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

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ABSTRACT: It is becoming increasingly evident that the use of organic nutrients is 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 dissolved organic phosphorus (DOP) in the onset and maintenance of an *O.* cf. *ovata* bloom, we investigated the growth rates in the presence of a range of phosphomonoesters (PMEs) (D-fructose 1,6-disphosphate, β-glycerophosphate, α-D-glucose 1-phosphate, guanosine 5′-monophosphate and phytic acid) and phosphodiesters (PDEs) (DNA and RNA). Levels of both phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities were assessed in the *O.* cf. *ovata* biofilms. The results showed that *O.* 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 extracellular polymeric substances (EPSs). Our findings suggest that the success of *O.* cf. *ovata* stems from the collective participation of all components of the biofilm (*O.* cf. *ovata*, EPSs and bacteria) that allows it to thrive in phosphorus-limited environments, but where the main source of phosphorus is organic.

KEY WORDS: *Ostreopsis* · Harmful benthic dinoflagellate · Phosphorus limitation · Phosphomonoesterase · Phosphodiesterase · Organic phosphorus

1. INTRODUCTION

During the last 2 decades, efforts to better understand the phenomenon of 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 with toxic blooms of these species that regularly occur in summer–autumn along rocky coasts (Accoroni et al. 2015, Jauzein et al. 2018, Vassalli et al. 2018). These blooms are often associated with noxious effects on human health through marine aerosol inhalation or direct contact (Pfannkuchen et

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al. 2012, Vila et al. 2016) and on 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). Hydrodynamics, water temperature and inorganic nutrients, among other environmental parameters, are the environmental factors that mainly influence bloom dynamics (Accoroni & Totti 2016, Fricke et al. 2018). Nevertheless, we still have no clear understanding of all factors (as well as the interactions among them) that drive bloom development throughout all of its phases. For example, although there is evidence of mixotrophic behaviour 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 & Björkman 2015).

Until recently, the status of aquatic phosphorus (P) in 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, Italy, 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). However, a recent study 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 P (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 phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities have been measured throughout the bloom cycle (Accoroni et al. 2017). In *O.* cf. *ovata* biofilms, intense activity was observed in exopolymeric substances (EPSs), 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 (Whitton 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 the potential of *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 EPSs 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 study was to investigate the ability of *O.* cf. *ovata* to use a variety of organic P forms (phosphomonoesters [PMEs] and phosphodiesters [PDEs]). This ability was assessed by comparing growth rates and rates of PMEase and PDEase with controls incubated with orthophosphate only. To determine the origin of the phosphatases within the *O.* cf. *ovata* biofilm samples, PMEase and PDEase assays were carried out on separate biofilm components (*O.* cf. *ovata*, bacteria and EPSs). Colorimetric staining of phosphatases was also carried out to observe the location of the PMEase within the biofilm. 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 as the Conero Riviera.

2. MATERIALS AND METHODS

2.1. Culture techniques

A non-axenic strain of *Ostreopsis* cf. *ovata* (OOAPS 0810/S3) was isolated from an epiphytic community found on seaweeds during a bloom in 2010 along the Conero Riviera (NW Adriatic Sea), Italy, 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 light:dark 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- to late-exponential phase were used as the inocula in the diverse experiments. At this phase, nutrient analyses showed that inorganic P levels were at the limits of detection for 5+ d. All treatments were carried out in triplicate. All flasks were washed with 30 % v/v HCl to remove substances such as ammonia and metals and were then rinsed 3 times with deionized water.

2.2. *O.* cf. *ovata* and bacterial growth on organic P

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 Section 2.1) and replacing it with singular PMEs or PDEs (see Section 2.3). The final P concentration in all treatments was 40 µmol l^{-1}. Mid- to 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]; β-glycerophosphate [GlyP]; α-D-glucose 1-phosphate [G1P]; guanosine 5′-monophosphate [GMP]; and phytic acid [PA]; all from Sigma-Aldrich) and 2 PDEs (DNA, Sigma-Aldrich; and 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; 3 aliquots (1 ml) were then taken from each flask every 3 d over a period of 39 d 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 *O.* cf. *ovata* biofilm

To elicit changes in the 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 (PME:PDE = 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 and 40 d after inoculation). From each sample, 1 ml was preserved for both bacterial and *O.* cf. *ovata* counts, 5 ml for the nutrient analysis (see Section 2.6) 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 h, and the reaction was stopped by adding 0.5 ml NaOH (1 mol l^{-1}). Each sample was then centrifuged for 9 s at 0.010 × *g*, and the absorbance of the supernatant was 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 (para-nitrophenol) ml^{-1} h^{-1} to avoid misinterpretations.

2.4. Phosphatase activity in the different components of the *O.* cf. *ovata* biofilm

In order to investigate the origin of phosphatase activity of the cultures, assays were performed on separated cells of *O.* cf. *ovata*, bacteria and on EPSs obtained from cultured non-axenic *O.* cf. *ovata* 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 pore size, and performing the phosphatase assay (see Section 2.3) on both the original culture and the 2 filtrates: (1) unfiltered culture, (2) 0.45 µm filtrate and (3) 0.22 µm filtrate. The phosphatase values of each component were calculated as follows: *O.* cf. *ovata* cells and closely associated bacteria = (1) – (2); loosely associated and free bacteria = (2) – (3); extracellular = (3).

2.5. Microscopy analyses

O. cf. *ovata* abundances were estimated using an inverted microscope (Zeiss Axiovert 135) at 200× 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, 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 by Accoroni et al. (2017). BCIP-NBT is a chromogenic phosphatase substrate that produces a blue-purple-coloured precipitate at the site of enzymatic activity. Samples of *O.* cf. *ovata* biofilm were incubated in 4 ml BCIP-NBT solution (Sigma-Aldrich) at room temperature (20°C) for around 15–20 min before terminating the reaction with 0.5 M NaOH and washing with deionized water. Images were taken using a Canon EOS 6D camera on the light microscope.

The bacterial populations within the EPS were analysed using 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 *O.* cf. *ovata* thecae. Moreover, observations in epifluorescence also allowed detection of the red autofluorescence of 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 P (FTP) was carried out spectrophotometrically using molybdate colorimetry (Eisenreich et al. 1975). Limits of detection for P were 0.03 µmol l^{-1}.

2.7. Calculations and statistical analyses

O. cf. *ovata* growth was expressed as specific growth rate (µ, d^{-1}) using:

$$\mu = (\ln N_1 - \ln N_0) / (t_2 - t_1) \quad (1)$$

where N_1 is the final cell density, N_0 is the initial cell density, and t is the number of days between 2 measurements.

The statistical analyses were conducted using Statistica 10.0 (StatSoft) software. The Shapiro-Wilks test was used to check data for normal distribution, while Levene's test was used to assess homogeneity of variance. When data were not normally distributed or when variances were not homogeneous, data were rank-transformed. Differences in growth rate, maximum yield and phosphatase activities between the different conditions of nutrient availability were assessed using 1-way ANOVA. When significant differences for the main effect were observed ($p < 0.05$), 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 OrthoP, the only exception being the FDP cultures (Fig. 1). The lowest growth rates (≤0.11 d^{-1}) and lowest 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 in the other PME cultures (Fig. 1, significantly compared to that in FDP, $p < 0.05$). Between the 2 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 d occurring in the sequence GMP (96%) > PA (95%) > GlyP (91%) > OrthoP (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. Effect of P source and concentration on PMEase and PDEase activities

ANOVA showed that same-substrate treatments at high and low concentrations did not elicit a significantly different response in either PMEase or PDEase (Fig. 2). The initial rates of PDEase and PMEase in the OrthoP and RNA cultures were very similar (2.7–3.6 compared to 2.7–3.9 µmol ρNP ml^{-1} h^{-1} over the first 20 d). 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 very

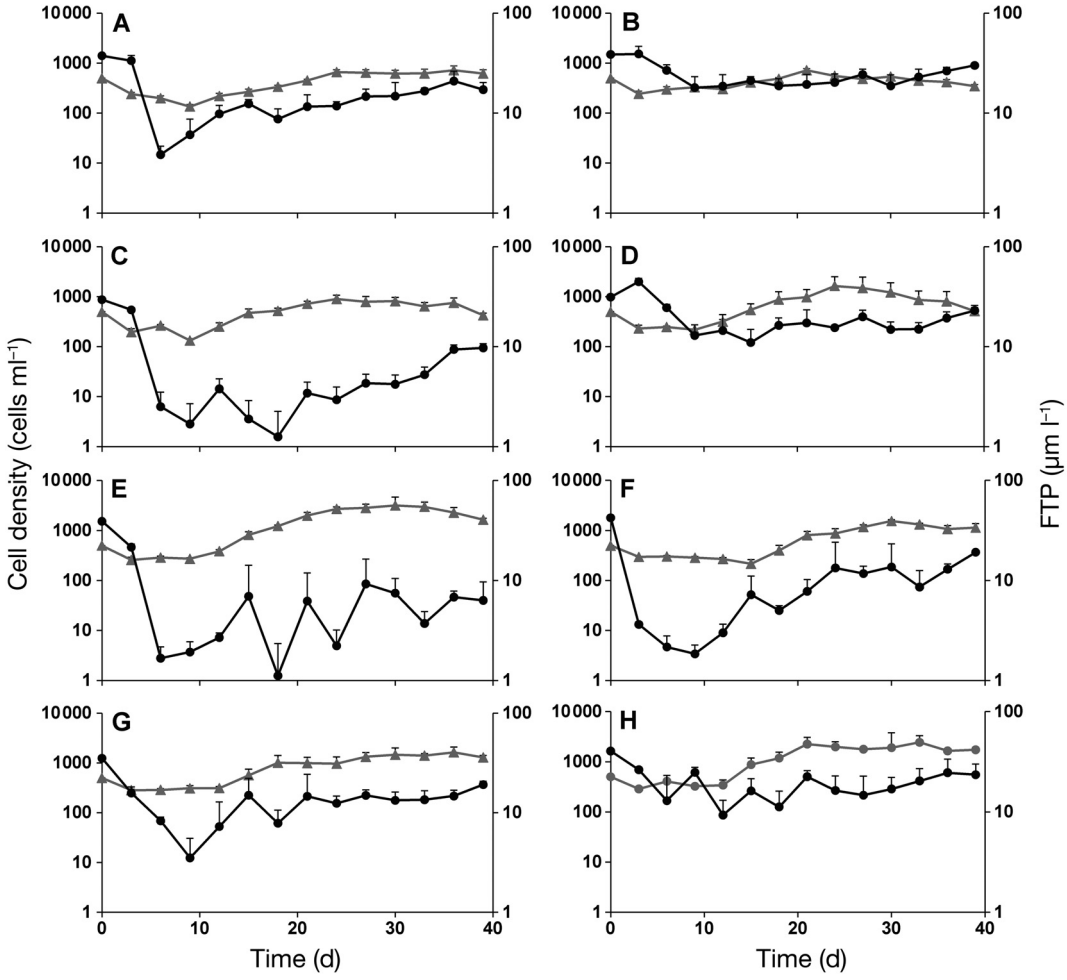

Fig. 1. Growth curves of *Ostreopsis* cf. *ovata* (grey triangles) and changes in medium filtrable total phosphorus (FTP, µmol l^{-1}) (black circles). *O.* cf. *ovata* cultures grown on diverse singular P substrates during a 39 d incubation period: (A) OrthoP, (B) FDP, (C) GlyP, (D) G1P, (E) GMP, (F) PA, (G) DNA and (H) RNA (for substrate abbreviations see Table 1 legend). Values are mean ± SD of 3 replicates

Table 1. Growth rates (µ) and maximum yield of (mean ± SD) *Ostreopsis* cf. *ovata* with various phosphorus sources (OrthoP: orthophosphate; FDP: D-fructose 1,6-bisphosphate; GlyP: β-glycerophosphate; G1P: α-D-glucose 1-phosphate; GMP: guanosine 5′-monophosphate; PA: phytic acid). PMEs: phosphomonoesters; PDEs: phosphodiesters

Growth medium	Exponential-phase timing (d)	µ (d^{-1})	Max. yield (cells ml^{-1})
Control			
OrthoP	9–24	0.11	718 ± 278
PMEs			
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
PDEs			
DNA	9–18	0.17	1629 ± 929
RNA	12–21	0.21	2433 ± 1224

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 (≈32 d), 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 the PDEase:PMEase ratio (Fig. 3), showing a decrease in the ratio in all treatments from between 1 and 2 down to between 0.2 and 0.3, between Days 26 and 32. Activity was always measurable in the inorganic P treatments.

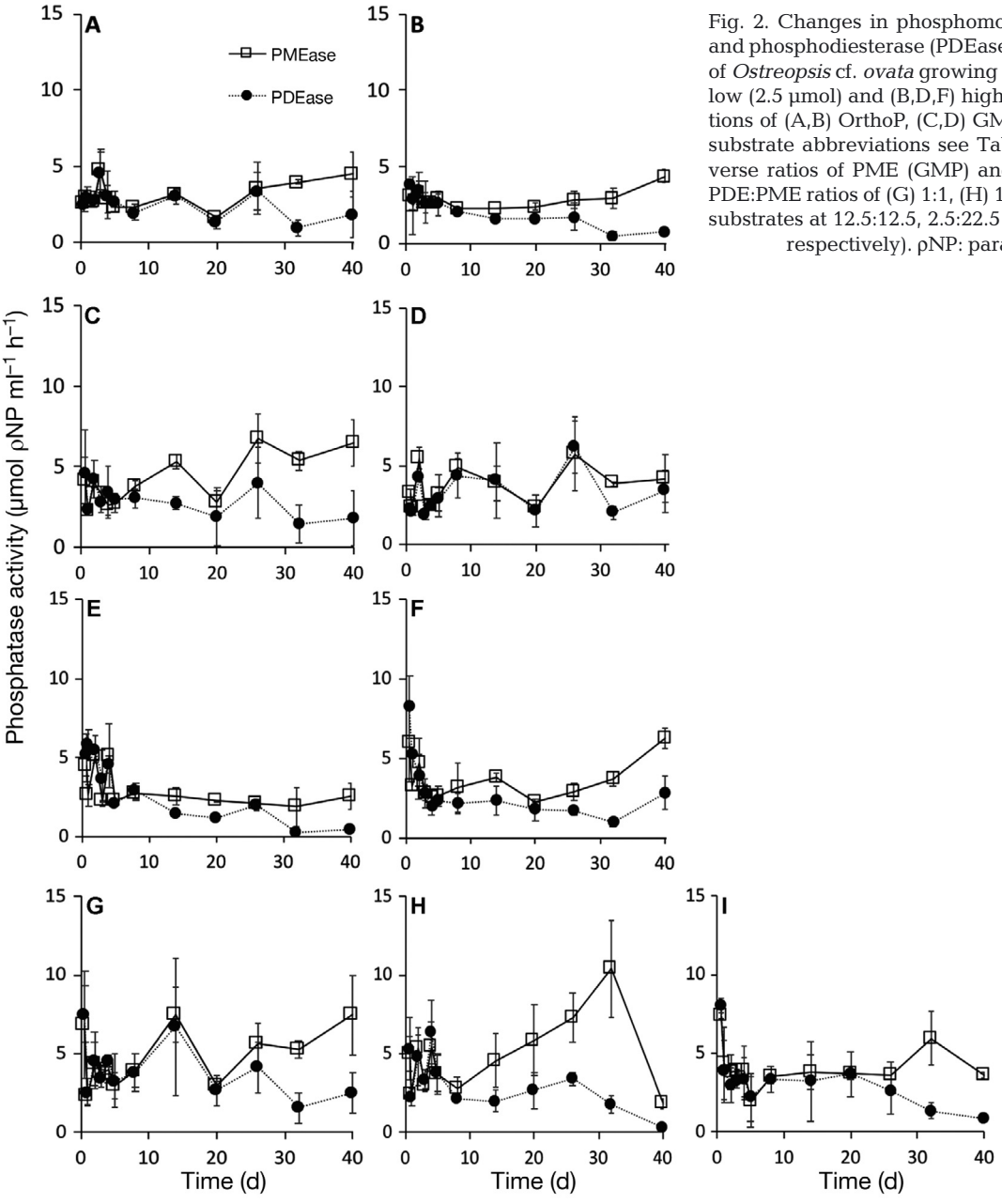

Fig. 2. Changes in phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activities in cultures of *Ostreopsis* cf. *ovata* growing in media with (A,C,E) low (2.5 µmol) and (B,D,F) high (75 µmol) concentrations of (A,B) OrthoP, (C,D) GMP and (E,F) RNA (for substrate abbreviations see Table 1 legend) and diverse ratios of PME (GMP) and PDE (RNA) to give PDE:PME ratios 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 µmol l^{-1}, respectively). ρNP: para-nitrophenol

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

In *O.* cf. *ovata* cultures, bacteria grew mostly in association with the EPSs, while few were detected as free-living in the growth media. Within the EPSs, there were bacteria more closely associated with the *O.* cf. *ovata* cells, some of which were closely surface-associated with the *O.* cf. *ovata* cells, while some others were more focussed on the ventral section of *O.* cf. *ovata* cells. The majority of bacterial cells, however, were concentrated within the EPSs far from *O.* cf. *ovata* cells (i.e. loosely associated with *O.* cf. *ovata* cells) (Fig. 4). PMEase and PDEase activities of cells (*O.* cf. *ovata* 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. Of the cellular activity, that of *O.* cf. *ovata* cells and their closely associated bacteria provided only 13.4 and 19.6% of the total activities, while the rest of the bacterial community were responsible for 43.4 and 19.7% of the total PMEase and PDEase activity, respectively (Fig. 5). Staining of *O.* cf. *ovata* PMEases with BCIP/NBT re-

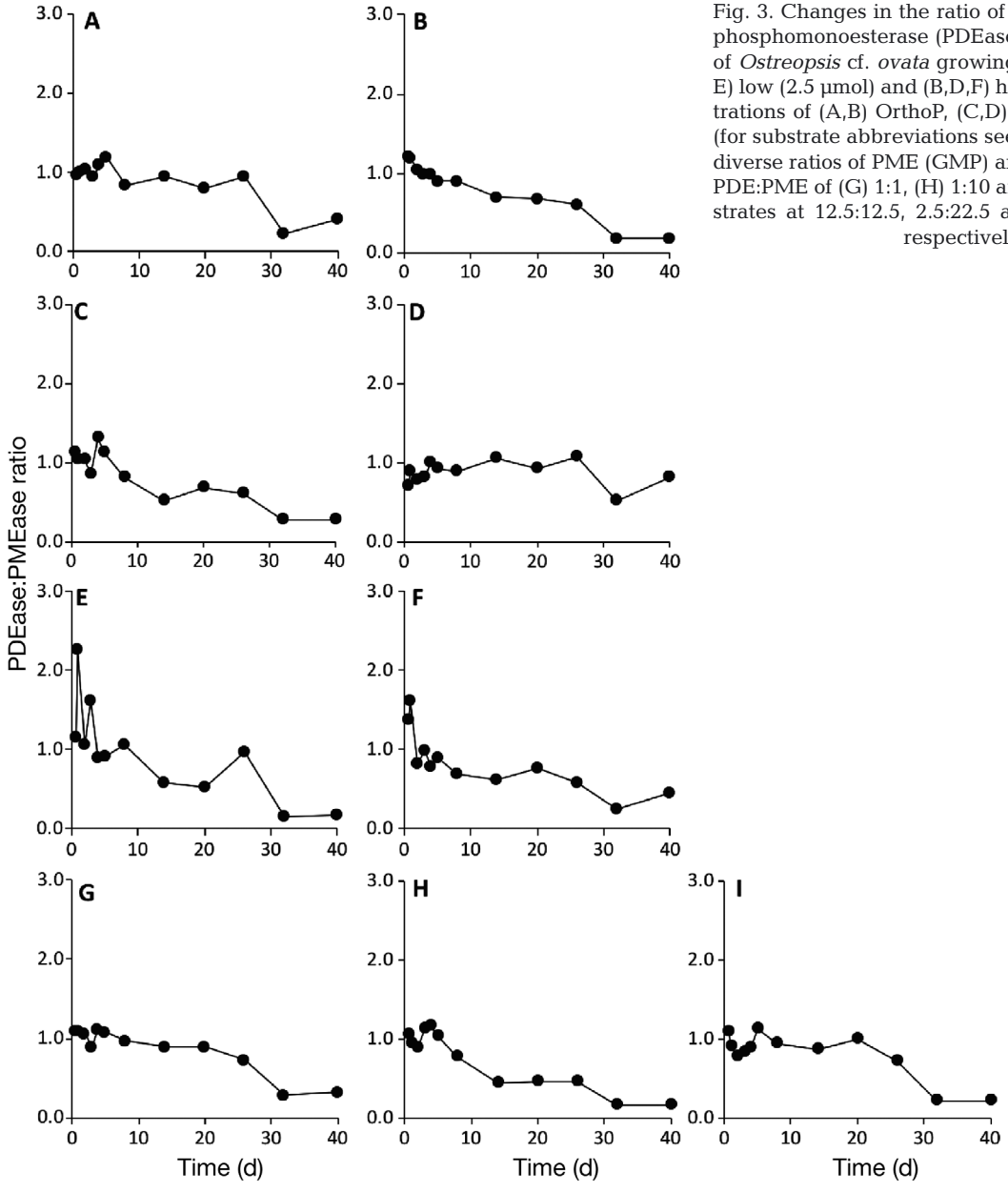

Fig. 3. Changes in the ratio of phosphodiesterase to phosphomonoesterase (PDEase:PMEase) in cultures of *Ostreopsis* cf. *ovata* growing in media with (A,C, E) low (2.5 µmol) and (B,D,F) high (75 µmol) concentrations of (A,B) OrthoP, (C,D) GMP and (E,F) RNA (for substrate abbreviations see Table 1 legend) 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 µmol l^{-1}, respectively)

vealed intracellular activity in the ventral part of the cells and extracellular activity associated with the cell surface, mostly within the EPSs, often close to the ventral section of *O.* cf. *ovata* cells (Fig. 6).

4. DISCUSSION

In this study, *Ostreopsis* cf. *ovata* was able to grow in media containing PME and PDE as the 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), even though it 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

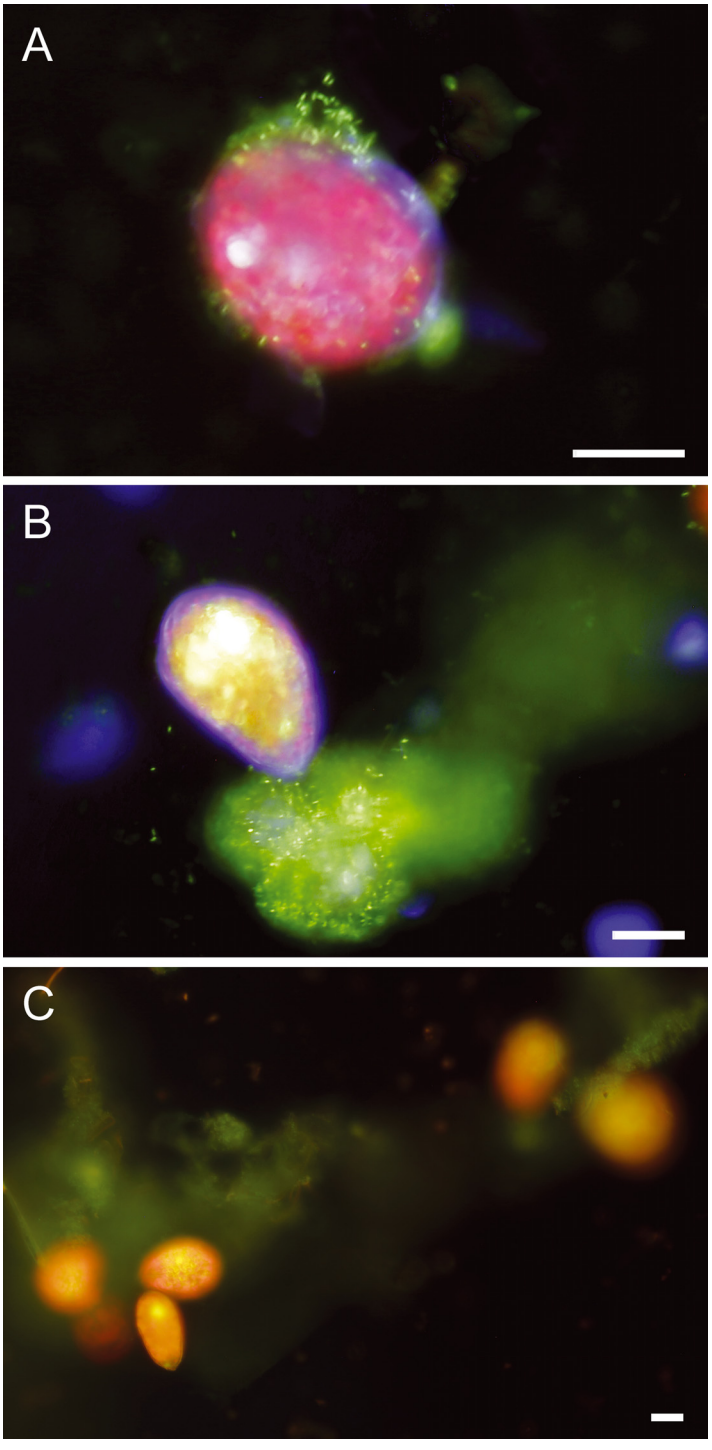

Fig. 4. Biofilm (extracellular polymeric substances [EPSs], *Ostreopsis* cf. *ovata* and bacterial cells) stained with SYBR Green I (green fluorescence of nucleic acids), Calcofluor-White M2R (blue fluorescence of cellulose) and red autofluorescence of the chlorophyll in *O.* cf. *ovata* is visible under epifluorescence microscopy: a certain amount of bacterial cells were closely associated with the *O.* cf. *ovata* cells, both with (A) the cell surface and (B) the ventral part of cells. (C) The majority of bacterial cells were located within the EPSs and loosely associated with *O.* cf. *ovata*. Scale bars = 20 µm

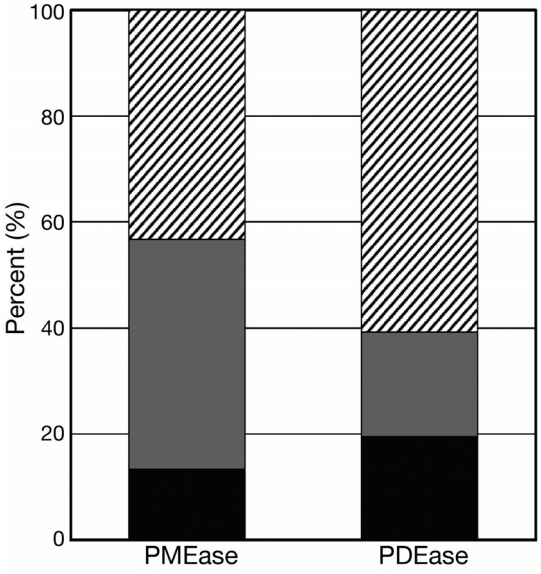

Fig. 5. Phosphatase activity (phosphomonoesterase [PMEase] and phosphodiesterase [PDEase]) of the different fractions of the culture of *Ostreopsis* cf. *ovata*. Phosphatase activities of *O.* cf. *ovata* cells and closely associated bacteria (black), loosely associated bacteria (grey) and extracellular (hatched) are given as a percentage of the total phosphatase activity of each sample

community dynamics, primary production and P 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 P 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 8 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 by the EPSs from moving towards the cells (Flemming 2011). Further-

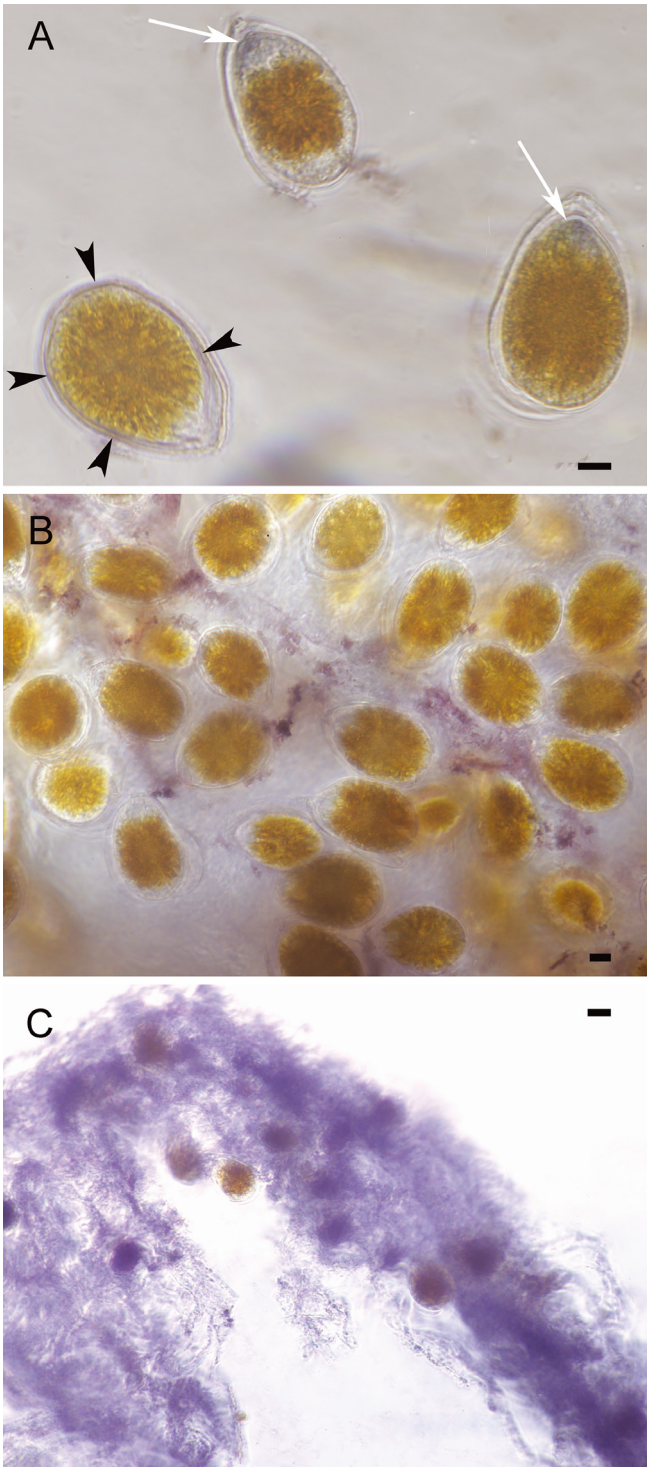

Fig. 6. BCIP-NBT staining of phosphomonoesterase (PMEase) activity of *Ostreopsis* cf. *ovata* and extracellular polymeric substances (EPSs). (A) Intracellular activity in the ventral part of the cells (white arrows) and associated with the cell surface (black arrowheads). (B) Extracellular activity within the EPSs, close to the ventral part of the cells. (C) The majority of activity was located within the EPSs. Scale bars = 20 µm (A,B); 40 µm (C)

more, bacteria 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 this is the case, 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 development 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 *O.* cf. *ovata* 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 identified within the mucocysts were suggested to be involved in the 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 may 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 (*O.* cf. *ovata* and bacteria), a large part of the overall activity was within the EPS.

The EPSs seem to be the main site of phosphatase activity and so function somewhat like an efficient external digestion system for the entire biofilm community (Flemming & Wingender 2010). The chemical and physical properties of EPSs 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 EPSs 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 the 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 6 d, there were large reductions in media P and lag phases in growth protracted up to 12 d, 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 EPSs (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 separately assess the EPS P content and the cellular content to determine where accumulation occurs, i.e. do EPSs behave as a functional immediate environment for the biofilm cells (Flemming 2011) or do the cells themselves take up luxury levels of P (Baek et al. 2008, Solovchenko et al. 2019) prior to subsequent growth?

The use of mid- to 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, and we only observed increases in activity toward the end of the incubation, when cellular activity likely added a more significant contribution to the overall activity. Within EPSs, 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 occurs 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 *O.* cf. *ovata* inocula, and the diminishing rates were an indication of a decrease in this stress over the incubation period.

As bacterial abundances during an *O.* 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 under non-axenic conditions with maximal bacterial densities of up to 10^7 cells ml^{-1} (Pezzolesi et al. 2016). The growth of the *O.* cf. *ovata* 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 bacteria–*Ostreopsis* interaction within the EPS should be conducted. We therefore propose 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 uncertain at present (Baldwin 2013), but it has been shown that leachate arriving from land is an important source of P to nearshore coastal environments (Suzumura & Kamatani 1995).

5. CONCLUSIONS

Growth of *Ostreopsis* cf. *ovata* is not limited when cultured in medium containing only organic P substrates. The efficient use of a range of PMEs and PDEs suggests a broad hydrolytic specificity of the PMEase and PDEase 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 activities coincided with the onset of an *O.* cf. *ovata* bloom that occurred in water where the main P source was organic.

Accumulation of phosphatases in the EPSs suggests that this was the main site of hydrolytic activity. The abundance of EPSs 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 EPSs essentially function like an external digestion system that benefits the whole biofilm/floc community. The EPSs trap and concentrate particulate and dissolved organics and provide a substrate that holds and binds enzymes; they also prevent the loss of hydrolytic products to ambient water and provide a medium through which nutrients can diffuse towards the cells.

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