Growth and phosphatase activities of Ostreopsis cf. ovata biofilms supplied with diverse dissolved organic phosphorus (DOP) compounds

Growth and phosphatase activities of *Ostreopsis* cf. *ovata* biofilm incubated under diverse dissolved organic phosphorus compounds

Neil Thomas William Ellwood[1], Marisa Margherita Pasella[2,3], Cecilia Totti[2,4], Stefano Accoroni[2,4*]

[1]Dipartimento di Scienze, Università Roma Tre, Viale G. Marconi 446, 00146 Roma, Italy

[2]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy

[3]School of BioSciencies, University of Melbourne, Victoria 3010, Australia.

[4]Consorzio Interuniversitario per le Scienze del Mare, CoNISMa, ULR Ancona, 60131 Ancona, Italy

*Corresponding Author:

Stefano Accoroni

s.accoroni@univpm.it

tel. +39 071 2204919

fax: +39 071 2204650

Abstract

It is becoming increasingly evident that the use of organic nutrients is actually widespread among many aquatic phototrophic organisms. Simultaneously, incidents of eutrophication of coastal waters are becoming more common due to rises in organic nutrient loads deriving from anthropogenic activities and natural terrestrial processes. In the northern Adriatic Sea, blooms of the toxic dinoflagellate *Ostreopsis* cf. *ovata* are reported as a frequent phenomenon linked to particular environmental conditions including increased organic nutrient loads. *Ostreopsis* blooms typically produce a mucilaginous biofilm that can cover all benthic substrata. In order to clarify the role of the Dissolved Organic Phosphorus (DOP) in the onset and maintenance of an *Ostreopsis* cf. *ovata* bloom, we investigated the growth rates in the presence of a range of phosphomonoesters (D-Fructose 1,6-disphosphate, β-Glycerophosphate, α-D-Glucose 1-phosphate, Guanosine 5'-monophosphate and Phytic acid) and phosphodiesters (DNA and RNA). Levels of both phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities were assessed in the *Ostreopsis* biofilms. The results showed that *Ostreopsis* cf. *ovata* growth is not inhibited in media containing a wide range of DOP and diverse ratios of PME:PDE compared to those containing inorganic phosphorus. Much of the hydrolytic activity was associated with bacteria and with the Extracellular Polymeric Substances (EPS). Based on the present findings, *O.* cf. *ovata* success seems to stem from the collective participation of all the components of the biofilm (*Ostreopsis*, EPS and bacteria) that allows it to thrive in P-limited environments, but where organic P is the main source of P.

Keywords

Ostreopsis; harmful benthic dinoflagellate; phosphorus limitation; phosphomonoesterase; phosphodiesterase; organic phosphorus

1. Introduction

During the last two decades, efforts to better understand the phenomenon of the Harmful Algal Blooms (HABs) have intensified mainly because of the increasing trend in the number of these events and their negative impact on human health, aquatic ecosystems, and the economy (Skinner et al. 2011, Wells et al. 2020).

In the Mediterranean Sea, some species of benthic dinoflagellates belonging to the genus *Ostreopsis* have gained particular attention, because of the numerous issues associated to their toxic blooms that regularly occur in summer-autumn in rocky coasts (Accoroni et al. 2015, Jauzein et al. 2018, Vassalli et al. 2018). These blooms are often associated with noxious effects through marine aerosol inhalation or direct contact on human health (Pfannkuchen et al. 2012, Vila et al. 2016), and marine organisms, causing suffering or mass mortalities (Faimali et al. 2012, Gorbi et al. 2013). These harmful effects are attributed to the production of toxins mostly belonging to the palytoxin group (Tartaglione et al. 2017).

Among the *Ostreopsis* species recorded in the Mediterranean Sea (Penna et al. 2012, Accoroni et al. 2016a), *O*. cf *ovata* is the most abundant and widely distributed (Battocchi et al. 2010). It has been shown that hydrodynamics, water temperature and inorganic nutrients, among other environmental parameters, are those environmental factors that mainly influence bloom dynamics (Accoroni & Totti 2016, Fricke et al. 2018). Nevertheless, there is still not a clear understanding of all the factors (as well as the interactions among them) that drive bloom development throughout all its phases. For example, although there is evidence of mixotrophic behavior in many HAB species (Burkholder et al. 2008) including *Ostreopsis* (Faust & Morton 1995), only a few studies have investigated the role of organic nutrients on growth and/or bloom dynamics (Accoroni et al. 2017, Jauzein et al. 2017). Moreover, it is now accepted that dissolved organic nutrients are quantitatively important in the phenomenon of coastal eutrophication (Suzumura et al. 1998, Heisler et al. 2008, Glibert & Burkholder 2011, Karl & Bjorkman 2015).

Until recently, the aquatic P status of coastal environments was nearly always assessed based on inorganic P levels (Dissolved Inorganic P; DIP), but it is becoming increasingly clear that inclusion of organic fractions (Dissolved Organic P; DOP) is of paramount importance. The DOP levels of coastal waters can be significantly higher than those of DIP, due to human-induced processes and natural events (Suzumura et al. 1998, Dyhrman & Ruttenberg 2006), and in these environments species which can use DOP would be more competitive and likely to reach bloom levels (Accoroni et al. 2017). Intense *Ostreopsis* blooms have occurred annually along the Conero Riviera, Ancona forming loose biofilms (sometimes referred to as mats) on the surface of macrophytes and most other benthic substrata (Accoroni et al. 2016b). Based on the DIP concentrations, this area, as well as in the entire northern Adriatic, is generally considered very P-limited (Cozzi & Giani 2011, Giani et al. 2012). A recent study however, showed that P limitation was much less pronounced with the inclusion of organic P fractions, which in the Conero Riviera often accounted for 85% of total phosphorus (Accoroni et al. 2017).

Recycling orthophosphate from organic substrates generally requires hydrolysis by a group of enzymes collectively known as the phosphatases that are produced by most organisms (bacteria, algae, animals). In mats of *O*. cf. *ovata*, elevated phosphomonoesterases (PMEase) and phosphodiesterases (PDEase) activities have been measured throughout the bloom cycle (Accoroni et al. 2017). In *O*. cf. *ovata* biofilms, intense activity was observed in the exopolymeric substances (EPS), likely resulting from an accumulation over time of extruded enzymes from both the phototrophic and heterotrophic component of the biofilm. The mat life-form was suggested to aid in efficient nutrient entrapment and processing, as well as to prevent loss of substrates and products to the surrounding water (Whìtton et al. 2009, Larned et al. 2011, Accoroni et al. 2017). Since PMEase and PDEase have been measured in the biofilms and both are linkage-specific, their presence signifies a potential of the *O*. cf. *ovata* biofilms to effectively hydrolyse a wide range of organic P compounds (Accoroni et al. 2017). The source of the enzymes in the EPS was assumed to originate

from both *O.* cf. *ovata* and the EPS bacterial community, but the contribution of each to the overall biofilm activity has not yet been determined.

The aim of the present work was to investigate the ability of *O.* cf. *ovata* to use a variety of organic P forms (phosphomonoesters [PME] and phosphodiesters [PDE]). This ability was assessed by comparing growth rates and rates of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) with controls incubated with orthophosphate only. To determine the origin of the phosphatases within the *Ostreopsis*-biofilm samples, PMEase and PDEase assays were carried out on separate biofilm components (*Ostreopsis*, bacteria and EPS). Colorimetric staining of phosphatases was also carried out to observe the location of the PMEase within the biofilm. By demonstrating the ability to utilize a range of organic substrates could help determine why *O.* cf. *ovata* repeatedly grows to bloom levels in such a strongly P-limited area such as the Conero Riviera.

2. Materials and methods

2.1. Culture techniques

A non-axenic strain of *Ostreopsis* cf. *ovata* (OOAPS0810/S3) was isolated from epiphytic community found on seaweeds during a bloom in 2010 along the Conero Riviera (NW Adriatic Sea) at the Passetto site (43°37'01.7"N 13°32'01.5"E), using a capillary pipette method (Hoshaw & Rosowski 1973). The cultures were grown and maintained at 21±0.1 °C under a 12:12 h L:D photoperiod at an irradiance of 90-100 µmol $m^{-2}s^{-1}$ in modified f/4 medium (-Si, +Se). The medium was prepared by adding macronutrients to give f/4 medium concentration (Guillard 1975), while, trace metals, vitamins and HEPES buffer (pH 7.1) were f/2 medium concentrations. Salinity was kept at 35 and pH at 8 ± 0.05. Cultures grown to the mid-late exponential phase were used as the inocula in the diverse experiments. At this phase, nutrient analyses showed inorganic P levels were at the limits of detection for 5+ days. All treatments were carried out in triplicate. All the flasks

were washed with 30% v/v HCl to remove substances such as ammonia and metals and were then rinsed three times with deionized water.

2.2. Ostreopsis *cf.* ovata *and bacterial growth on organic phosphorus*

The ability of *O.* cf. *ovata* to grow using DOP as a P source was investigated omitting the orthophosphate ($NaH_2PO_4 \cdot H_2O$, OrthoP) in the modified f/4 medium (see above) and replacing it with singular PMEs or PDEs (see below). The final P concentration in all treatments was 40 μmol l^{-1}. Mid-late exponential phase cells of *O.* cf. *ovata* were used as the inocula at a starting density of around 500 cells ml^{-1} in 250 ml flasks. Five PMEs [D-Fructose 1,6-bisphosphate (FDP; Sigma-Aldrich), β-Glycerophosphate (GlyP; Sigma-Aldrich), α-D-Glucose 1-phosphate (G1P; Sigma-Aldrich), Guanosine 5'-monophosphate (GMP; Sigma-Aldrich) and Phytic acid (PA; Sigma-Aldrich)] and two PDEs [Deoxyribonucleic acid (DNA; Sigma-Aldrich) and Ribonucleic acid (RNA: Roche Diagnostics)] were used. Control culture media contained OrthoP at the same final concentration (40 μmol l^{-1}). Sampling involved homogenization of the medium, three aliquots (1 ml) were taken from each flask every three days over a period of 39 days and preserved with 0.8% neutralized formaldehyde and stored in the dark at -4 °C until cell counts were made.

2.3. Effect of DOP source on phosphatase activities of the Ostreopsis *cf.* ovata *biofilm*

To elicit changes in phosphatase response, singular and mixed (PME and PDE) incubations were repeated using GMP (PME) and RNA (PDE) as model substrates. High (75 μmol l^{-1}) and low (2.5 μmol l^{-1}) concentrations of each single substrate and diverse ratios of PME and PDE (PDE:PME= 1:10, 10:1 and 1:1 using substrates at 2.5:22.5, 22.5:2.5 and 12.5:12.5 μmol l^{-1}, respectively) were tested. Samples of 9 ml were collected from each repeat culture after homogenization at discrete times (0.6, 1, 2, 3, 4, 5, 8, 14, 20, 26, 32, 40 days after inoculation). From each sample, 1 ml was

preserved for both bacterial and *O.* cf. *ovata* counts, 5 ml for the nutrient analysis (see below) and 3 ml for PMEase and PDEase assays (1.5 ml for each substrate type).

The procedure used for the alkaline phosphatase activity assay broadly followed that of Turner et al. (2001). The colorimetric substrates *para*-nitrophenyl phosphate (ρNPP) and bis-*para*-nitrophenyl phosphate (bis-ρNPP) were used as analogue substrates for PMEase and PDEase activities, respectively. Assays consisted of 4.32 ml of sample (for each substrate, 1.5 ml subsamples were made up to 4.32 ml with fresh medium) dispensed in 15 ml glass tubes; the samples were then incubated in a shaking incubator for 20 min at 25 °C. The assay was then started by the addition of 0.18 ml of ρNPP or bis-ρNPP (1000 µmol l^{-1}) and timed precisely. The assay in general lasted 1 hour, and the reaction was stopped by adding 0.5 ml NaOH (1 mol l^{-1}). Then, each sample was centrifuged for 9 seconds at 0.010 x g, the absorbance of the supernatant was then read at 405 nm using a Varian Cary 100 Scan spectrophotometer. As total activity of the biofilm came from enzymes deriving from diverse origins, the units given for activity are given as µmol ρNP ml^{-1} h^{-1} to avoid misinterpretations.

2.4. Phosphatase activity in the different components of the Ostreopsis *biofilm*

In order to investigate the origin of phosphatase activity of the cultures, assays were performed on separated cells of *Ostreopsis*, bacteria and on EPS obtained from cultured non-axenic *Ostreopsis* biofilm grown in modified f/4 medium. This was done by filtering 5 ml of sample through membrane filters of 0.45 and 0.22 µm poresize, and performing the phosphatase assay (see above) on both the original culture and the two filtrates: (A) unfiltered culture, (B) 0.45 µm filtrate and (C) 0.22 µm filtrate. The phosphatase values of each component were calculated as follows: *Ostreopsis* cells and closely-associated bacteria= A-B; loosely-associated and free bacteria = B-C; Extracellular= C.

2.5. Microscopy analyses

Ostreopsis cf. *ovata* abundances were estimated using an inverted microscope (Zeiss Axiovert 135) at 200x magnification. Samples were settled in counting chambers after homogenization, according to the Utermöhl sedimentation method (Edler & Elbrächter 2010). Counts were made on either a half or a whole sedimentation chamber to give a representative cell number, which were expressed as cells ml^{-1}. Locating PMEase activity involved staining with 5-bromo-4-chloro-3'-indolyphosphate-nitro blue tetrazolium (BCIP-NBT) following the procedure described in Accoroni et al. (2017). BCIP-NBT is a chromogenic phosphatase substrate that produces a blue-purple-colored precipitate at the site of enzymatic activity. Samples of *Ostreopsis* biofilm were incubated in 4 ml BCIP-NBT solution (Sigma-Aldrich Chemicals, Poole, UK) at room temperature (20 °C) for around 15–20 min before terminating the reaction with 0.5 M NaOH and washing with de-ionised water. Images were taken using a Canon EOS 6D camera (Canon Inc., Tokyo, Japan) at the light microscope.

The bacterial populations within the EPS were analysed by epifluorescence after staining samples with the green-fluorescing SYBR Green I (Molecular Probes; dilution 1:2000) to visualize nucleic acids and the cellulose specific dye Calcofluor-White M2R to distinguish *Ostreopsis* thecae. Moreover, observations in epifluorescence allowed detecting also the red autofluorescence of the chlorophyll.

2.6. Nutrient analyses

Water samples (5 ml) were filtered using 25-mm GFF filters (Whatman) and the filtrates were then persulphate digested at 1 bar (120 °C) for 45 min (Langner & Hendrix 1982). Analysis of Filtrable Total Phosphorus (FTP) was carried out spectrophotometrically using molybdate colorimetry (Eisenreich et al. 1975). Limits of detection for P were 0.03 $\mu mol\ l^{-1}$.

2.7. Calculations and statistical analyses

Ostreopsis cf. *ovata* growth was expressed as specific growth rate (μ, day^{-1}) using the equation

$\mu = (\ln N_1 - \ln N_0) / (t_2 - t_1)$

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

The statistical analyses were conducted using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA) software. The Shapiro-Wilks test was used to check data for normal distribution, while the Levene's test was used to assess homogeneity of variance. When data were not normally distributed nor homogeneity of variances were respected, data were rank-trasformed. Differences in growth rate, maximum yield and phosphatase activities between the different conditions of nutrient availability were assessed using one-way analysis of variance (ANOVA). When significant differences for the main effect were observed ($p < 0.05$), a Tukey's pairwise comparison test was also performed.

3. Results

3.1. Effect of P source on growth of Ostreopsis *cf.* ovata

In general the growth rates of *O.* cf. *ovata* in media containing DOP were higher than those supplied with orthophosphate, the only exception being the FDP cultures (Fig. 1). The lowest values of growth rate (≤ 0.11 d^{-1}) and maximum yield (*ca.* 700 cells ml^{-1}) were recorded in OrthoP and FDP cultures (Table 1). The mean cell abundances throughout the entire experiment in OrthoP cultures were significantly lower than those in GMP and RNA ($p < 0.05$).

The mean cell abundances throughout the entire experiment of *O.* cf. *ovata* with GMP were higher than the other PME cultures (Fig. 1, significantly compared to that in FDP, $p < 0.05$). Between the two PDE cultures, growth rates and maximum yield were highest in the RNA cultures, but were not significantly different.

Although the exponential growth of *O.* cf. *ovata* among the different DOP-containing media did not occur until day 9, the reduction in FTP in all the diverse media was rapid across the first 3 to 9 days occurring in the sequence GMP (96%) > PA (95%) > GlyP (91%) > OrthP (90%) > DNA (76%) > RNA (68%) > G1P (45%) > FDP (31%) based on percent P removal by day 6 (Fig. 1). After this initial period, in most cases there was little change in concentration, but in some instances there were small subsequent increases of media P concentration.

3.2. The effect of P source and concentration on PMEase and PDEase activities

ANOVA analysis showed that same-substrate treatments at high and low concentrations did not elicit a significantly different response in neither PMEase nor PDEase (Fig. 2). The initial rates of PDEase and PMEase in the OrthoP and RNA cultures were very similar (from 2.7 to 3.6 compared to 2.7-3.9 μmol ρNP ml^{-1} h^{-1} over the first 20 days). In the latter half of the incubation, PDEase rates decreased (1.0 – 1.8 μmol ρNP ml^{-1} h^{-1}), whilst those of PMEase remained the very similar or slightly increased (mean values of 2.2 – 3.8 μmol ρNP ml^{-1} h^{-1}). The PDEase rates in the GMP cultures did not show any significant variations, while PMEase mainly increased in the lower GMP concentration and was significantly higher than PDEase by day 32 ($p < 0.05$). In the higher GMP cultures, there was no significant difference between PMEase and PDEase. The rates of PMEase and PDEase were mainly similar in each treatment for the main part of the incubation period (≈32d), after which PMEase tended to be higher than PDEase in 7 out of 9 treatments ($p < 0.05$). This shift of importance to PMEase from PDEase was also indicated by the plots of PDEase:PMEase (Fig. 3) showing a decrease in the ratio in all treatments from between 1 and 2 down to between 0.2 to 0.3, between day 26 and 32. Activity was always measurable in the inorganic P treatments.

3.3. Bacteria within the Ostreopsis*-biofilm and alkaline phosphatase activity in the different components of the culture*

In *Ostreopsis* cf. *ovata* cultures, bacteria grown mostly associate to the EPS, while few of them were detected as free-living in the growth media. Within the EPS, there were bacteria more closely associated to the *Ostreopsis* cells, some of them closely-surface associated to the *Ostrepsis* cells, while some others more focused to the ventral section of *Ostreopsis* cells. The main part of bacterial cells, however, were gatherated within the EPS far from *Ostreopsis* cells (i.e. loosely-associated to *Ostreopsis* cell) (Fig. 4). PMEase and PDEase activities of cells (*Ostreopsis* and bacteria) made up 56.8% and 39.3% of the total activity, while the extracellular activities made up 43.2% and 60.7%, respectively. Within the cells activities, those of *Ostreopsis* cells and their closely-associated bacteria providing only 13.4% and 19.6% of the total activities, while the rest of the bacteria community made up 43.4% and 19.7% of the total PMEase and PDEase activity, respectively (Fig. 5). Staining of *Ostreopsis* PMEases with BCIP/NBT located an intracellular activity in the ventral part of the cells and an extracellular activity associated with the cell surface, mostly within the EPS often close to the ventral section of *Ostreopsis* cells (Fig. 6).

4. Discussion

In this study, it was shown that *Ostreopsis* cf. *ovata* was able to grow in media containing PME and PDE as sole P sources. The presence of PMEase and PDEase activities suggests that mineralisation of the dissolved organic P substrates provided enough P to sustain the growth of *O*. cf. *ovata*. Both phosphatase activities were measurable in all treatments and for the whole incubation period. Several studies of algal growth on PMEs have been conducted, but those on PDEs as potential P-sources are rare (Whitton et al. 1991, Yamaguchi et al. 2014), despite this has been shown that nucleic acids (DNA and RNA), or their partially degraded derivatives, are very common in aquatic environments (Suzumura et al. 1998, Baldwin 2013). In the open oceans, PDEs can play an

important role in diatom community dynamics, primary production and phosphorus cycles (Yamaguchi et al. 2014), and results obtained in this study show that the PDEase activity can be significant in coastal environments as well. *O.* cf. *ovata* was efficient in using nucleic acids (PDE) as phosphorus sources under non-axenic conditions, with the RNA-grown cultures having the highest growth rates and the second highest maximum cell yield out of the eight P substrates tested. To better understand the ecology of this species, or more precisely the biofilm community, it is crucial to quantify the environmental concentrations of PMEs and PDEs and the potential of organisms to use them.

In light of evidence showing relationships among organic P, phosphatase activities and *O*. cf. *ovata* blooms (Accoroni et al. 2017), it was still surprising to find comparable or even higher growth rates and cell abundances on organic P substrates compared to those with inorganic P. This finding suggests that *O*. cf. *ovata* biofilms are able to process organics rapidly, and that the products of the extracellular (EPS-associated) hydrolysis are not diffusionally hindered through the EPS towards the cells (Flemming 2011). Furthermore, bacteria are known to play a key role in providing phytohormones and/or macro- and micronutrients (i.e. not only P) to algae which can enhance algal growth rates (Ramanan et al. 2016). If so, it could be that the higher growth rates in organic compared to inorganic P media may be due to the complete breakdown of the organic substrates by the EPS-associated bacteria and the supply of additional N and C derived from the breakdown of the nucleic acid substrates (not present in the OrthoP cultures). An interesting advancement to this experiment would be to compare these growth rates with those of cultures grown in media where N and C levels are also maintained at the same level in the organic and inorganic treatments.

Although the observed BCIP-NBT staining in the ventral region of *Ostreopsis* cells may be just an optical artefact from staining of external membrane bound enzyme activity, the staining pattern outside the cells (which extends from the ventral opening) does lend some weight to the idea of intracellular activity. In the euglenoid *Peranera trichophorum* phosphatases have been identified

within the mucocysts and were suggested to be involved in release of exoenzymes (Hilenski & Walne 1983). In *O*. cf. *ovata*, many mucocysts are closely associated with a mucus canal, that collects their contents and leads to the ventral opening (Escalera et al. 2014), so the intracellular staining of phosphatases in the ventral section of the cell maybe be located in this mucus canal, or possibly up to the discharging mucocysts. If this is the case, it also signifies that simple organics, such as the BCIP-NBT stain used here, can enter the cell and be processed intracellularly. From the observations of stained samples, and also the assays, it seems that although some activity remained close to the cells (*Ostreopsis* and bacteria) a large part of the overall activity was within the EPS. The EPS seems to be the main site of phosphatase activity and so functions something like an efficient external digestion system for the entire biofilm community (Flemming & Wingender 2010). The chemical and physical properties of EPS lead to entrapment and accumulation of extruded exoenzymes, hydrolytically active cells, substrates (allochthonous and autochthonous) and hydrolytic products and will minimise any loss to the surrounding water and maintain a short diffusional distance towards the cells (e.g. approximately 30 μm; Decho & Gutierrez 2017). The abundant hydrolysis of substrates in the EPS would create steep diffusional gradients of phosphate toward the cells, and in some instances toward the bulk medium (Stewart 2003, Flemming et al., 2016). In latter case, if cells were P-satiated, the diffusional gradient would be inverted and losses to the ambient water from the biofilm may occur, which would explain those occasions where increases in media P concentrations were observed during the latter part of the incubation period. In fact, over the first six days there were large reductions in media P and lag phases in growth protracted up to 12 days suggesting that at that time the cells ceased to become sinks of P and the EPS may have been accumulating P prior to its eventual release to the media during the latter part of the incubation period. It must be noted that the filtration process for P analysis may have contributed to the rapid reduction in the media FTP levels, where losses of organic P compounds associated with the EPS (bound or interstitial) may have over estimated the biological removal rate

(that which enters the cells of the biofilm). However, it would be interesting to assess separately the EPS-P content and the cellular content to determine where accumulation occurs, i.e. does EPS behave as a functional immediate environment for the biofilm cells (Flemming 2011) or if the cells themselves are taking up luxury levels of P (Baek et al. 2008, Solovchenko et al. 2019) prior subsequent growth.

The use of mid-late exponential phase cells for the inocula (P stressed) led to elevated phosphatase activities in the cultures at the onset of the experiment. However, the significant EPS contribution to the overall phosphatase activity of the biofilm (i.e. activity not under direct cellular control) likely masked the cellular response in the diverse P sources and concentrations. Indeed the initial rates were quite similar in all treatments, only toward the end of the incubation were increases in activity observed, where cellular activity likely added a more significant contribution to the overall activity. Within the EPS, non-cellular control of PMEase activities can occur from a build-up of phosphate product near the active sites of the enzymes, causing competitive inhibition, which can be overcome by cellular P uptake or loss to the media (Sakshaug et al. 1984, Jansson et al. 1988). Competitive product inhibition has not yet been identified for PDEase and so the latter-stage increase in PMEase over PDEase could possibly arise from them having different controls over their rates. In aquatic bryophytes (Christmas & Whitton 1998, Whitton et al. 2005, Ellwood et al. 2008), diatoms (Yamaguchi et al. 2014) and some cyanobacteria (Whitton et al. 1990) an increase in PDEase activity is shown to occur at higher P stress than that of PMEase. The high initial PDEase rates were probably a result of elevated P-stress that occurred during preparation of the *Ostreopsis* inocula and that the diminishing rates were an indication of a decrease in this stress over the incubation period. As bacteria abundances during an *Ostreopsis* cf. *ovata* bloom are normally about 10^6 cells ml^{-1} (Vanucci et al. 2016), it is quite obvious that their contribution to overall biofilm phosphatase activities could be significant. The intimate relationships between *Ostreopsis* and bacteria have not been elucidated and could be commensal, symbiotic or pathogenic, but considering the bloom

history of this species, the latter association cannot be quantitatively important. In the laboratory, *Ostreopsis* is generally cultivated in non-axenic conditions with maximal bacterial densities of up to 10^7 cells ml^{-1} (Pezzolesi et al. 2016). The growth of the *Ostreopsis*-biofilm cultured under phytic acid is very interesting as this is indicative of phytase activity. Phytase production is normally associated with filamentous fungi, bacteria, yeasts, some cyanobacteria and higher plants (Whitton et al. 1991, Mullaney et al. 2000), but has not been associated with eukaryotic algae. Phytic acid cannot be hydrolysed by PMEase or PDEase (Turner et al. 2002) so this P source is generally considered biologically unavailable to phytoplankton or microphytobenthos. In light of this evidence, further studies with a focus on the bacterial-*Ostreopsis* interaction within the EPS should be conducted. It is therefore proposed that the EPS matrix allows *O.* cf. *ovata* to form stable synergistic relationships with bacteria and to provide a protective environment to maintain the activity of a collective group of extracellular enzymes from diverse origins that may function similar to an active external digestion system (Flemming 2011). The ecological significance of phytate in the marine environment is at present uncertain (Baldwin 2013), however it has been shown that leachate arriving from land is an important source of phosphorus to the near shore coastal environments (Suzumura & Kamatani 1995).

5. Conclusions

Growth of *O.* cf. *ovata* is not limited when cultured in medium containing only organic P substrates. The efficient use of a range of phosphomonoesters and phosphodiesters suggests a broad hydrolytic specificity of the phosphomonoesterases and phosphodiesterases within the biofilm. Phytase activity was also indicated by the unimpeded growth when the cultures were supplied with phytic acid, although the source of these enzymes was not identified. The findings shown here integrate well with previous field observations where elevated activies coincided with the onset of an *O.* cf. *ovata* bloom occured in water where the main P source was organic.

Accumulation of phosphatases in the EPS suggests that this was the main site of hydrolytic activity. The abundance of the EPS also suggested that much of the overall activity was not under cellular control, and possibly explains why there are no detectable differences in the PMEase and PDEase response to changes in the substrate concentration or type. It is proposed that the EPS essentially functions like an external digestion system that benefit the whole biofilm/floc community. The EPS traps and concentrates particulate and dissolved organics and provides a substrate that holds and binds enzymes, it also prevents the loss hydrolytic products to ambient water and providing a medium through which nutrients can diffuse towards the cells.

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Table 1. Growth rates (μ) and maximum yield (max-yld) of *Ostreopsis* cf. *ovata* with various phosphorus sources (Orthophosphate, OrthoP; D-Fructose 1,6-bisphosphate, FDP; β-Glycerophosphate, GlyP; α-D-Glucose 1-phosphate, G1P; Guanosine 5'-monophosphate, GMP; Phytic acid, PA; deoxyribonucleic acid, DNA; ribonucleic acid, RNA)

Growth medium	Exponential-phase timing (days)	μ (day^{-1})	max-yld (cells ml^{-1})
Control			
OrthoP	9–24	0.11	718 ± 278
PME			
FDP	12–21	0.10	721 ± 103
GlyP	9–24	0.13	901 ± 382
G1P	9–18	0.15	1487 ± 211
GMP	12–21	0.18	3179 ± 282
PA	15–30	0.13	1550 ± 403
PDE			
DNA	9–18	0.17	1629 ± 929
RNA	12–21	0.21	2433 ± 1224

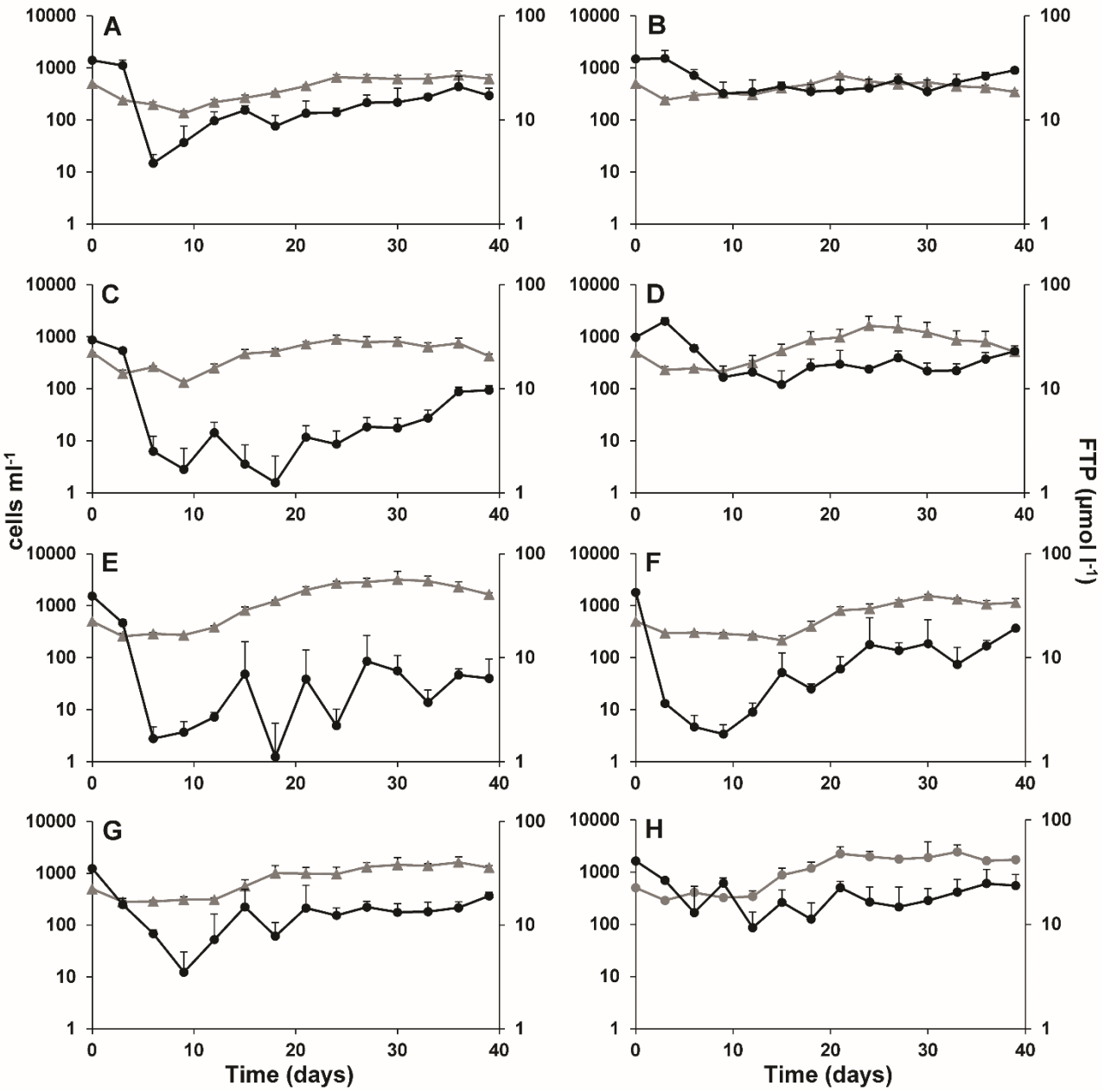

Fig. 1. Growth curves of *Ostreopsis* cf. *ovata* (grey triangles) and changes in mdium Filtrable Total Phosphorus (FTP, µmol l^{-1}) (black circles). *O.* cf. *ovata* cultures grown on diverse singular P substrates during a 39 day incubation period: (A) OrthoP, (B) FDP, (C) GlyP, (D) G1P, (E) GMP, (F) PA, (G) DNA and (H) RNA (for substrate abbreviations see Table 1 legend). Values are means and standard deviations of three replicates

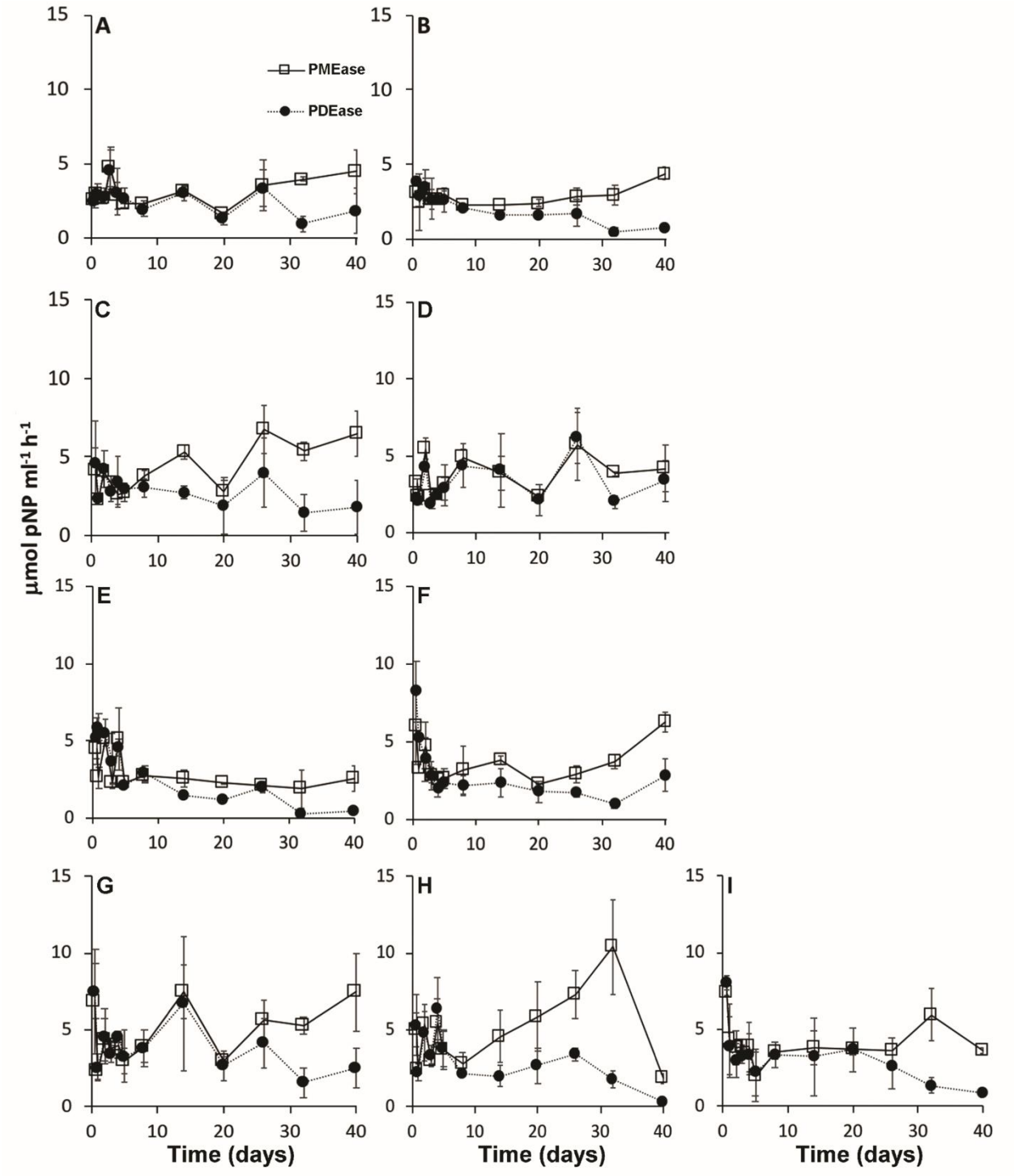

Fig. 2. Changes in PMEase and PDEase activities in cultures of *O.* cf. *ovata* growing media with low (A, C, E; 2.5µmol) and high (B, D, F; 75 µmol) concentrations of (A, B) OrthoP, (C, D) GMP and (E, F) RNA (for substrate abbreviations see Table 1 legend) and diverse ratios of PME (GMP) and PDE (RNA) to give PDE:PME of (G) 1:1, (H) 1:10 and (I) 10:1 (using substrates at 12.5:12.5, 2.5:22.5 and 22.5:2.5 and µmol l^{-1}, respectively).

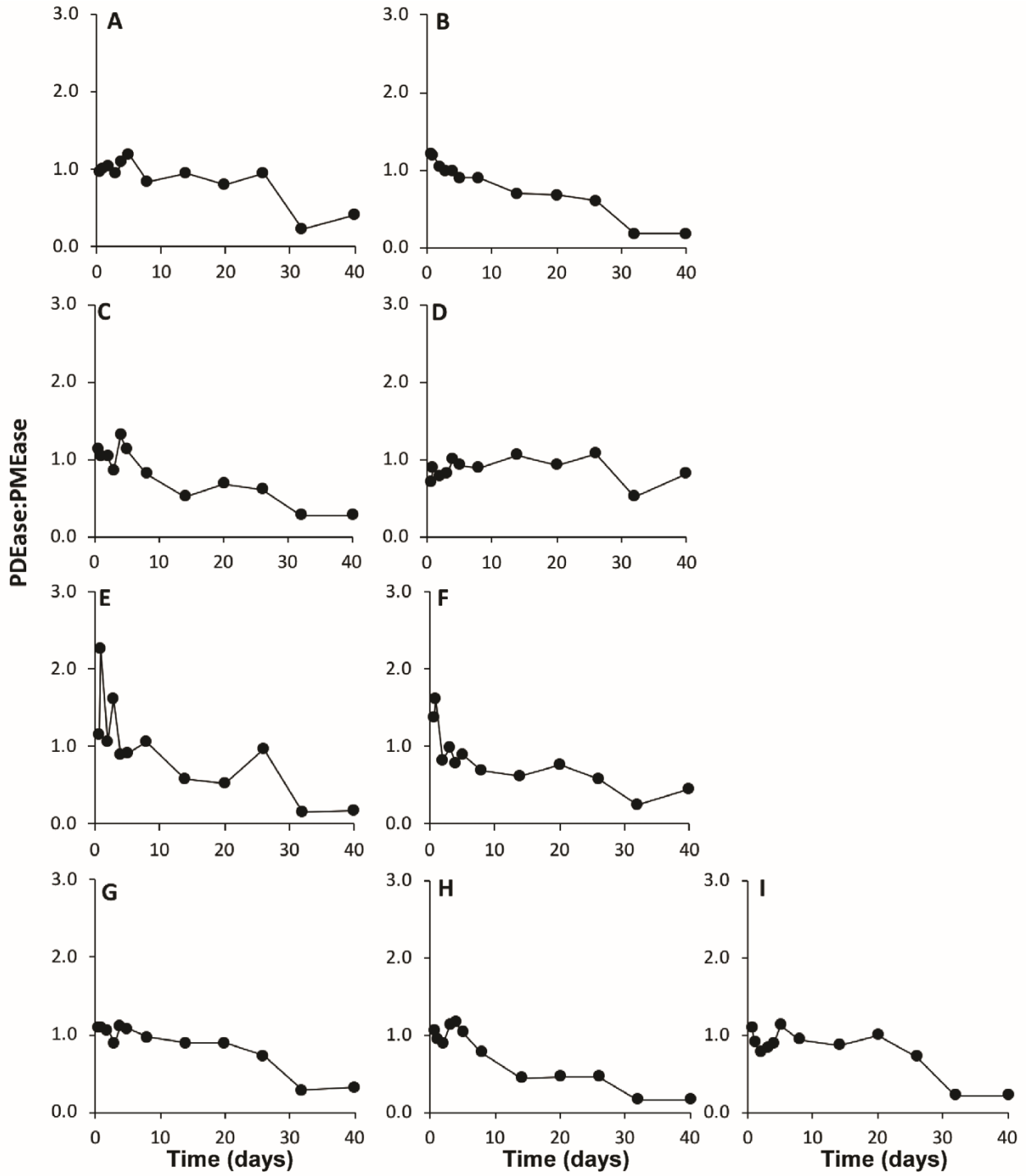

Fig. 3. Changes in the ratio of PDEase to PMEase (PDEase:PMEase) in cultures of *O*. cf. *ovata* growing media with low (A, C, E; 2.5μmol) and high (B, D, F; 75 μmol) concentrations of (A, B) OrthoP, (C, D) GMP and (E, F) RNA (for substrate abbreviations see Table 1 legend) and diverse ratios of PME (GMP) and PDE (RNA) to give PDE:PME of (G) 1:1, (H) 1:10 and (I) 10:1 (using substrates at 12.5:12.5, 2.5:22.5 and 22.5:2.5 and μmol l^{-1}, respectively).

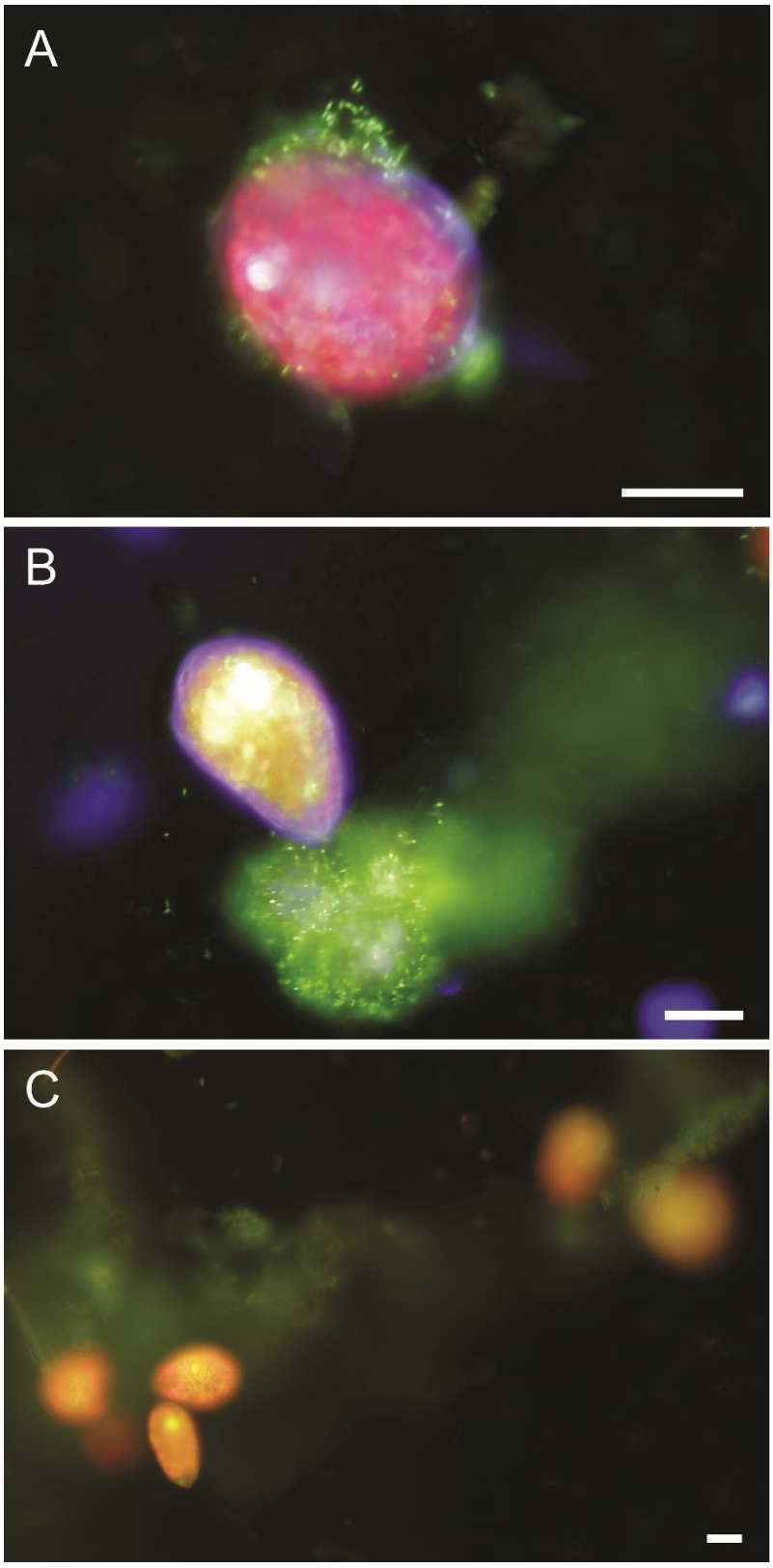

Fig. 4. Biofilm (EPS, *Ostreopsis* cf. *ovata* and bacterial cells) stained with SYBR Green I (green fluorescence of nucleic acids), Calcofluor-White M2R (blue fluorescence of cellulose) and red

1 autofluorescence of the chlorophyll in *Ostreopsis* is visible under epifluorescence microscopy: a
2 certain amount of bacteria cells were closely associated to the *Ostreopsis* cells, both (A) to cell
3 surface and (B) to the ventral part of cells of *Ostreopsis*. (C). The majority of bacterial cells were
4 located within the EPS and loosely associated with *Ostreopsis*. Scale bars = 20 µm.
5

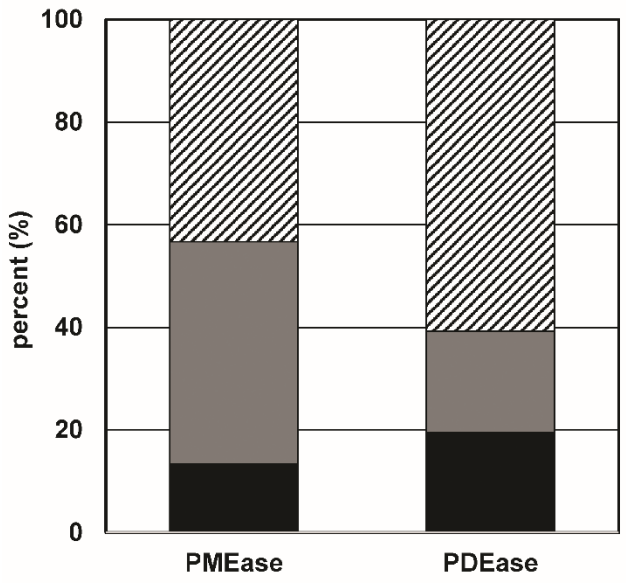

Fig. 5. Phosphatase activity (PMEase and PDEase) of the different fractions of the culture of *Ostreopsis* cf. *ovata*. Phosphastase activities of *Ostreopsis* cells and closely-associated bacteria (black), loosely-associated bacteria (grey) and extracellular (diagonal lines) are given as a percentage of the total phosphatase activity of each sample.

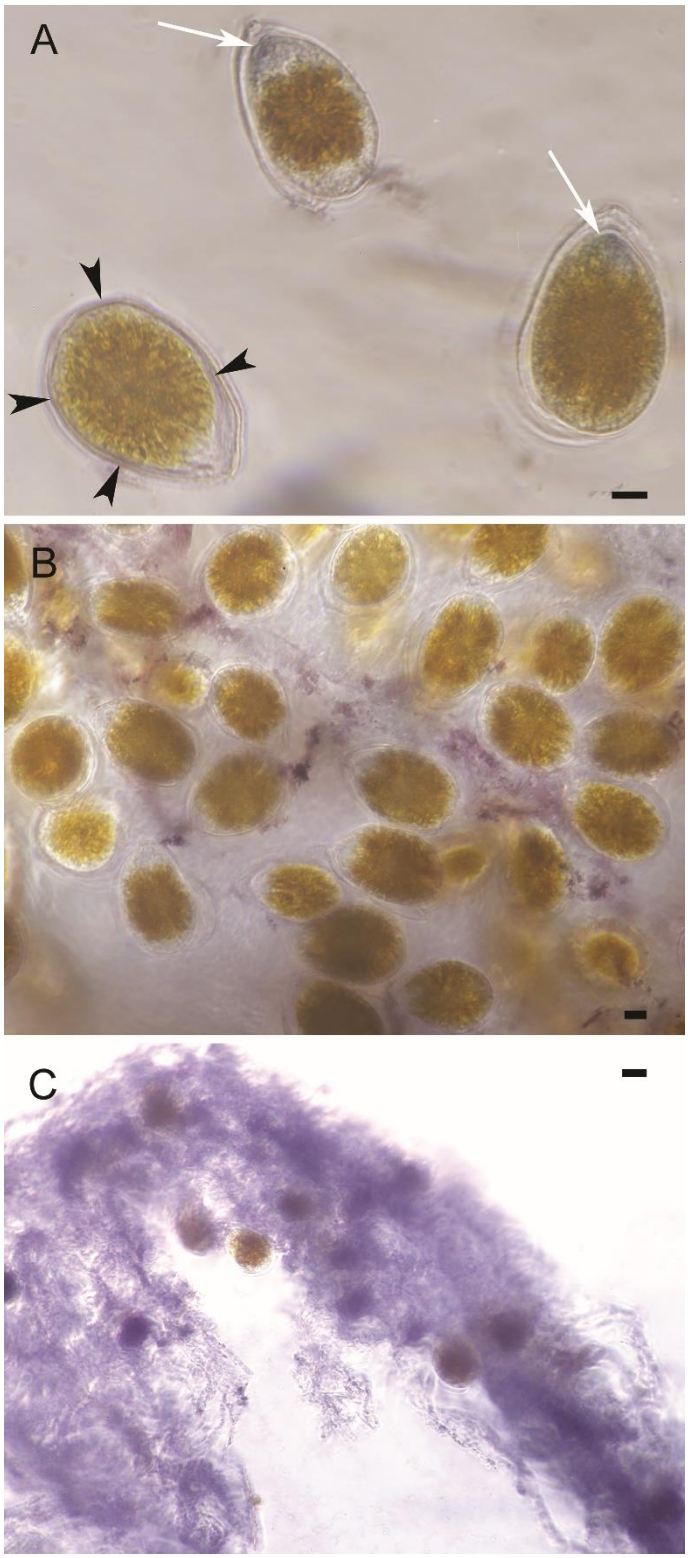

Fig. 6. BCIP-NBT staining of PMEase activity of *Ostreopsis* cf. *ovata* and EPS: (A) Intracellular activity in the ventral part of the cells (white arrows) and cell surface associated (black arrowheads).

(B) Extracellular activity pushed out within the EPS close to the ventral part of the cells. (C) The majority of activity located within the EPS. Scale bars = 20 µm (A, B); 40 µm (C).