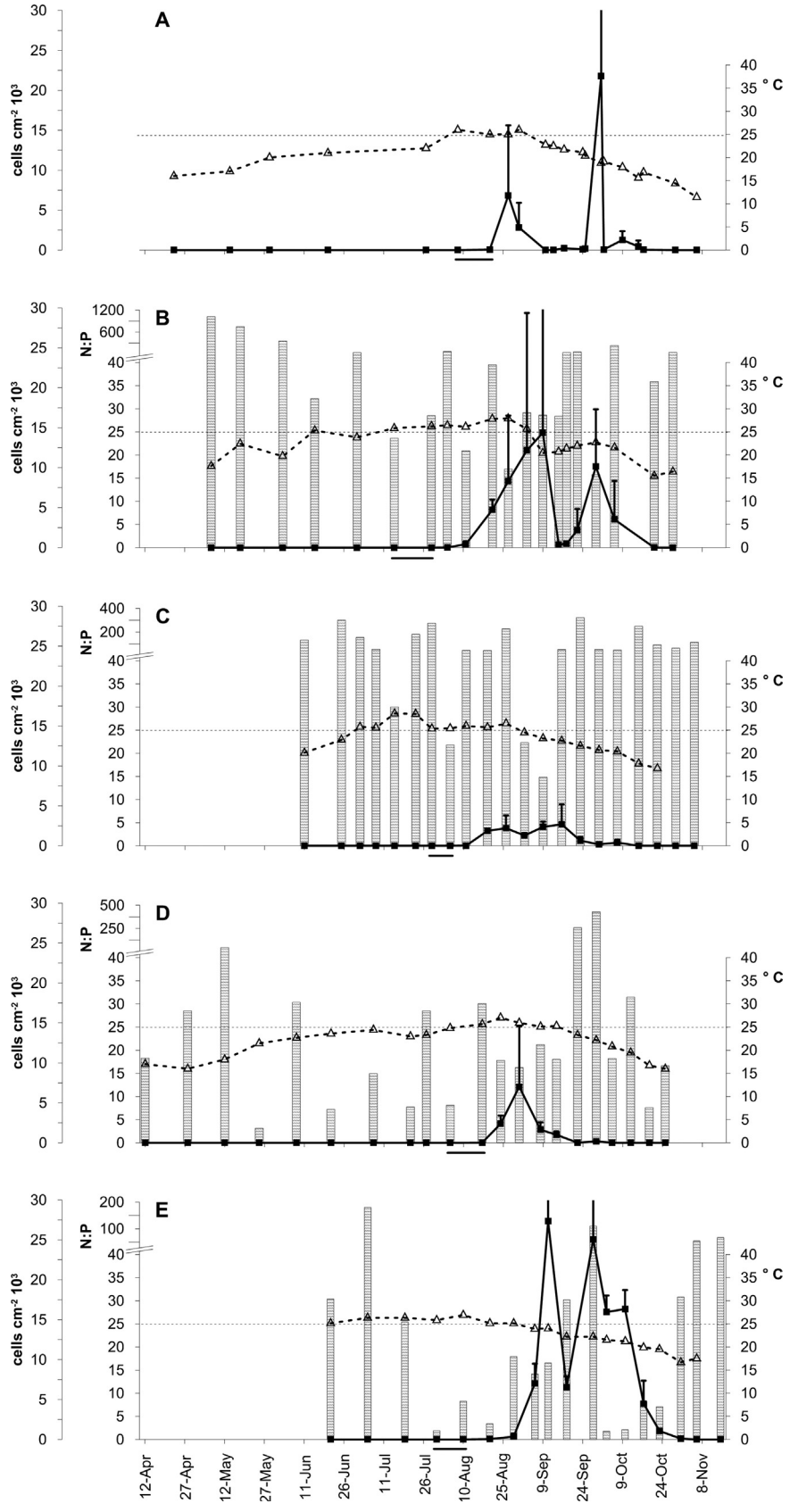

Fig. 2. Trend in *Ostreopsis* cf. *ovata* abundance on seaweeds expressed in cells cm^{-2} (bold lines, 1st left *y*-axis), N:P ratio (bars, 2nd left *y*-axis) and water temperature (dotted lines, right *y*-axis), in years 2007 (A), 2009 (B), 2010 (C), 2011 (D) and 2012 (E). N:P ratio in 2007 is missing. 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 *O.* cf. *ovata* growth.

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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).

In all years, *Ostreopsis* cf. *ovata* was also observed in the water 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, 10.2×10^4 cells l^{-1}in 2010, 39.5×10^3 cells l^{-1} in 2011 and 77.9×10^3 cells l^{-1} in 2012). The correlation between *O.* cf. *ovata* abundances on benthic substrata and those in water column was positive and significant ($n = 98$; $r = 0.44$; $p < 0.001$; Fig. 3A). Hydrodynamic events influenced this correlation as, excluding sampling days when sea state was not calm (Douglas scale >1), the correlation improved ($n = 32$; $r = 0.66$; $p < 0.001$; Fig. 3B).

Among co-occurring benthic dinoflagellates, three species of potentially toxic microalgae were most often found with *Ostreopsis* cf. *ovata*, although with abundances 1–3 orders of magnitude lower: *Prorocentrum lima* and *Amphidinum* cf. *carterae* were found on all the analyzed substrata (6 ± 1 and 16 ± 10 cells cm^{-2}, respectively) and they did not display any clear seasonal pattern. *Coolia monotis* was found as well (104 ± 20 cells cm^{-2}), but this species has been recently removed from the list of harmful algae (www.marinespecies.org/hab/index.php).

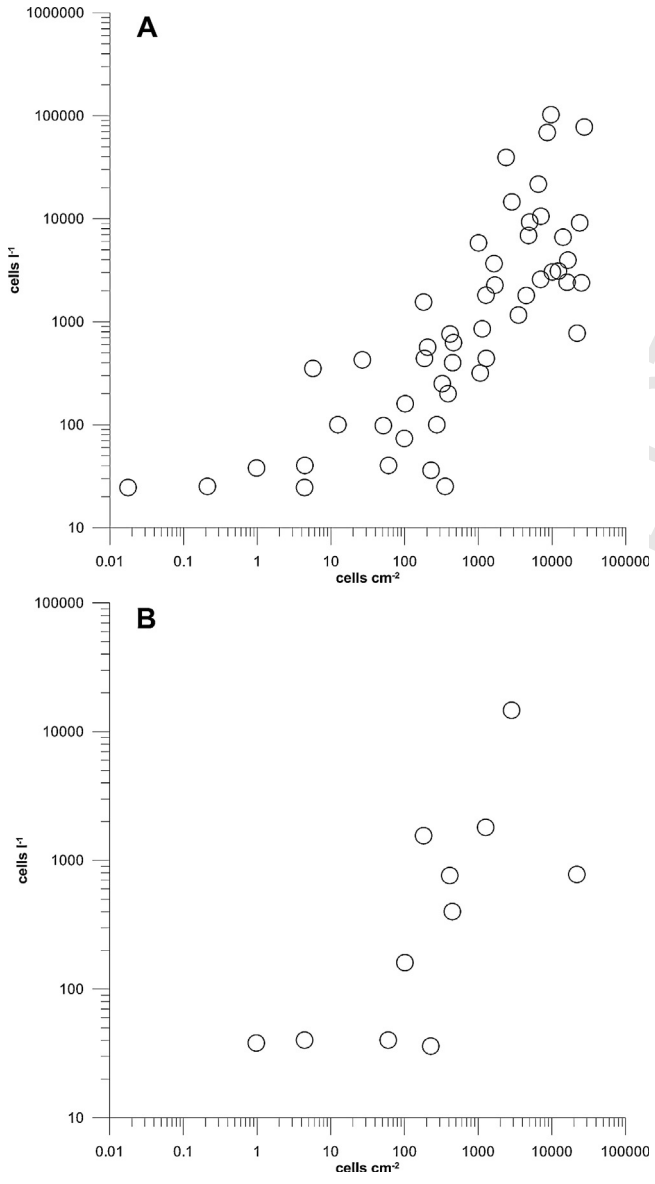

Fig. 3. Correlation between *Ostreopsis* cf. *ovata* abundances on benthic substrata (*y*-axis, cells cm^{-2}) and in water column (*y*-axis, cells l^{-1}) for all the study years, on a logarithmic scale, considering all the hydrodynamic conditions (A) and excluding sampling days when sea state was not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

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

The bloom peak was mainly observed when surface temperature was decreasing from the seasonal maximum (18.8–24 °C), with values in the middle of the two previously described extremes. However, in summer 2011 the early bloom peak was recorded close to the seasonal maximum (27 °C) with water temperature of 25.9 °C. Water temperature was significantly lower in bloom maintenance phase (22.7 ± 3.3 °C) than in the onset (26.5 ± 1.1 °C, Tukey HSD test, $p < 0.05$). Lowest temperature values were recorded at the end of the bloom, with values ranging from 14.4 to 17.5 °C (Fig. 5).

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

3.2.2. Nutrients

Concentrations of DIN were highly variable between and within the *Ostreopsis* cf. *ovata* blooms, ranging from 0.55 to 19.40 μmol l^{-1} (Fig. 6). In 2009, 2010 and 2011, NH_4^+ was the major contributor to DIN from June to early-October (Fig. 6A–C), while in 2012 NO_3^- represented the dominant N source for the entire sampling period (Fig. 6D).

Concentrations of PO_4^{3-} ranged from 0.02 to 0.49 μmol l^{-1} during the blooms (Fig. 6). PO_4^{3-} concentrations were significantly higher in the bloom onset periods (0.35 ± 0.27 μmol l^{-1}) than in pre-bloom (0.10 ± 0.09 μmol l^{-1}, Tukey HSD test, $p < 0.001$) or bloom maintenance periods (0.18 ± 0.10 μmol l^{-1}, Tukey HSD test, $p < 0.01$). A recognizable PO_4^{3-} seasonal trend was observed in 2009, when an increase in concentrations from the end of May to mid-August was followed by a decrease until early October and a new increase at the end of October when the bloom declined (Fig. 6A), while during the other studied years, a clear seasonal trend was not observed (Fig. 6B, C and D).

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

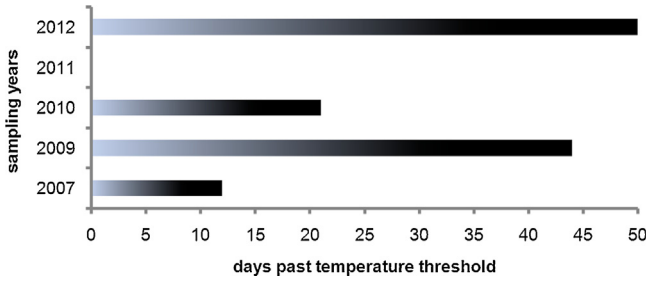

Fig. 4. Timing of the bloom onset of *Ostreopsis* cf. *ovata* blooms in terms of days past temperature threshold of 25 °C, in each sampling year.

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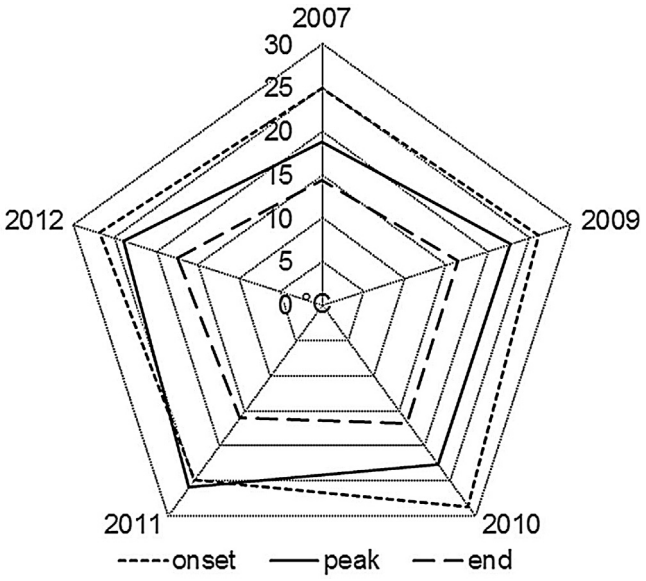

Fig. 5. Water temperature recorded each year at bloom onset, peak and end. Each radius represents the temperature values axis (°C) of year indicated at the corner.

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

3.2.3. Relationship between environmental parameters and the in situ growth rate

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

Water temperatures affected the *Ostreopsis* cf. *ovata in situ* growth rates: at temperatures ≤20 °C, *O.* cf. *ovata* average net growth rates were significantly lower (−0.23 ± 0.05 day^{-1}) than at temperatures ≥20 °C (0.03 ± 0.03 day^{-1} Tukey HSD test, $p < 0.01$). Assuming the average N:P ratio recorded during bloom onset (24.5) as the threshold below which better nutrient conditions are supplied to *O.* cf. *ovata* growth, *O.* cf. *ovata* showed growth rate higher in optimal nutrient conditions (0.06 ± 0.04 day^{-1}) than in higher N:P ratio values (−0.02 ± 0.03 day^{-1}), although not significantly. However, analyzing these data considering also the effect of the temperature, *O.* cf. *ovata* show a significantly higher growth rate in optimal nutrient conditions and temperature ≥20 °C (0.09 ± 0.04 day^{-1}) than at higher N:P ratios and temperatures <20 °C (−0.31 ± 0.10 day^{-1}, Tukey HSD test, $p < 0.05$).

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 10^4 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 relationships between *Ostreopsis* bloom dynamics and both water temperature and nutrient concentrations. *Ostreopsis* blooms are summer events in temperate areas and maximum abundances have been associated with high temperatures (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008), except for the northern Adriatic Sea (Monti et al., 2007; Accoroni et al., 2012a) and the Sea of Japan (Selina et al., 2014). Our data from the Conero Riviera show that the bloom onset was always observed at high temperature (25–28.6 °C). This seems to suggest that *Ostreopsis* needs to reach a temperature threshold to start its bloom, probably linked to cyst germination that generally occurs around values of 25 °C (Accoroni et al., 2014). Once *Ostreopsis* cysts are germinated, its vegetative forms seem to actively proliferate even if temperature values decrease. In this area, the peak in bloom abundance was found to occur in the late summer when temperatures were decreasing from the seasonal maximum (18.8–24 °C) and water temperatures were significantly lower in the bloom maintenance phase (22.7 ± 3.3 °C) than in the bloom onset period (26.5 ± 1.1 °C). In this area, *Ostreopsis* cf. *ovata* blooms can persist on benthic substrata

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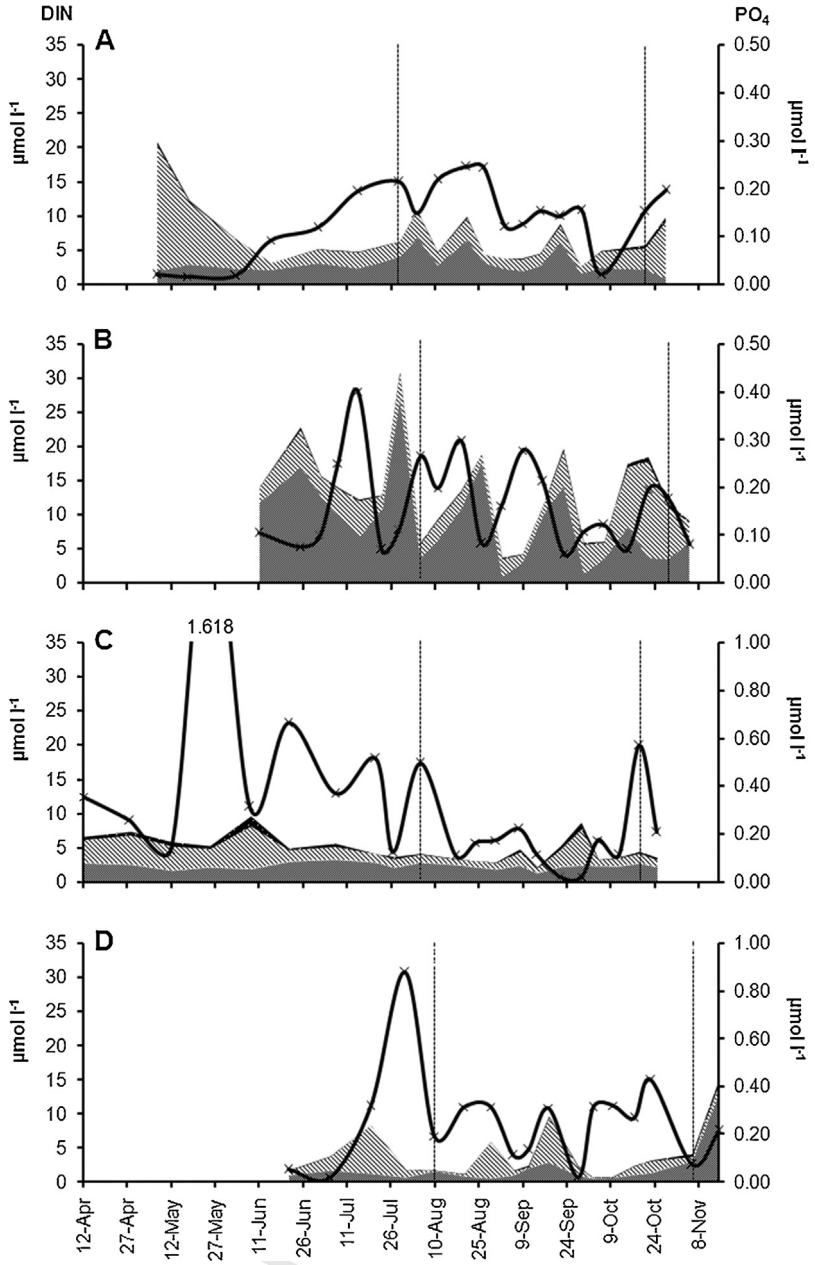

Fig. 6. Temporal variability of nutrient concentration (µmol l^{-1}) in years 2009 (A), 2010 (B), 2011 (C) and 2012 (D): dissolved inorganic nitrogen (DIN) with the detail of NO_3^- (crossed), NO_2^- (black) and NH_4^+ (gray) contribution (left *y*-axis) and PO_4^{3-} (bold line, right *y*-axis). Vertical bars indicate the period of *Ostreopsis* cf. *ovata* blooms. (A): modified from Accoroni et al., 2011; (B) modified from Accoroni et al. (2012a).

until temperature values decrease to values ranging from 14.4 to 17.5 °C. These observations completely agree with what observed in experimental conditions about the ecophysiology of *O.* cf. *ovata* from the northern Adriatic Sea: although *Ostreopsis* strains can growth at 20, 25, and 30 °C, the highest growth rates were recorded at 20 °C (Pezzolesi et al., 2012) and *O.* cf. *ovata* resting cysts were able to germinate after a 5-month-dormancy period only at 25 °C and not at 21 °C (Accoroni et al., 2014). Binder and Anderson (1987) also found that in experimental conditions the optimal temperature range for the germination of the dinoflagellate *Scrippsiella trochoidea* does not 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 (anthropogenic eutrophication) and harmful algal events (Glibert and Burkholder, 2006; Heisler et al., 2008; Glibert et al., 2010). However, there is very limited information on relationships between nutrient concentrations and trends in development of benthic dinoflagellate blooms. Vila et al. (2001) and Cohu et al. (2011) in the NW Mediterranean Sea and Shears and Ross (2009) in NE New Zealand, did not find any relation between epiphytic *O.* cf. *ovata* and nutrients, while Parsons and Preskitt (2007) found that *Ostreopsis* sp.1 abundance was positively correlated with all inorganic nutrients concentrations in the waters surrounding Hawaii. Cohu et al. (2013) reported that PO_4^{3-} concentration, rather than N or $Si(OH)_4$, was also positively associated with *O.* cf. *ovata* abundances and that N:P ratio was significantly and negatively related to *Ostreopsis* abundances in the northwestern Mediterranean Sea. In fact, if the bloom cells are consuming nutrients, one

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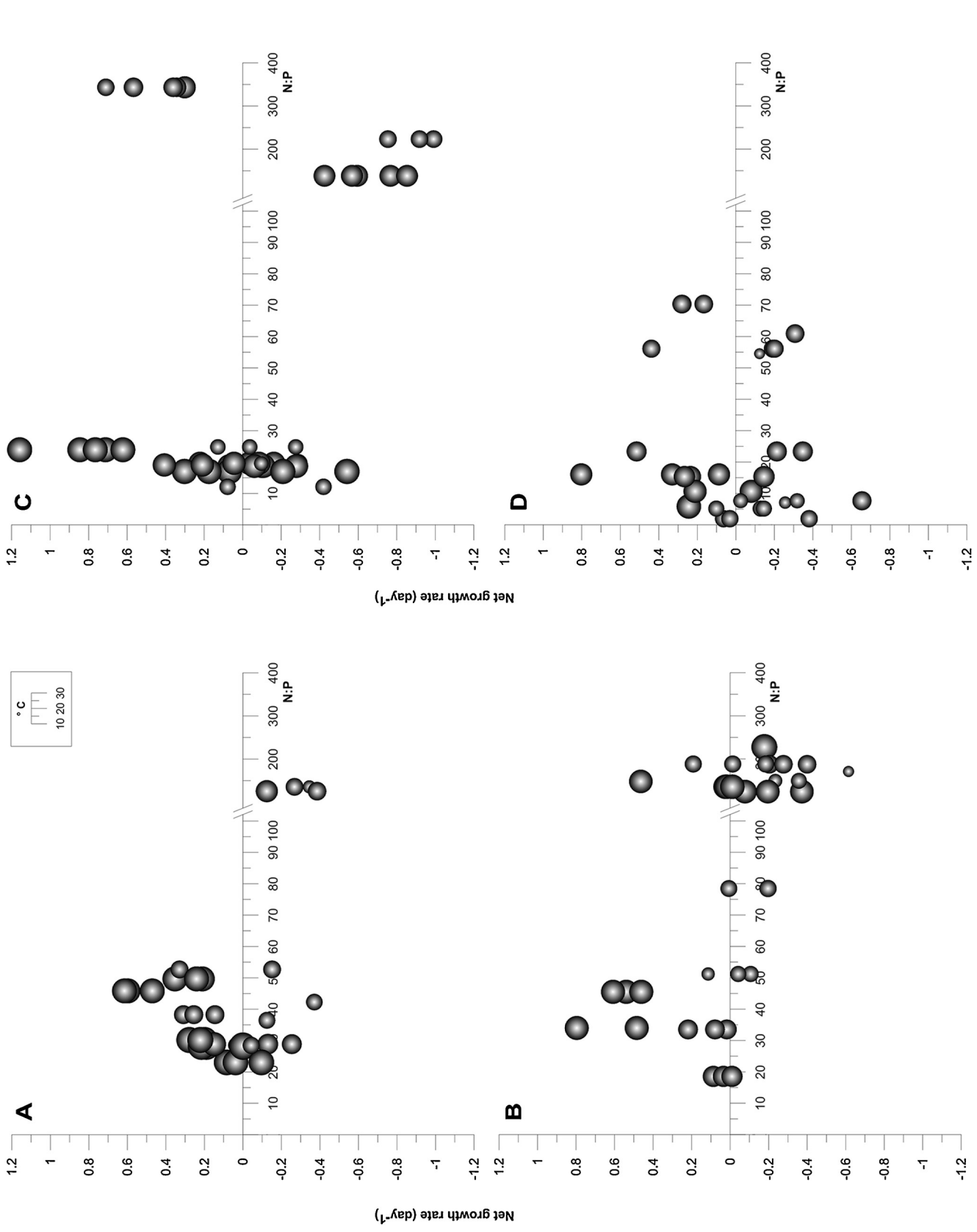

Fig. 7. *In situ* net growth rates of *Ostreopsis* cf. *ovata* expressed in day^{-1} (*y*-axis), in relation to N:P ratio (*x*-axis) and temperature values (size of circles, scale in the inset) in 2009 (A), 2010 (B), 2011 (C) and 2012 (D).

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

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

Interestingly, blooms were maintained in a condition of elevated N:P ratios, well above Redfield proportions, a condition normally taken to be indicative of P limitation. However, PO_4^{3-} was never depleted from the water column, and thus true P limitation likely never developed physiologically. However, net growth rates were slower than during the initial phase of blooms development. Such a progression of bloom growth in relation to N:P ratios would be consistent with the progression of *Prorocentrum* blooms previously described by Glibert et al. (2012). They suggested that such blooms may be initiated at low N:P levels (even less than Redfield ratio, but not in severely N limiting conditions), often stimulated by a "flush" of nutrients or organic materials that may allow a latent resident population (in our case, the newly germinating cells) to increase growth rate, as observed in blooms in Tolo Harbor, Chesapeake Bay, and Neuse River Estuary. After that growth rate increase, bloom biomass is able to increase often accompanied by N:P values greater than Redfield. At this point, these blooms are able to sustain high biomass levels, not necessarily by high growth rates, but also through other mechanisms, including (i) metabolic dissipatory strategies that allow the maintenance of cellular nutrient and energy balance in an environment where energy flow and nutrients are provided at unbalanced proportions and (ii) allelopathic and mixotrophic interactions with other species. Thus, as suggested by our observations on *Ostreopsis* blooms as well, while high growth rates (stimulated by an injection of P-rich water) may allow blooms to initiate, adaptive physiology would enable the maintenance of blooms at less than maximal growth rates and at non-optimal N:P ratios.

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

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

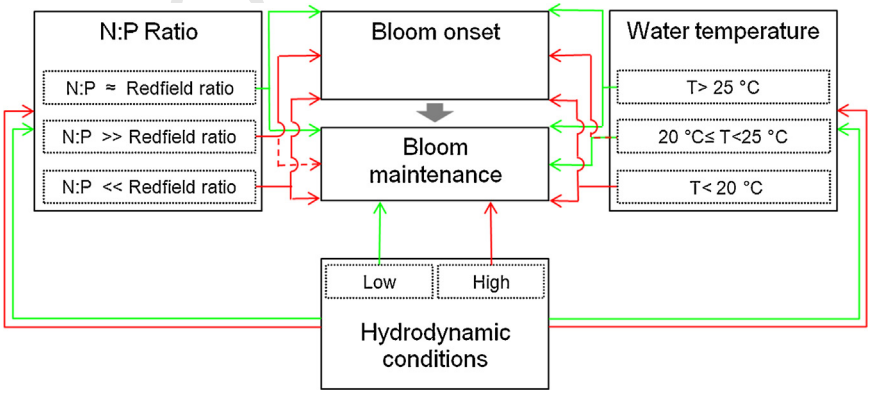

Fig. 8. Conceptual model of the effect of N:P ratio, water temperature and hydrodynamic conditions on both bloom onset and maintenance of *Ostreopsis* cf. *ovata*. Green and red arrows symbolize positive and negative effect, respectively. Dotted line represents a slight effect relative to solid line.

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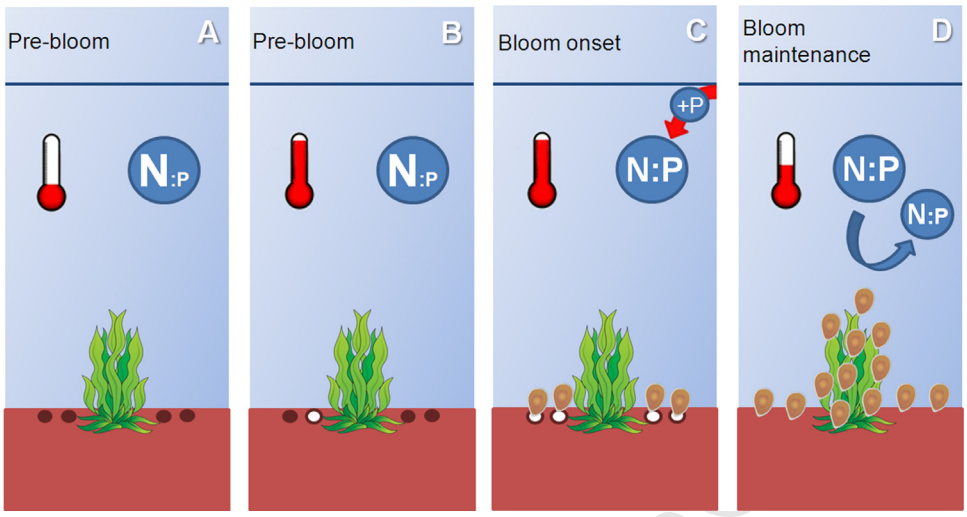

Fig. 9. Conceptual diagram of the effect of N:P ratio and water temperature on *Ostreopsis* cf. *ovata* bloom. Panel A represents the typical pre-bloom condition, showing low temperature and very high N:P ratio. Panel B represents the pre-bloom condition just before the bloom onset: although the temperature (>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.

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

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

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