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Effects of N and P availability on carbon allocation in the toxic dinoflagellate*Ostreopsis*cf.*ovata*

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ARTICLE INFO

Keywords:
Ostreopsis
Ovatoxins
Nutrients
Carbon content
C:N
C:P
N:P ratio

ABSTRACT

Blooms of the toxic dinoflagellate *Ostreopsis* cf. *ovata* are usually associated with shallow and calm coastal waters, characterized by low nutrient concentrations. The algal cells typically cover the benthic substrates, such as the macroalgal and invertebrate communities and rocks, forming a mucilaginous film. Data reported on *O.* cf. *ovata* toxin production observed under both field and culture conditions show high variability in terms of toxic profile and cellular content; little is known about the environmental and physiological aspects which regulate the toxin dynamics. In this study, *O.* cf. *ovata* physiology was investigated using batch cultures supplied with nutrient concentrations similar to those found in the Adriatic Sea during the recurrent blooms and the observed cellular dynamics were compared with those found in a culture grown under optimal conditions, used as a reference. Data on the cellular C, N and P content during the growth highlighted a possible important role of the cellular nutritional status in regulating the toxin production that resulted to be promoted under specific intervals of the C:N and C:P ratios. The variable toxicity found for *O.* cf. *ovata* in various geographic areas could be related to the different *in situ* prevalent environmental conditions (e.g., nutrient concentrations) which affect the cellular elemental composition and carbon allocation. The obtained results strongly suggest that in the environment toxin production is steadily sustained by a low and constant nutrient supply, able to maintain appropriate cellular C:N (>12) or C:P (>170) ratios for a long period. These results explain to some extent the variability in toxicity and growth dynamics observed in blooms occurring in the different coastal areas.

1. Introduction

Field and laboratory studies on the toxic dinoflagellate *Ostreopsis* cf. *ovata* have increased substantially in the last years, concomitantly to the spread of its blooms (Zina et al., 2012; Cohu et al., 2013; Casabianca et al., 2014; Escalera et al., 2014; Selina et al., 2014; Accoroni et al., 2015; Brissard et al., 2015). Attention has been posed to the development of rapid and valuable methods for the detection of toxins produced by this species (Ciminiello et al., 2010; Ciminiello et al., 2015; Garcia Altares et al., 2015), named isobaric palytoxin (isoPLTX) and ovatoxins (OVTXs), leading to the on going identification of new analogs in algal extracts (Brissard et al., 2015; Garcìa Altares et al., 2015). So far, no human intoxications ascribed to these toxins have been documen ted in the Mediterranean area, where *O.* cf. *ovata* causes recurrent blooms in the last decade, despite the presence of high amounts of OVTX a in seafood (Brissard et al., 2014) and respiratory problems due to inhalation of marine aerosol (Tichadou et al., 2010) have been detected. At the same time, studies have been focused on the toxicity of these compounds towards several organisms (Simonini et al., 2011; Gorbi et al., 2012; Faimali et al., 2012; Pagliara and Caroppo, 2012; Privitera et al., 2012) as well as on studies on the toxic mechanism of palytoxins (Bellocci et al., 2008; Crinelli et al., 2012; Del Favero et al., 2013, 2014; Pelin et al., 2013, 2014). Ovatoxins are complex polyketides (e.g., $C_{129}H_{223}N_3O_{52}$, molecular formula of the OVTX a) presenting a long and highly functionalized chain with both hydrophilic and lipophilic parts. Recently, a 100 fold lower OVTX a toxicity than that of the PLTX parent compound has been reported

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(Pelin et al., 2015); however, following the adverse effects ascribed to OVTXs detected in both algal cells and marine aerosols in the Mediterranean Sea (Durando et al., 2007; Ciminiello et al., 2008; Tichadou et al., 2010), it is very important to define the conditions promoting the toxin production in this dinoflagellate. With regards to the algal growth, *in situ* investigations on the environmental parameters characterizing *O.* cf. *ovata* blooms (Vila et al., 2001; Penna et al., 2005; Totti et al., 2010; Accoroni et al., 2011, 2012) as well as batch culture studies aimed to investigate *O.* cf. *ovata* physiological dynamics have been carried out. In particular, abiotic (e.g., nutrient, temperature, salinity and irradiance) and biotic (e.g., influence of the bacterial community) factors have all been shown to affect either *O.* cf. *ovata* growth and the ovatoxin content (Pistocchi et al., 2011; Pezzolesi et al., 2012; Scalco et al., 2012; Vanucci et al., 2012a,b; Yamaguchi et al., 2012, 2014; Tanimoto et al., 2013; Vidyarathna and Granéli, 2013). Nutrient depletion has been reported to affect toxicity with contrasting results, either decreasing the toxin content in an Adriatic strain (Vanucci et al., 2012b) and/or increasing the haemolytic activity of a Tyrrhenian strain, particularly in N deficient cultures (Vidyarathna and Granéli, 2013). The role of bacteria in remineralizing the organic matter, likely releasing N and P, resulted important in the growth population dynamics and in the toxin degradation (Vanucci et al., 2012a; Pinna et al., 2015). On one hand, environmental factors have been observed to substantially alter *O.* cf. *ovata* toxicity, on the other hand strain differences in toxicity and growth have been also noted (Ciminiello et al., 2012a,b; Brissard et al., 2015). More recently the growth and toxin dynamics of *O.* cf. *ovata* were studied in culture (Pezzolesi et al., 2014), focusing on the production of the main cellular compounds (polysaccharides, proteins and lipids) within the growth period in relation to the ovatoxin production and to the nutrient uptake. A rapid uptake of the external nutrients (P in particular) was observed, as well as the toxin, extracellular and total polysaccharide and lipid content increases during the growth: these physiological dynamics resulted ecologically convenient for the dinoflagellate, as a strategy to colonize the substrate and deter grazing, taking also into account that mucus has been recently hypothesized as a vehicle through which toxins are released into external medium (Giussani et al., 2015) .

The dinoflagellate *Ostreopsis* cf. *ovata* is usually found in shallow coastal waters in association with macroalgae; in well developed blooms cell aggregates are formed creating a mucilaginous film which covers the substrate, such as rocks and/or benthic organisms. Prior or during blooms either low N or P concentrations are usually found in the water column (Vila et al., 2001; Accoroni et al., 2011, 2014), and it has been suggested that nutrient availability alone is not the sole prerequisite for its bloom development (Accoroni et al., 2015). Nutrient availability and growth phase can alter toxicity, as already reported (Vanucci et al., 2012b; Vidyarathna and Granéli, 2013) and also observed for other dinoflagellates (e.g., *Alexandrium* spp., Anderson et al., 1990; *Karenia brevis*, Brown et al., 2006; *Gambierdiscus toxicus*, Lartigue et al., 2009). Recently, Pistocchi et al. (2014) varied inorganic N:P ratio at two different nutrient levels (a high and a low concentration) in batch cultures of *O.* cf. *ovata* and found (i) that the best condition for its growth was represented by high macronutrient levels in a balanced ratio (between 16 and 30) and (ii) that alkaline phosphatase activity was induced when external P was depleted or present in low amounts.

Culture media that are usually prepared for algal culturing contain high concentrations of N and P relative to C; however, environmental conditions which are usually found during algal blooms are quite different, particularly in terms of N and P concentrations, which are typically at least one order of magnitude lower. At these "nutrient stress" conditions the cellular dynamics are different, as well as the carbon acquisition and allocation by the cells, resulting in variations of primary and then secondary metabolic processes. As reported by Flynn and Flynn (1995), perturbations due to nutrient depletion induce stress and cause variations in the rate of synthesis of secondary metabolites, such as toxins produced by dinoflagellates. The effects of P stress has to be particularly taken into account, as the phosphorylation of metabolic intermediates and enzymes represent a mode of metabolic regulation. On the other hand, cellular N goes through amino metabolites, thus variations in the N status of the cell affect the intracellular free amino acid pool. Thus nutrient availability results to affect the algal metabolism and, as for *Ostreopsis* cf. *ovata*, could potentially affect the toxin production dynamics either as fast P utilizer organism and as N containing toxin dinoflagellate. Recently, it has been developed a conceptual model linking toxin production and nutritional status in *O.* cf. *ovata* and observed a toxin increase when both C:P and C:N were higher than the Redfield ratio (Pinna et al., 2015), supporting the idea that the cellular accumulation of C rich toxins (i.e., with C:N > 6.6) can be stimulated by both N and P deficiency, in accordance with the literature reviewed by Van de Waal et al. (2014) for most algal toxins.

As the patterns of change in the cellular concentration of N, P, C and toxin are closely linked, it is important to investigate the physiological aspects of ovatoxin production during the growth at nutrient stress conditions, in order to understand the cellular metabolism at nutrient concentrations comparable to those found during *Ostreopsis* cf. *ovata* blooms.

In the present study, the dinoflagellate *Ostreopsis* cf. *ovata* (strain OOAB0801) was grown under N,P depleted condition, i.e., at low nutrient concentrations comparable to the ones measured during algal blooms of this species in the Adriatic coastal areas (Accoroni et al., 2011); the nutrients were supplied with a balanced N:P ratio (e.g., 24) as this condition was supposed to be among the triggering factors for bloom onset (Accoroni et al., 2015). The results were compared with those of a culture performed in standard medium (N, P five fold diluted f/2), usually employed for *O.* cf. *ovata* in previous studies, used as control. Toxin content and growth dynamics were monitored as well as the N, P and C assimilation and allocation by the cells in the different phases of growth.

2. Materials and methods

2.1. Experimental setup and culture conditions

This study was performed using the strain OOAB0801 of *Ostreopsis* cf. *ovata* studied in Pezzolesi et al. (2014). Two sets of cultures, obtained from cultures previously acclimatized to the different growth conditions, were tested, differing only in the nitrate and phosphate concentrations: one set was cultured at a final N and P concentration of 176.6 μM and 7.3 μM, respectively (named "control"), the second set was cultured at 16.3 and 0.69 μM of N and P, respectively (named "N,P depletion"). The experimental setup was the same as described in Pezzolesi et al. (2014). Briefly, three replicate flasks (final culture volume of 2700 mL) for each condition were established at salinity 36 and maintained under illumination from cool white light at a photon flux density of 110 120 $\mu mol\ m^{-2}\ s^{-1}$, at $20 \pm 1\ ^{\circ}C$ on a 16:8 h light/dark cycle in a growth chamber. An aliquot for the analyses was collected from each flask at the sampling days (i.e., day 0, 1, 2, 6, 7, 9, 13, 15, 21, 27 and 35).

Control flasks were analysed for growth, elemental composition, toxin content, PAM measurement and bacterial abundance, while the other parameters were compared with those obtained in the previous study (Pezzolesi et al., 2014).

The presence of bacteria and their growth in the algal cultures were assessed by direct bacteria counts using epifluorescence

microscopy after staining with SYBR gold (Shibata et al., 2006), as reported in Vanucci et al. (2012a).

2.2. Nutrient determination

Nitrate and phosphate analyses were performed on filtered culture medium aliquots (Whatman GF/F filters, pore size 0.7 μm) and analysed spectrophotometrically (UV/VIS/NIR, JASCO V 650, Tokyo, Japan) according to Strickland and Parsons (1972).

2.3. Chlorophyll a determination

Chlorophyll a was extracted and determined as reported in Pezzolesi et al. (2014), using the equation reported by Ritchie (2006).

2.4. PAM fluorometry measurements

Kinetics and parameters of Photosystem II (PSII) were measured by means of pulse amplitude modulated fluorometry. The model used is: 101 PAM (H. Walz, Effeltrich, Germany) connected to a PDA 100 data acquisition system, high power LED Lamp Control unit HPL C and LED Array Cone HPL 470 to supply saturated pulses, US L665 and 102 FR to provide far red light and measuring light, respectively. During the growth a volume of algal culture (3 mL) was analysed in cuvettes (10 × 10 mm) mounted on an optical unit ED 101US/M.

2.5. Determination of carbohydrate content

Total polysaccharides were extracted following the Myklestad and Haug protocol (1972) and the total carbohydrate content of microalgae was determined by the Phenol Sulfuric Method (Dubois et al., 1956; Hellebust and Craigie, 1973) using glucose as the standard, as reported in Pezzolesi et al. (2014).

2.6. Cellular C, N and P analysis

Measurements of carbon (C) and nitrogen (N) in the cell were performed filtering culture aliquots (10 mL) on 1 cm GF/F glass fiber filters (Whatman) precombusted at 550 °C for 20 min. The filters were stored at 20 °C until analyses. The filters were dried in an oven at 60 °C for 30 min and encapsulated into tin containers. Elemental analysis was conducted using a ThermoFisher organic elemental analyzer (Flash 2000) configured for CHNS O determination using a copper/copper oxide column. The standard 2,5 bis (5 tert butyl 2 benzooxazol 2 yl) thiophene (BBOT) was used for calibration. Particulate phosphate (P) was measured photometrically (UV/VIS/NIR, JASCO V 650, Tokyo, Japan) after digestion with a solution of 5% potassium peroxide using the method described by Menzel and Corwin (1965).

C:N, C:P and N:P ratios were calculated in terms of atoms.

2.7. Toxin analysis

2.7.1. Sample extraction

Cell samples were obtained by filtering an aliquot of culture (150 250 mL) using glass fiber filters (Whatman GF/F, pore size 0.7 μm), and the obtained filters were stored at 80 °C until the extraction. All organic solvents used for the toxin extraction and analysis were of distilled in glass grade (Carlo Erba, Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). For the toxin extraction, 1 3 mL of a methanol/water (1:1, v/v) solution was added to each filter and then sonicated for 2 min in pulse mode, while cooling in an ice bath. The mixture was centrifuged at 3000*g* for 15 min, the supernatant was decanted and the pellet was washed twice with 0.5 1 mL of methanol/water (1:1, v/v). The extracts were combined and the volume adjusted to 2 5 mL with extracting solvent. The obtained mixture was analysed directly by LC HRMS (5 μL injected). Recovery percentages of the above extraction procedures were estimated to be 98% (Ciminiello et al., 2006). Each growth medium (V = 100 mL) was extracted five times with an equal volume of butanol. The butanol layer was evaporated to dryness, dissolved in 2 mL of methanol/water (1:1, v/v) and analysed directly by LC HRMS (5 μL injected). Recovery percentage of this extraction procedure was estimated to be 75% (Ciminiello et al., 2006).

2.7.2. Liquid chromatography high resolution mass spectrometry (LC HRMS)

The LC HRMS analyses were carried out on an Agilent 1100 LC binary system (Palo Alto, CA, USA) coupled to a hybrid linear ion trap LTQ Orbitrap XL™ Fourier Transform MS (FTMS) equipped with an ESI ION MAX™ source (Thermo Fisher, San Josè, CA, USA). Chromatographic separation was accomplished by using a 3 μm Gemini C18 (150 × 2.00 mm) column (Phenomenex, Torrance, CA, USA) maintained at room temperature and eluted at 0.2 mL min ^{1} with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid (Laboratory grade, Carlo Erba). A slow gradient elution was used: 20 50% B over 20 min, 50 80% B over 10 min, 80 100% B in 1 min, and hold 5 min. This gradient system allowed a partial chromatographic separation of most palytoxin like compounds.

High resolution full mass spectrometry experiments (positive ions) were acquired in the range m/z 800 1400 at a resolving power of 60,000. The following source settings were used in all LC HRMS experiments: a spray voltage of 4.8 kV, a capillary temperature of 290 °C, a capillary voltage of 35 V, a sheath gas and an auxiliary gas flow of 29 and 17 (arbitrary units). The tube lens voltage was set at 145 V.

Quantitative determinations of isobaric palytoxin, ovatoxin a, b, c, d and e in the extracts were carried out using a calibration curve (triplicate injection) of palytoxin standards (Wako Chemicals GmbH, Neuss, Germany) at four levels of concentration (100, 50, 25, 12.5 and 6.25 ng mL ^{1}) and assuming that their molar responses were similar to that of palytoxin. Calibration curve equation was $y = 9698.2372x$ 56140.7464 and its linearity was expressed by $R^2 = 0.999$. Extracted ion chromatograms (XIC) for palytoxin and each ovatoxins were obtained by selecting the most abundant ion peaks of both $[M + 2H \quad H_2O]^{2+}$ and $[M + H + Ca]^{3+}$ ion clusters. A mass tolerance of 5 ppm was used. Instrumental limit of detection for palytoxin was 6.25 ng mL ^{1}. Based on extraction volume, the presence of toxins in the growth medium (100 mL each) could be estimated at level 0.125 ng mL ^{1}.

2.8. Calculations

Specific growth rate (μ, day ^{1}) was calculated using the following equation:

$$\mu \quad \frac{\ln N_1 \quad \ln N_0}{t_1 \quad t_0} \tag{1}$$

where, N_0 and N_1 were cell density (cell mL ^{1}) values at time t_0 and t_1.

Calculations of cell volume were performed with the assumption of ellipsoid shape using the following equation (Sun and Liu, 2003):

$$V \quad \frac{\pi}{6} abc \tag{2}$$

where, a = dorsoventral diameter (DV, μm), b = width (W, μm) and c = mean anteriorposterior diameter (AP, μm).

The nutrient (NO_3 N and PO_4 P) uptake rate (U, pmol day ^{1} cell ^{1}) was calculated according to Lim et al. (2006) from the residual nutrient concentrations in the medium (C) and the difference in cell densities (γ) between days, when the depletion of nutrients was linear. The following equations were used:

$$U \quad \frac{C_1 \quad C_0}{\gamma \Delta t} \tag{3}$$

$$\gamma \quad \frac{N_1 \quad N_0}{\ln N_1 \quad \ln N_0} \tag{4}$$

where, C_0 and C_1 were the nutrient concentrations (μM) at time t_0 and t_1, and N_0 and N_1 were the corresponding cell densities (cell mL ^{1}).

Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (Φ_{PSII}) (Bolhar Nordenkampf and Oquist, 1993) and effective quantum yield of PSII (Φ'_{PSII}) (Genty et al., 1989), calculated as:

$$\Phi_{PSII} \quad \frac{F_m \quad F_0}{F_m} \quad \frac{F_V}{F_m} \tag{5}$$

$$\Phi'_{PSII} \quad \frac{F'_m \quad F}{F'_m} \quad \frac{\Delta F}{F'_m} \tag{6}$$

where, all parameters were given by induction curve measure ments. The minimal fluorescence (F_0) was measured on dark adapted cultures for 20 min, by using modulated light of low intensity (2 μmol m ^{2} s ^{1}). Then, a short saturating pulse of 3000 μmol m ^{2} s ^{1} for 0.8 s induced the maximal fluorescence yield (F_m). Changes of the maximal fluorescence yield (F'_m) were induced on light adapted cultures by saturating flashes given periodically every 60 s. The steady state value of fluorescence immediately prior to the flash is termed F.

The total toxin production rate μ_{TOX} (day ^{1}) in the cultures throughout the growth phase was calculated using the following equation:

$$\mu_{TOX} \quad \frac{\ln\left(\frac{N_1 T_1}{N_0 T_0}\right)}{t_1 \quad t_0} \tag{7}$$

where, $N_t T_t$ was the toxin concentration (pg PLTXs mL ^{1}) calculat ed by multiplying the cell concentration N_t (cells mL ^{1}) by the cellular toxin content T_t (pg cell ^{1}) at time t.

To account for the effect of cell growth rates on toxin production, the net toxin production rate R_{TOX} (pg PLTXs cell ^{1} day ^{1}) was determined over each growth phase according to Anderson et al. (1990):

$$R_{TOR} \quad \frac{N_1 T_1 \quad N_0 T_0}{\gamma (t_1 \quad t_0)} \tag{8}$$

where, γ is the same as in Eq. (4).

2.9. Statistical analysis

Temporal differences in cell abundance, cell growth rate, cell size, cell volume, cell content and concentrations of the various compounds (i.e., polysaccharides and toxins) between the two conditions were tested by a repeated measures analysis of variance (ANOVA), using STATISTICA (StatSoft) software. Whenever a significant difference for the main effect was observed ($p < 0.05$) a Tukey's pairwise comparison test was also performed.

3. Results

3.1. Cell growth and dimensions

Growth curves of *Ostreopsis* cf. *ovata* reported at the two different conditions (i.e., nutrient replete and N,P depleted condition) are shown in Fig. 1. Cultures had initial cell densities of about 160 cells mL ^{1} and reported different growth patterns as a consequence of the differences in the N and P initial concentra tions. The control curve had a pattern similar to that described in Pezzolesi et al. (2014), where the same *O.* cf. *ovata* strain was studied: the exponential phase lasted till day 7, with a mean growth rate of 0.46 ± 0.00 day ^{1} and a final yield in the stationary phase of 6500 7000 cell mL ^{1}. Cells grown under N,P depletion had a shortened exponential phase and entered in the stationary phase between day 2 and 6, concomitantly with the exhaustion of both nutrients (NO_3 N and PO_4 P, Fig. 2), reporting a significantly (ANOVA, $p < 0.05$) lower growth rate (0.27 ± 0.02 day ^{1}) and max cell densities around 1000 cell mL ^{1}. Nutrients were rapidly taken up by cells during the first day of growth, resulting in high uptake rates (U) of 26.4 ± 0.6 and 2.5 ± 0.4 pmol day ^{1} cell ^{1} for N and P, respectively, which then decreased to 10.2 ± 1.2 and 0.2 ± 0.1 pmol day ^{1} cell ^{1} between day 1 and 2.

In N,P depletion, *Ostreopsis* cf. *ovata* cell dimensions and volumes varied significantly (ANOVA, $p < 0.05$) during the growth (Fig. 3), reporting a decreasing trend (from about 37,000 to 27,000 μm^3) during the exponential phase, as a consequence of cell division, followed by an increase to about 35,000 μm^3 in the stationary phase. In the late stationary phase cell volumes decreased again, reaching values about 28,000 30,000 μm^3. The dorsoventral to width (DV/W) ratio was not significantly different over the growth and had values around 1.2 1.3; the dorsoventral to anteroposterior (DV/AP) ratio had values of around 1.6 at the beginning of the growth and significantly (ANOVA, $p < 0.05$) increased to 1.8 during active cell division, reaching a value of 1.9 in the stationary phase.

In the N,P depleted condition, maximum chlorophyll a con centration (Fig. 4) was about 15 μg L ^{1} measured while cells were in the early stationary phase (day 6 9), then it decreased to a value of about 5 μg L ^{1} in the late stationary phase. On a cell basis the maximum value was reported at the beginning of the growth (about 20.5 pg cell ^{1}, day 2), then it decreased to a value of about 5 pg cell ^{1} in the stationary phase.

3.2. Cellular components

3.2.1. Polysaccharides

Total polysaccharides content in N,P depleted condition is reported in Fig. 5. Polysaccharides were produced from the first days of growth till reaching a maximum concentration of 6.23 μg mL ^{1} corresponding to 5.77 ng cell ^{1} at day 13. From

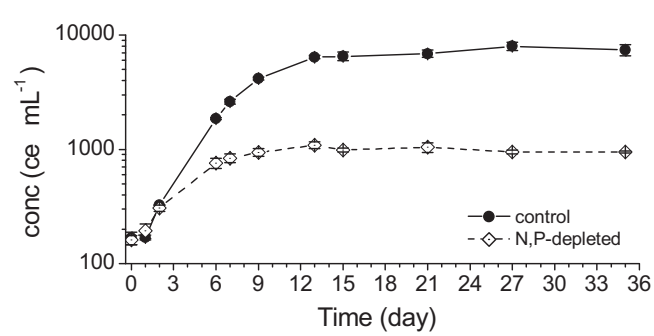


Fig. 1. Growth pattern of *O.* cf. *ovata* cells in control and N,P-depleted conditions. Bars indicate standard deviation (mean of three replicates).

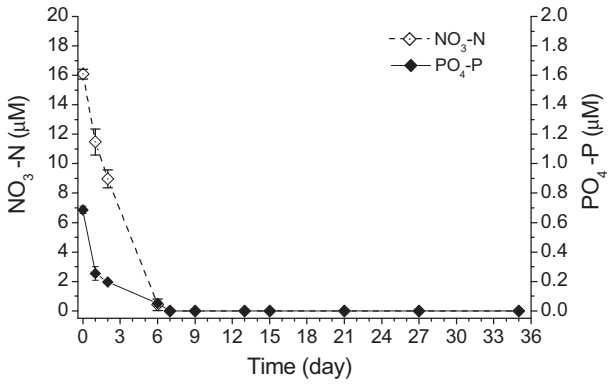


Fig. 2. Nitrogen (NO_3–N) and phosphorus (PO_4–P) concentrations measured in *O.* cf. *ovata* culture medium under N,P-depleted condition. Bars indicate standard deviation (mean of three replicates).

the mid to late stationary phase their amount significantly decreased (ANOVA, $p < 0.01$) either per cell or per culture volume (ng cell ^{1} or μg mL ^{1}).

3.2.2. Toxins

Toxin content was investigated either in the algal extracts and in the extracellular medium, under both N,P depleted and control conditions. The LC HRMS analysis of the algal extracts of the N,P depleted condition revealed the presence of several ovatoxins (OVTX a, b, c, d, e; Fig. 6). The concentration of all the individual toxins increased from the beginning of the growth, starting from 2.5 pg cell ^{1} at day 2 to 5.28 pg cell ^{1} at day 6. At the beginning of the stationary phase (day 6 9) total toxin content remained almost constant (5.36 pg cell ^{1}, day 9), but subsequently increased significantly (ANOVA, $p < 0.01$) to a value of about 10 pg cell ^{1} in the late stationary phase (day 21 35). The relative percentage of each single toxins remained almost constant throughout the growth curve: the most abundant toxin was ovatoxin a (56 59%), followed by ovatoxin b (25 29%), d + e (11%) and c (4%). Total toxin content expressed on a culture volume basis (Fig. 7) increased significantly (ANOVA, $p < 0.01$) from the beginning of the growth till day 21, ranging from 0.77 to 9.8 ng mL ^{1}, then it remained constant till day 35.

In the control condition the toxin amount increased mainly during the stationary phase, in fact it was 46.7 ng mL ^{1} at day 9, and increased to 127.8 and 155.6 ng mL ^{1} at day 21 and 27, respectively. In this condition, ovatoxins were released extracellularly during the growth: analyses showed the presence of toxins

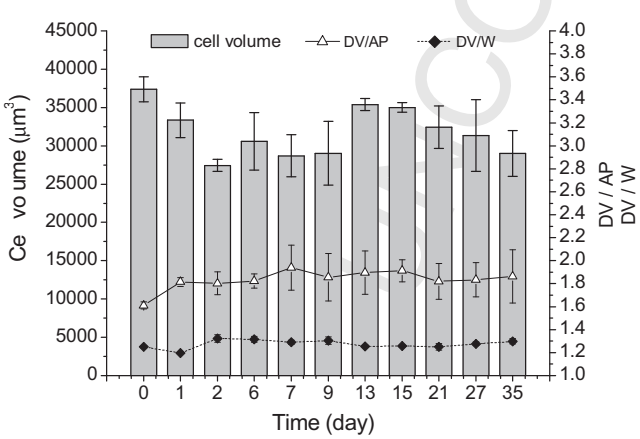


Fig. 3. Mean cell volume, dorsoventral to anteroposterior (DV/AP) ratio and dorsoventral to width (DV/W) ratio of *O.* cf. *ovata* cells grown under N,P-depleted condition. Bars indicate standard deviation (mean of three replicates).

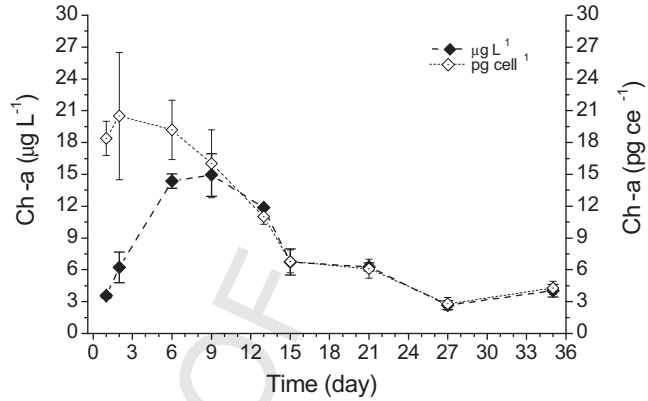


Fig. 4. Content of chlorophyll-a (Chl-a) expressed either on a culture volume (μg L ^{1}) and on a cell basis (pg cell ^{1}) in *O.* cf. *ovata* cells grown under N,P-depleted condition. Bars indicate standard deviation (mean of three replicates).

in the medium starting from day 9, when the amount released was about 6%, then it increased to about 9% at the beginning of the stationary phase and reached 27% in late stationary phase (day 27). In the N,P depleted condition, extracellular toxins were not detectable (values <LOD, 0.125 ng mL ^{1}).

Under nutrient stress the highest toxin production rate (μ_{TOX}) was reported during the exponential phase (Fig. 6, inset), with a maximum value of about 0.41 ± 0.04 day ^{1} between days 2 and 6, then it decreased to 0.08 ± 0.03 day ^{1} at the early stationary phase (days 6 9) and to a minimum value of 0.03 ± 0.00 day ^{1} during the late stationary phase. The net toxin production rate (R_{TOX}), which considers the effect of cell growth rates on toxin production, reported the same trend in fact R_{TOX} values varied from 1.6 ± 0.1 to 0.4 ± 0.2 pg PLTXs cell ^{1} day ^{1} between days 2 6 and days 6 9, respectively, to 0.2 ± 0.0 pg PLTXs cell ^{1} day ^{1} in the stationary phase (days 9 35).

3.3. Cellular content: Carbon, nitrogen and phosphorus

Cells of *Ostreopsis* cf. *ovata* grown under N,P depleted condition displayed differences in the cellular content and C:N, C:P and N:P ratios (Figs. 8 and 9) with respect to the control. Nitrogen and phosphorus contents (Fig. 8) were significantly higher in exponential phase cells than in stationary phase cells in both conditions (ANOVA, $p < 0.05$). The initial (day 1) cellular N content ranged from 1.26 ng cell ^{1} to 1.12 ng cell ^{1} in N,P depletion and control, with no significant differences between the two conditions. N content decreased along the growth to values of about 0.35 0.40 ng cell ^{1} in

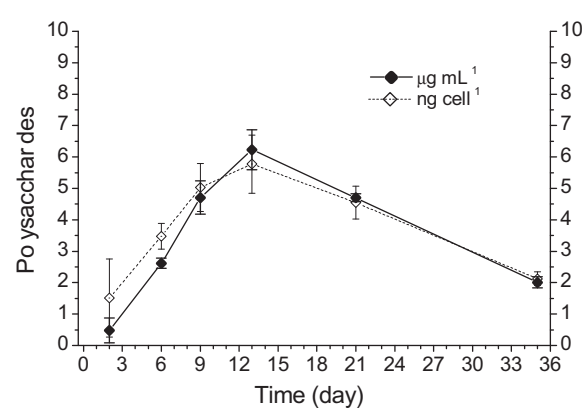
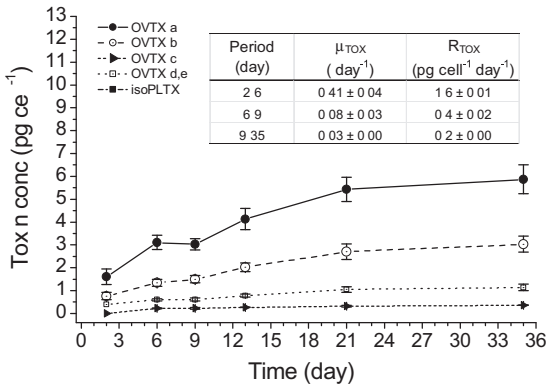


Fig. 5. Total polysaccharides content in *O.* cf. *ovata* cells grown under N,P-depleted condition, expressed either on a culture volume (μg mL ^{1}) and on a cell basis (ng cell ^{1}). Bars indicate standard deviation (mean of three replicates).



Period (day)	μ_{TOX} (day^{-1})	R_{TOX} (pg cell^{-1} day^{-1})
2 6	0 41 ± 0 04	1 6 ± 0 01
6 9	0 08 ± 0 03	0 4 ± 0 02
9 35	0 03 ± 0 00	0 2 ± 0 00

Fig. 6. Individual toxin content of isobaric palytoxin (isoPLTX), ovatoxin (OVTX)-a, -b, -c and -d plus -e of *O.* cf. *ovata* cells grown under N,P-depleted condition, expressed on a cell basis (pg cell ^{1}). Bars indicate standard deviation (mean of three replicates). Inset: total (μ_{TOX}) and net (R_{TOX}) toxin production rates calculated between different days during the growth curve.

the stationary phase. On the contrary, cellular P at day 1 was 0.12 and 0.38 ng cell ^{1} in the nutrient stressed and in the control condition, respectively; it decreased rapidly to 0.04 ng cell ^{1} at day 6 when cells were nutrient limited, while it decreased more gradually in the control and reached 0.04 ng cell ^{1} only in the stationary phase (day 13 35).

As for cellular C, at day 1 values were 10.91 ng cell ^{1} and 10.77 ng cell ^{1} in N,P depletion and control, respectively. In the control, there was an initial cellular C drop to 3.17 ng cell ^{1} (day 7) while cells were dividing (exponential phase), then from day 9 cellular C content increased to a final value about 7.52 ng cell ^{1} (day 35). In nutrient stressed cultures, cellular C content decreased as well in the exponential early stationary phase (5.84 ng cell ^{1} at day 7), however it maintained higher values than control cells. Conversely, in the stationary phase, C content increased to values around 7 ng cell ^{1}.

The molar C:N ratio had an initial value of 11 in both conditions (Fig. 9), then it decreased in the control to a value of 6.9 in the exponential phase (day 7 9) and to 8.6 under nutrient stress at day 2. C:N ratios increased to final values of about 25 in both nutrient conditions, as a result of C accumulation within the cell. N:P ratios reflected the initial nutritional status of the cells: values were about 24 at the beginning of the growth under nutrient stress, while in the control N:P ratio had initial lower values (about 7 8) which then increased during the exponential phase. In the stationary phase N:P ratio stabilized to a value around 24, which corresponded to the N:P ratio supplied initially in the growth medium in both conditions.

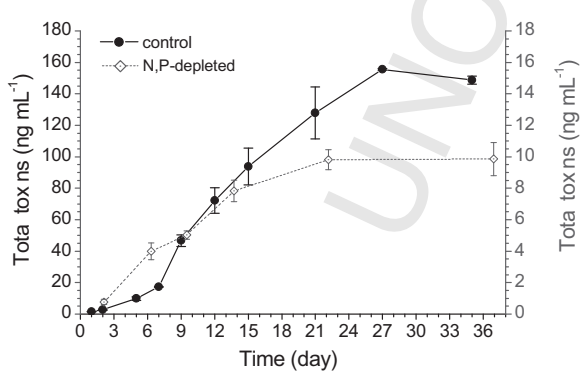


Fig. 7. Total toxin content of *O.* cf. *ovata* cells in control and N,P-depleted conditions during the growth, expressed on a culture volume basis (ng mL ^{1}). Bars indicate standard deviation (mean of three replicates).

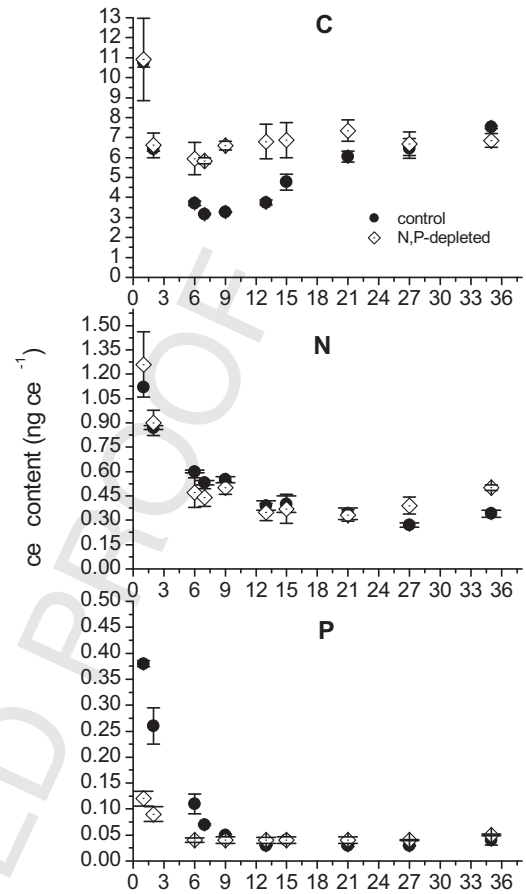


Fig. 8. Elemental composition (ng cell ^{1}) in terms of carbon (C), nitrogen (N) and phosphorus (P) of *O.* cf. *ovata* cells in control and N,P-depleted conditions during the growth. Bars indicate standard deviation (mean of three replicates).

C:P ratios increased during the growth, as a consequence of cellular C accumulation, leading to values significantly higher than the Redfield ratio (106): C:P ratios of 450:500 were found in both conditions in the stationary phase.

3.4. PAM

Max (Φ_{PSII}) and effective (Φ'_{PSII}) quantum yields followed the growth pattern observed for *Ostreopsis* cf. *ovata* cells, reporting a similar trend (Fig. 10). It is noticeable as the max quantum yields increased during the exponential phase till day 6 and 9 with the highest values of 0.20 and 0.45 in nutrient stress and control conditions, respectively, then yields decreased in the stationary phase (0.02 and 0.23, respectively). Under nutrient limited conditions the PSII efficiency showed the lowest values.

3.5. Bacterial growth

The bacterial growth pattern was investigated throughout *Ostreopsis* cf. *ovata* growth in both N,P depleted and control conditions (Fig. 11). In the control bacterial initial densities were on average about 260,000 cell mL ^{1}, while in N,P depletion about 460,000 cell mL ^{1}. Growth patterns were not significantly differ ent till day 5, when bacteria were about 2.3×10^{6} cell mL ^{1}, then in the control bacterial concentration increased more sharply reaching a value of about 2.3×10^{7} cell mL ^{1} at day 35, while in N,P depletion cell numbers were about one order of magnitude lower (7.5×10^{6} cell mL ^{1}).

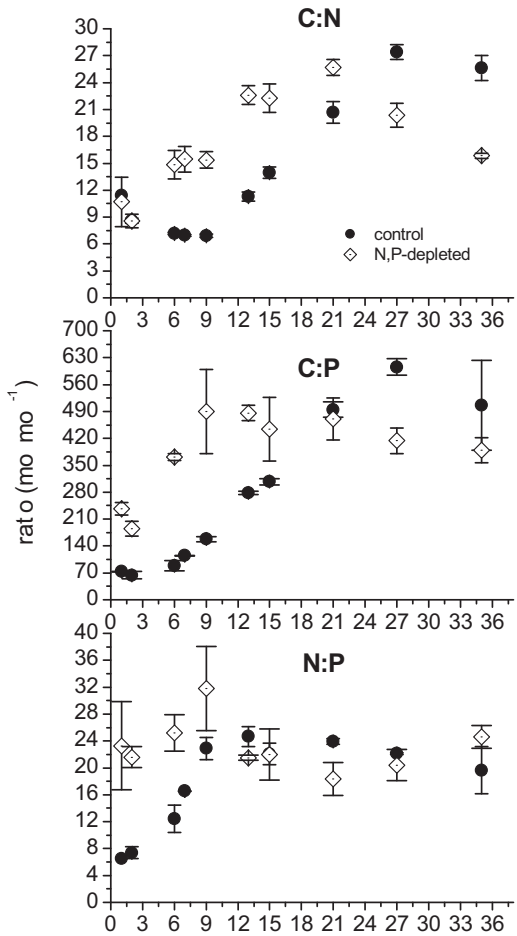


Fig. 9. Cellular C:N, C:P and N:P ratios (mol mol $^{-1}$) of *O.* cf. *ovata* during the growth in control and N,P-depleted conditions. Bars indicate standard deviation (mean of three replicates).

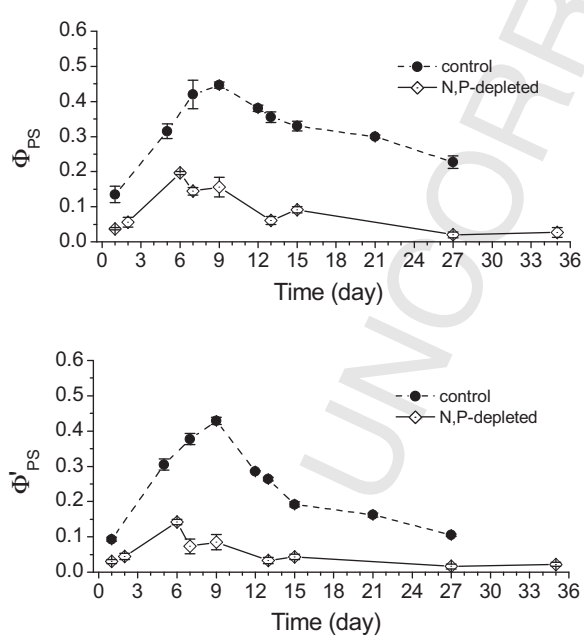


Fig. 10. Maximum (Φ_{PSII}) and effective (Φ'_{PSII}) quantum yields determined from active fluorometry (PAM) during *O.* cf. *ovata* growth in control and N,P-depleted conditions. Bars indicate standard deviation (mean of three replicates).

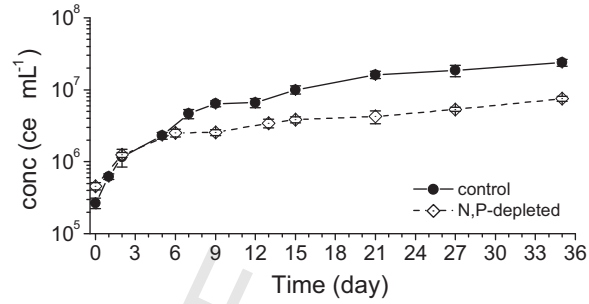


Fig. 11. Growth of bacteria in *O.* cf. *ovata* cultures in control and N,P-depleted conditions. Bars indicate standard deviation (mean of three replicates).

4. Discussion

Growth curves of *Ostreopsis* cf. *ovata* obtained in the present study under nutrient replete conditions confirmed the growth pattern previously reported in several studies (Pezzolesi et al., 2012, 2014; Vanucci et al., 2012b; Vidyarathna and Granéli, 2012, 2013) and can be considered as optimal reference values. Growth rates in the newly studied N,P depleted condition were signifi cantly lower than in the control, in agreement with results reported in Vanucci et al. (2012b), where cultures were performed under unbalanced conditions (N or P limitation), evidencing as nutrient limitation affects either growth rates and final cell yields. The environmental nutrient concentrations were previously reported to interfere with nutrient uptake dynamics and timing (John and Flynn, 2000; Vanucci et al., 2012b), and this is confirmed also by the results obtained in the present study: nutrient uptake rates within the first days were comparable between control (Pezzolesi et al., 2014) and N,P depletion, attesting that cells rapidly consume nutrients (P in particular) when available but, as the nutrient concentrations become low, the uptake rates drop.

In cultures under nutrient limitation, during the late stationary phase, cells were 17% smaller than those found in the controls. In *Ostreopsis* cf. *ovata* variations in cell size appear as a usual response to environmental conditions: anomalous round forms were observed, for example, in media with high ionic strength (Aligizaki and Nikolaidis, 2006; Pagliara and Caroppo, 2012; and personal observations); small cells have been interpreted as (i) gametes (Giacobbe and Yang, 1999; Bravo et al., 2012), or (ii) fusing gametes (Accoroni et al., 2014) or (iii) related to adverse conditions: for example small cells were detected under unfavorable light and temperature (Scalco et al., 2012), in nutrient limited cultures (Vanucci et al., 2012b) and in the absence of bacteria (Vanucci et al., 2012a). Finally, size variations observed in standard growth conditions were also highly reproducible, and characterized by small cells during active divisions and large cells in the stationary phase (Pezzolesi et al., 2014). This last increasing decreasing cell volume trend was reported also during field surveys performed in different bloom periods (Accoroni et al., 2012), thus comparing cell size variations, as well as their average values, measured in the field with cell sizes observed in nutrient replete cultures, it is possible to hypothesize that natural population develop under nutrient replete conditions.

The growth kinetics and size of microalgae also influence the biochemical composition and reserve accumulation in microalgal cells: for instance the microalgal population growth rate affects metabolites (e.g., proteins, pigments, carbohydrates and lipids) accumulation rate, and the nutrient condition determines their productivity and content per microalgal biomass. A rapid and large decrease in chlorophyll content, following nutrient exhaustion, was already observed in control cultures (Pezzolesi et al., 2014); in the present study under nutrient stress the decrease was more

marked, as expected. On the contrary, the polysaccharides pattern along the growth in the nutrient limited condition highly differed from that reported for the control (Pezzolesi et al., 2014). In the exponential phase, the polysaccharides increased in both conditions and their content per cell was comparable, then it differed in the stationary phase, steadily decreasing and being significantly lower in the nutrient stressed condition. This behaviour is opposite to the one usually observed in microalgae exposed to N and P depletion where the reduced carbon compounds are increasingly accumulated or excreted (Myklestad and Haug, 1972; Guerrini et al., 2000). The decreasing trend observed in the stationary phase of the present cultures might be related to the lower photosynthetic activity (discussed below) as well as be linked, in a certain way, to the lower toxin content of the algal cells in N,P limitation, which did not report any extracellular release of toxins. A recent study (Giussani et al., 2015) reported the direct role of *Ostreopsis* cf. *ovata* mucous filaments in conveying toxicity, as the mucous matrix could interlock the organisms, enhance the surface of the contact area, and actively disseminate toxins. These results confirmed the potential role of the mucilaginous mat in defence against grazing (Liu and Buskey, 2000; Honsell et al., 2013) and/or in adaptation to live in different benthic habitats (Totti et al., 2010; Parsons et al., 2012). As reported for this species (Giussani et al., 2015) and for other microalgal species (Blossom et al., 2012), mucus could be a vehicle through which toxins are released into external medium. Present results evidenced as in nutrient limited condition toxins were not found in detectable amounts in the extracellular medium, probably as the total toxin amount was significantly lower (about 16 fold) than in the control. Moreover, it could be hypothesized that under nutrient stress the toxin fraction released extracellularly was even lower (<1.3%) than the one (up to 27%, Vanucci et al., 2012b; Pezzolesi et al., 2012; present study) usually found under growth conditions which promote toxin production, as reported in literature under N deficiency (3% in the stationary phase, Vanucci et al., 2012b).

Results of this study confirmed the qualitatively constant toxin profile and the quantitative increase in toxin content during the growth (Brissard et al., 2014; Pezzolesi et al., 2014), either in nutrient limited or in control conditions. On the contrary, in this study it was not observed a decrease of the cellular toxin content, usually associated to the cellular division which divide the "mother" toxin content between the "daughters" cells (Granéli and Flynn,2006; Pezzolesi et al., 2014), during the exponential phase, probably due to the lower division rates (0.27 vs 0.49 day ^{1} in nutrient limitation and control, respectively) of the cells in nutrient stress. The strain used in this study produced several of the most common ovatoxins (i.e., OVTX a, b, c, d, e), but not the more recently discovered OVTX f, g and h (Ciminiello et al., 2012b; Brissard et al., 2015; Garcìa Altares et al., 2015). Under nutrient limitation, cells confirmed a significantly lower toxin production, in agreement with a previous study on this species (Vanucci et al., 2012b) or studies on other dinoflagellates (John and Flynn, 2000; Granéli and Flynn, 2006). Toxin content per cell was about 2.5 fold lower than in control while when expressed per culture volume (ng mL ^{1}), it was about 16 fold lower, attesting that toxin production and growth dynamics are strictly linked and could severely affect the toxicity of a bloom. On the contrary, *Alexandrium* spp. (John and Flynn, 2000; Touzet et al., 2007; Xu et al., 2012) reported enhanced toxin synthesis in response to nutrient deprivation: in particular, when cells were P limited, alkaline phosphatase activity in *Alexandrium minutum* developed only after several days of P deprivation. On the contrary, in *Ostreopsis* cf. *ovata* it has been reported that alkaline phosphatase activity increased immediately after P depletion (Pistocchi et al., 2014), thus this different response in terms of enzyme synthesis could affect the cellular metabolism and toxin biosynthesis, especially considering that "the effects of P depletion may be complex through the involvement of the phosphorylation of metabolic intermediates and enzymes as a common mode of metabolic regulation" (Flynn and Flynn, 1995). In N,P limited cultures, toxins were produced mainly during the exponential phase, then the toxin production rate decreased. Although slightly lower under nutrient limitation (2.2. vs 1.6 pg toxin cell ^{1} day ^{1}), the maximum net toxin production rate *R* was comparable under both conditions, meaning that in the first days nutrients are immediately taken up by cells and used for the cell metabolism, independently by their initial concentrations. Moreover, this suggests that cellular toxin content is directly influenced by time dependent variations of nutrients rather than by the indirect impact of nutrients on growth rates. In the field variable toxin amounts per cell were measured due to either genetic variability among the single clones, and the complexity of the sampling method and sample treatment. Actually, toxin content reported in wild cells of the Adriatic Sea ranged between 14 and 72 pg cell ^{1} in the western coast (Accoroni et al., 2011), and 22 and 75 pg cell ^{1} in the eastern coast samples (Pfannkuchen et al., 2012).

Generally, C:N ratio can be used as an indicative index for nutritional status and physiological behaviour (Cullen and Horrigan, 1981), and it is attested that N metabolism plays a role in regulating growth rate (Flynn and Flynn, 1995). At the beginning of the growth, low C:N ratios suggest that a phototrophic metabolism is prevailing which is able to maintain high growth rates, while the increase in C:N ratios in the stationary phase may reflect the enhanced energy reserve polymers (Geider et al., 1998; Geider and La Roche, 2002). C:N ratio in *Alexandrium tamarense* cultured with nitrate was about 6 10 and cellular N about 0.4 0.5 ng cell ^{1}, while cellular C was about 3 3.5 ng cell ^{1} (Leong and Taguchi, 2004; Leong et al., 2010), in agreement with the reported results on nutrient replete cultures. C:N and C:P ratios, which represent important factors for cell physiology (John and Flynn, 2000), found in the present study were much higher than those usually considered for cultured phytoplankton (e.g., Redfield ratio) where the N:P ratio can range from 5 to 20 under extreme nutrient limitation (Ryther and Dunstan, 1971; Nalewajko and Lean, 1980). An optimum stoichiometric C:N:P ratio of 119:17:1 has been reported for benthic microalgae (Hillebrand and Sommer, 1999); however *Ostreopsis* cf. *ovata* grown in optimum conditions reported a C:N:P ratio of 186:17:1 (Vidyarathna and Granéli, 2013). In this study, values closed to those proposed for benthic microalgae at the beginning of the stationary phase in control condition (124:17:1) were observed, then the atomic ratios increased significantly attesting the ability of *O.* cf. *ovata* to accumulate C rich compounds which could be also partially extruded from the cells (e.g., polysaccharides, toxins) as an ecological strategy.

Values of C:N and C:P ratios measured in N,P depleted condition were close to optimal values only at day 2 of growth, then both steadily increased up to day 21 after which declined to values significantly lower than those in the controls. On the contrary, N:P values which were also higher than in the controls in the first days of growth, attested at values between 18 and 26 for all the growth period.

The ratios of C:N and C:P higher than 12 and 170, respectively, were previously linked to increased toxin production (Pinna et al., 2015) and the present study confirmed this assumption: the supposed critical C:N and C:P values were reached from day 6 of growth and a parallel increase in toxin content per cell was observed, however the toxin synthesis stopped after day 21 concomitantly with the C:N and C:P ratios decrease, contrarily to the control condition where toxins continue to be produced. This result is in accordance with the hypothesis proposed by Ianora et al. (2006) attesting that defence compounds (e.g., toxins) are produced under high carbon conditions.

Changes in the overall photosynthetic capacity during *Ostreopsis* cf. *ovata* growth in control and nutrient stress conditions were investigated, to the best of our knowledge, for the first time and could provide additional insights into the observed metabolic behaviour. The F_v/F_m parameter (Φ_{PSII}) provides an estimate of the maximum quantum yield of PSII. Control cells maintained a high Φ_{PSII} (>0.3) for the whole growth period, while nutrient stressed cells reported a low value (<0.2) along the growth and a progressive decrease to values below 0.1 after day 13 (mid late stationary phase), indicating a progressive reduction of their PSII activity. The decrease in Φ_{PSII} measured in the stationary phase of both culture conditions may correspond to a higher proportion of damaged PSII reaction centres due to impaired repair mechanisms (Lippemeier et al., 2003) or to down regulation of photosynthesis associated processes in non growing cells. Changes in Φ_{PSII} could in fact indicate either a direct modification of the reaction centre amount/activity (e.g., increase of the F_0 or decrease of F_m parameter) or an energetic uncoupling of the antenna from the reaction centre. Cells exposed to different nutrient concentrations exhibit physiological acclimation by regulating their cellular materials to optimize the growth. Previous results on nitrogen starved algal cells (Berges et al., 1996; Simionato et al., 2013) showed a significant reduction in the amount and/or activity of PSII reaction centres in agreement with the decreased Φ_{PSII}. Nitrogen deprivation has an important impact on the chloroplast, which in fact showed a strongly reduced chl a content, and a general depression of the photosynthetic machinery. This energetic change could have important physiological effects: primarily an alteration of carbon metabolism that would consequently affect the synthesis of primary metabolites (polysaccharides) and of secondary metabolites (toxins). As observed for other compounds (e.g., lipids), toxin biosynthesis has a high NADPH demand and since the biosynthetic path of many compounds occurs in the chloroplast, this NADPH should probably mostly be of photosynthetic origin. Therefore, the photosynthetic changes might affect the toxin biosynthesis, as confirmed by the reduced toxin amount under nutrient stress, while maintaining a relatively constant production of each individual toxin along the growth curve. Geider et al. (1998) reported that the components of light harvesting apparatus change in abundance with increasing N concentrations in order to optimize growth. During N depletion, photosynthesis efficiency is clearly limited, as suggested in the present study by the low Φ_{PSII}, and intra cellular pools of nitrogen are allocated to the maintenance of basic and essential cellular functions, discouraging the energetically costly metabolic pathways (e.g., toxin synthesis)

The reduction in metabolite production by *Ostreopsis* cf. *ovata* exerts an influence on the number of bacterial cells associated with the cultures: their amount, during the exponential phase, was similar in the two conditions but it was significantly lower in N,P depleted cultures respect to control ones in the stationary phase. This decrease might presumably be related to the low release of carbon compounds, such as polysaccharides and toxins (Vanucci et al., 2012b; Pezzolesi et al., 2014; Pinna et al., 2015). It is important to point out as in both nutrient conditions, it was observed a slightly but constant increase of bacteria biomass during the growth curve; under N,P limitation either cellular N and chlorophyll a increased in the late stationary phase, highlighting the mutualistic relationship which exist between bacteria and microalgae, as previously hypothesized by Pinna et al. (2015).

5. Conclusions

The results of the present study, compared with previous studies performed under nutrient replete conditions and with reported *in situ* observations (Vila et al., 2001; Vanucci et al., 2012b; Cohu et al., 2013; Pezzolesi et al., 2014; Accoroni et al., 2011, 2012, 2015), are important for understanding the physiological response of *Ostreopsis* cf. *ovata* to variable nutrient concentrations and its nutritional status. The nutrient regime has implications on one hand on the bloom evolution and on the toxicity of the algal cells, as observed in the field (Accoroni et al., 2015) and as emerged also in this study by the reduced final cell yields and toxin production rates, on the other hand on the carbon allocation within the cells. Although nutrient concentrations prior or during blooms are usually not reported to be high (Vila et al., 2001; Accoroni et al., 2011; Carnicer et al., 2015), the observed cell size and growth pattern (high cell numbers, long lasting events and quite high toxicity) lead to hypothesize that *O.* cf. *ovata* biomass develops under nutrient replete conditions, which can be achieved by a rapid uptake of available N and P derived by occasionally nutrient inputs, as suggested by Accoroni et al. (2015), or by mixotrophy and/or commensalistic interactions with other organisms (e.g., algae and bacteria).

The variable and sometime unusual high cell toxin content found for some *Ostreopsis* cf. *ovata* extracts from various geographic areas could represent an effect of the different *in situ* prevalent environmental conditions (e.g., nutrient concentrations) on cellular elemental composition and carbon allocation, so that, for instance, under a low and constant nutrient supply, able to maintain appropriate cellular C:N (>12) or C:P (>170) ratios for a long period, toxin production could be steadily sustained. Conversely, in batch cultures nutrients are only initially supplied and consequently their exhaustion leads to the end of toxin synthesis.

In conclusion, it was demonstrated that *Ostreopsis* cf. *ovata* nutrient requirement is a key factor in determining its growth and toxic dynamics, and that information from physiological studies could provide useful tools for controlling and preventing harmful blooms and toxic events.

Uncited references

Falkowski and Kolber (1995), Sakshaug et al. (1997) and Vassiliev et al. (1995).

Acknowledgments

This research was supported by Italian PRIN2009 project from MIUR.[CG]

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