Phosphonate utilization by eukaryotic phytoplankton

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Scientific Significance Statement
Comprising up to 10% of the marine dissolved organic phosphorus pool, phosphonates have been shown to be a dynamic phosphorus source both being assimilated and produced by marine cyanobacteria. The ability of eukaryotic phytoplankton to supplement growth with phosphonates found naturally in the marine environment remains vastly under explored. Here, we show phosphonate utilization may no longer be restricted to the realm of prokaryotes as at least some eukaryotic phytoplankton were shown to exhibit growth on these substrates, suggesting those able to utilize this phosphorus source may have an advantage over those that cannot thereby influencing carbon production and export.

Abstract
Phytoplankton readily assimilate dissolved inorganic phosphorus (phosphate; $P_i$) into essential biomolecules, making it their preferred form of phosphorus ($P$). In low $P_i$ marine environments, phytoplankton also utilize dissolved organic $P$ (DOP) compounds to sustain primary productivity. Phosphonates comprise up to 10% of the DOP pool and have been shown to be an important source of $P$ in the nutrition of marine prokaryotic phytoplankton. To date, the ability of eukaryotic phytoplankton to supplement growth with natural phosphonates remains unknown. Here, we show that the growth rates of two eukaryotic phytoplankton species grown on phosphonate(s) as the sole source of $P$ were significantly greater than growth rates under $P_i$-deficient conditions. Phosphonate did not support growth of a third species, indicating utilization is not universal among eukaryotic phytoplankton. Taken together, these results indicate that direct uptake and utilization of extracellular phosphonates may no longer be restricted to the realm of prokaryotes.

Phosphorus ($P$) is an essential macronutrient utilized by phytoplankton. As such, $P$ availability exerts an important control on oceanic primary production (Benitez-Nelson 2000; Ammerman et al. 2003; Dyhrman et al. 2007; Mather et al. 2008; Lomas et al. 2010). Phytoplankton can easily transport and assimilate dissolved inorganic phosphorus ($P_i$) directly into needed biomolecules, making it their preferred form of $P$. However, in many ocean regions, in particular, the oligotrophic North Atlantic and the eastern Mediterranean Sea, $P_i$ concentrations are consistently low (< 10 nmol L$^{-1}$) and its availability may restrict primary production (Wu et al. 2000; Mather et al. 2008; Torres-Valdes et al. 2009; Krom et al. 2010; Lomas et al. 2010). In these regions, dissolved organic phosphorus (DOP) is an important nutrient source. The composition of the DOP pool is complex and while largely unknown, it can generally be divided into two major groups based on bond types: $P$ esters (C-O-P bonds) and phosphonates (C-P bonds) (Clark et al. 1999; Kolowith et al. 2001).

All marine microbes are capable of utilizing $P$ esters through the activity of enzymes like alkaline phosphatase (Karl and Bjorkman 2002). In contrast, phosphonates, which...
can comprise up to 10% of the total DOP pool (Clark et al. 1999; Kolowith et al. 2001), have only been shown to be an important source of P in the nutrition of marine cyanobacteria (Dyhman et al. 2006; Ilikchyan et al. 2009; Ilikchyan et al. 2010). Our understanding of naturally occurring phosphonate utilization by eukaryotic phytoplankton is severely limited. In the open ocean, phytoplankton likely encounter methylphosphonate (MPN), a phosphonate synthesized by marine microbes (Metcalfe et al. 2012), and 2-aminoethylphosphonate (2-AEPN), the most common form of phosphonate found in nature (McGrath et al. 2013). Dinoflagellates, in general, are unable to use 2-AEPN as a P source (Cui et al. 2016); however, other eukaryotic phytoplankton have been shown to utilize glyphosate, a chemically synthesized herbicidal phosphonate, as the sole source of P for growth (Wang et al. 2016). It is worth noting that with the exception of dinoflagellates not being able to use either 2-AEPN or glyphosate, there does not appear to be a consistent taxonomic pattern to phosphonate utilization (e.g., some diatoms can while others cannot utilize glyphosate; Wang et al. 2016). These prior studies demonstrate that the potential for phosphonate utilization in support of growth exists in at least some eukaryotic phytoplankton.

In this study, we examined the growth of three eukaryotic phytoplankton species grown on MPN and 2-AEPN as the sole source of P. We sought to characterize differences in phosphonate utilization and cellular physiology among the targeted species. To do this, we compared the growth and intracellular P content of cultures grown with phosphonate as a P source to cultures grown under Pi-deficient and Pi-replete conditions.

Materials and methods

Experimental culture conditions

Axenic cultures of the pico-prasinophyte Micromonas commoda (CCMP 2709), the coccolithophore Emiliania huxleyi (CCMP 2090), and the diatom Phaeodactylum tricornutum (CCMP 2561) were obtained from the National Center for Marine Algae and Microbiota (Bigelow Laboratories). The cultures remained axenic throughout the experiments as determined by SYTO staining and flow cytometric counting (Gal et al. and DelGiorgio 2000) on a BD FACS Jazz cell sorter. Phytoplankton were grown in artificial seawater (Worden et al. 2009) amended with L1 media (Guillard and Hargraves 1993) with (P. tricornutum) or without (M. commoda and E. huxleyi) silica. The P source was added separately to achieve the desired growth conditions; Pi-replete media contained 36 μmol L⁻¹ PO₄³⁻, the Pi-deficient condition received 0.1 μmol L⁻¹ PO₄³⁻, and the phosphonate treatments received either 36 μmol L⁻¹ MPN or 2-AEPN. The Pi-deficient treatment (0.1 μmol L⁻¹) represents a control for the low level of contaminating Pi measured in the phosphonate media; thus, an increase in growth in the MPN and 2-AEPN conditions above that measured in the Pi-deficient condition is due to phosphonate utilization. The potential for abiotic breakdown of phosphonate to Pi was investigated in media-only tubes exposed to the experimental temperature and light conditions for 10 d. Pi levels did not change throughout the experimental period (MPN average Pi = 0.11 μmol L⁻¹ ± 0.02, 2-AEPN average Pi = 0.10 μmol L⁻¹ ± 0.02), strongly supporting the notion of active enzymatic breakdown of phosphonates for growth. Natural P concentrations are much lower than those used in this study; the replete nutrient concentrations were used to support high cell yields necessary for analytical measurements. Cultures were acclimated to the four growth conditions described above as they had been maintained in each P treatment for a minimum of two transfers (20 d). Cultures were grown at 20°C in a 14 h light/10 h dark cycle at ~100 μE m⁻² s⁻¹ with a starting concentration of ~1 × 10⁴ cells mL⁻¹ in 25 mL culture volumes. Phytoplankton growth was monitored by fluorescence measurements using a Turner TD-700 fluorometer and cell counts analyzed by flow cytometry. Specific growth rates (μ) were calculated from the linear regression of the natural log of cell counts during the exponential growth phase of cultures. Quadruplicate cultures were setup for each treatment; three replicates were harvested in the late exponential phase of growth for physiological measurements, while the fourth was used to monitor cell abundances later in the growth cycle.

Physiological measurements

Cell samples (5 mL culture volume) for particulate P were collected onto precombusted 25-mm-Whatman glass fiber filters (GE Healthcare Bio-Sciences), rinsed with 0.17 mol L⁻¹ Na₂SO₄, and stored frozen at ~20°C until analysis. Determinations were made as previously described (Lomas et al. 2010). Briefly, filters were rinsed with 0.017 mol L⁻¹ MgSO₄ dried at 90°C, and combusted at 500°C for 2 h. Upon cooling, 0.2 mol L⁻¹ HCl was added and hydrolyzed at 80°C for 30 min. After cooling, mixed reagent (Strickland and Parsons 1972) was added, the samples were centrifuged, and absorbance was read at 885 nm using a Genesys 10 spectrophotometer (Thermo Scientific). The particulate P determinations were used to determine and compare the cellular P levels among the treatments; it was not used to infer P acquired from the phosphonate source. Because particulate P measurements were made from a single time point and Pi and total dissolved P were not measured, we are unable to construct a measurement-based P mass balance. Future experiments should include monitoring these P pools to fully explore the potential impact of eukaryotic phosphonate consumption on marine P biogeochemistry.

Alkaline phosphatase activity (APA) measurements were made by quantifying the hydrolysis of 6,8-difluoro-4-methylumbelliferyl phosphate (Life Technologies) using a Molecular Devices FilterMax F5 microplate reader. Abiotic substrate hydrolysis was accounted for in killed controls that were boiled and cooled prior to substrate addition, as well as in media-only controls. The fluorescent reference standard 6,8-difluoro-4-methylcoumarin (Life Technologies) was used to calculate the rate of hydrolysis, which was then normalized to cell abundance to determine APA rates per cell.
Statistical analysis

For all parameters, one-way analysis of variance (ANOVA) tests were conducted using SigmaStat (version 3.5; Systat Software) to determine statistically significant differences. All pairwise comparisons were made using the Student–Newman–Keuls test.

Results and discussion

We investigated phosphonate utilization in axenic cultures of three species of eukaryotic phytoplankton, which represent ecologically important functional groups: M. commoda (picoeukaryote), E. huxleyi (cocolithophore), and P. tricornutum (diatom). These species were selected as potential phosphonate transporter genes (phnC) were identified in the E. huxleyi and P. tricornutum genomes (Wang et al. 2016); putative phnC genes were also detected in the M. commoda genome (this study; data not shown). The phytoplankton used in this study were acclimated to four P treatments: P-replete, P-deficient, MPN, and 2-AEPN as the sole source of P at replete concentrations.

Phosphonates supported growth of the picoeukaryote M. commoda

The growth efficiency, with regards to cell yield and growth rate, of M. commoda on MPN was significantly greater than cells grown under P-deficient conditions (p < 0.05; one-way ANOVA); MPN cell yields were nearly six-fold greater and achieved a growth rate nearly double that of P-deficient cells (Fig. 1a,b). Phosphonate utilization by eukaryotic phytoplankton has been previously demonstrated; a two-fold increase in cell yield was seen in the prymnesiophyte, Isochrysis galbana, grown on glyphosate when compared to growth in Pi-deplete conditions (Wang et al. 2016). The cell density of M. commoda supplied with 2-AEPN as the sole source of P did not differ significantly from the P-deficient treatment until the experiment concluded, at which point cell yields were similar to those seen in the MPN treatment and nearly 8-fold greater than the Pi-deficient treatment (Fig. 1a). The growth rate on 2-AEPN was also significantly greater than the Pi-deficient treatment (Fig. 1b). The delay in cell growth may suggest M. commoda cannot utilize 2-AEPN in the presence of Pi, even at low levels. The presence or absence of Pi has been shown to differentially impact the ability of other eukaryotic phytoplankton to utilize glyphosate (Wang et al. 2016). A delay was not seen in the MPN treatment suggesting M. commoda can utilize both MPN and Pi simultaneously as has been previously shown in the cyanobacterium Trichodesmium (Beversdorf et al. 2010). The experiment concluded while the Pi-replete cultures were still in exponential growth; we have measured higher growth rates in previous experiments (Whitney and Lomas 2016) and assume that a higher growth rate would have been achieved in this study had the investigation continued. Thus, when compared to the Pi-replete treatment, reduced cell yields and growth rates of the MPN and 2-AEPN treatments suggests M. commoda cannot extract P from phosphonates as efficiently as it can utilize Pi (Fig. 1a,b).

Despite reaching a relatively high cell density and growth rate, M. commoda cells grown on MPN had severely reduced cellular P levels (Fig. 1c). A similar response was seen in Trichodesmium cultures given MPN (White et al. 2010). Synechococcus cells maintained in MPN specifically reduced their polyphosphate pool, polymers of Pi molecules joined by phosphoanhydride bonds (Adams et al. 2008). The decrease in P quota measured in this study may also be a consequence of M. commoda cells modulating their intracellular P storage pools. While there is precedent for MPN utilization coupled with reduced P storage pools and thus total cellular P levels in cyanobacteria, further study looking into the cellular mass balance for P is required to support this hypothesis in eukaryotic phytoplankton and evaluate the impact of this physiological observation on marine P biogeochemistry. Concurrent with the decrease in cellular P in M. commoda grown under MPN conditions was a significant increase in APA when compared to the other P treatments, including a 10-fold increase over the Pi-deficient condition (p < 0.05; one-way ANOVA; Fig. 1d). The strong induction of APA may be in response to cells detecting both internal and external Pi-deplete conditions. The data suggest that M. commoda cells acclimated to grow on MPN are P stressed; however, the P acquired from MPN is enough to support critical cellular processes, growth, and cell accumulation. The cellular P content and APA of cells grown on 2-AEPN did not differ from those measured in the Pi-deficient condition (Fig. 1c,d). The similarities in growth and physiology between the 2-AEPN and Pi-deficient treatments suggest that M. commoda cannot access 2-AEPN until Pi drawdown is complete.

The reduced cellular P and corresponding elevated APA levels of M. commoda could also be a response to high absolute or relative (i.e., DOP : Pi) concentrations of a DOP source that is energetically difficult to hydrolyze. It is possible that in the presence of phosphonates, such as MPN, M. commoda induces high-affinity Pi transporters to more efficiently transport liberated Pi at very low levels, when compared to Pi-deficiency without phosphonates. High-affinity Pi transporters have been identified in several species of eukaryotic phytoplankton, including dinoflagellates, diatoms, as well as chlorophytes, but their regulation is poorly characterized (Lin et al. 2016). To the best of our knowledge, this phenomenon has never been described in M. commoda, and the most parsimonious explanation remains that this eukaryotic alga can hydrolyze and grow on phosphonates. The reduction in cellular P content in M. commoda grown on MPN, as was seen Trichodesmium (White et al. 2010), coupled with the demonstrated ability of several eukaryotic phytoplankton to use a chemically synthesized phosphonate as a P source (Wang et al. 2016), strongly supports the notion of enzymatic phosphonate utilization.
Growth patterns on phosphonates differ in other eukaryotic phytoplankton

*E. huxleyi* was also able to use MPN as a P source; the cell density and growth rate were both significantly greater than those measured in the P-i-deficient treatment (*p* < 0.05; one-way ANOVA; Fig. 2a,b). Cell yields were approximately two-fold different between the MPN and P-i-deficient treatments. While the absolute cell yield was less than that observed in *M. commoda*, given the differences in cell P quota, the total amount of P extracted from the MPN pool during growth was similar between these two species. The cellular P content of cells grown on MPN was also reduced when compared to the other treatments (Fig. 2c), as was seen in *M. commoda*, suggesting a similar response to internal/external P* i* availability and mobilization. We hypothesize that *E. huxleyi* and *M. commoda*, and perhaps other eukaryotic phytoplankton, are capable of utilizing only a fraction of the available MPN. This hypothesis is supported by recent work that shows for *I. galbana* and *E. huxleyi*, both of which can use glyphosate as a sole P source, there is no difference in cell yield or growth rate when provided 3.6 and 36 μmol L* 1* glyphosate (Wang et al. 2016). Unlike *M. commoda*, *E. huxleyi* was not able to use 2-AEPN as a P source; its cell yield and growth rate did not differ from the P-i-deficient condition (Fig. 2a,b). There was no observed increase in APA in the 2-AEPN culture; rather, high levels were detected in the P-i-deficient and both phosphonate grown cultures (Fig. 2d). The cause of the differing APA patterns between *E. huxleyi* and *M. commoda* cells given MPN as the sole source

Fig. 1. (a) Growth of axenic *M. commoda* acclimated to P-i-replete (+Pi, 36 μmol L* 1* Pi, black circles), P-i-deficient (−Pi, 0.1 μmol L* 1* Pi, white circles), MPN replete (+MPN, 36 μmol L* 1* MPN, triangles), and 2-AEPN replete (+2-AEPN, 36 μmol L* 1* 2-AEPN, squares) conditions. Four biological replicates were used for each condition; on day 9 (ellipse), three replicates were harvested for additional analyses. (b) *M. commoda* specific growth rates for each P treatment as determined from days 3–9. (c) Cellular P quotas (QP) for each treatment. No significant differences between the growth conditions were found due to variability among the biological replicates. (d) APA for each P treatment. Error bars represent standard deviation of biological replicates. Samples that lack a common letter are significantly different (*p* < 0.05).
of P is unknown. The alkaline phosphatase enzyme is particularly complex as there are many isoforms that can vary in cellular localization, metal cofactor, and regulation (Lin et al. 2016). Notably, *E. huxleyi* uses a novel alkaline phosphatase (Xu et al. 2006).

In contrast to prior work (Wang et al. 2016), neither of the naturally occurring phosphonates used in this study supported significantly higher growth and cell yields of the diatom, *P. tricornutum*. Cell yields and growth rates did not differ among MPN, 2-AEPN, and Pi-deficient conditions (Supporting Information Fig. S1a,b) indicating the growth observed in these treatments was due to the low concentration of Pi contamination available in the media. Similarly, the cellular P content and APA levels did not differ among the phosphonate and Pi-deficient treatments suggesting that there was no physiological response to the presence of phosphonates (Supporting Information Fig. S1c,d). These results clearly show that the use of phosphonates is not universal within the eukaryotes or within a specific phosphonate compound. Further work is needed to

Fig. 2. (a) Growth of axenic *E. huxleyi* acclimated to Pi-replete (+Pi, 36 μmol L⁻¹ Pi, black circles), Pi-deficient (−Pi, 0.1 μmol L⁻¹ Pi, white circles), MPN replete (+MPN, 36 μmol L⁻¹ MPN, triangles), and 2-AEPN replete (+2-AEPN, 36 μmol L⁻¹ 2-AEPN, squares) conditions. Four biological replicates were used for each condition; on day 5 (ellipse), three replicates were harvested for additional analyses. (b) *E. huxleyi* specific growth rates for each P treatment as determined from days 0 to 5. (c) Cellular P quotas (Q_p) for each treatment. No significant differences between the growth conditions were found due to variability among the biological replicates. (d) APA for each P treatment. Error bars represent standard deviation of biological replicates. Samples that lack a common letter are significantly different (p < 0.05).
elucidate the molecular underpinnings that permit growth on phosphonates and why it appears that only a fraction of a total compound pool is available.

**Ecological implications**

These data suggest that phosphonate utilization may be important in regulating phytoplankton community composition and biogeochemical cycling, especially in marine environments like the Sargasso Sea (Benitez-Nelson 2000; Ammerman et al. 2003; Dyhrman et al. 2007; Mather et al. 2008) and the eastern Mediterranean Sea (Krom et al. 2010), where Pi levels are chronically low. Phosphonates, in combination with another reduced P species phosphite, represent up to 3% of microbial P uptake in the Sargasso Sea (Van Mooy et al. 2015) and could be a currently unrecognized source of P to the small eukaryotic phytoplankton which make significant contributions to autotrophic biomass and primary productivity in these oligotrophic waters (Li 1994; Li 1995; Man-Aharonovich et al. 2010). Species like *M. commoda*, which can utilize two forms of phosphonate (this study) and grow well on other forms of DOP (Whitney and Lomas 2016), may have a competitive advantage over species like *P. tricornutum*, which have reduced access to or ability to hydrolyze the DOP pool. Additionally, we have shown eukaryotic phytoplankton growing on phosphonates can alter the particulate P pools as cells growing on MPN were found to have severely reduced cellular P quotas. These findings profoundly change our understanding of phosphonate availability to the diverse phytoplankton assemblages in ocean regimes. This is an especially timely finding as the surface ocean is expected to warm and become increasingly stratified, leading to a reduction in vertical nutrient supplies, a shift in phytoplankton community composition, and possibly an increase in ocean regimes. This is an especially timely finding as the surface ocean is expected to warm and become increasingly stratified, leading to a reduction in vertical nutrient supplies, a shift in phytoplankton community composition, and possibly an increase in ocean regimes. This is an especially timely finding as the surface ocean is expected to warm and become increasingly stratified, leading to a reduction in vertical nutrient supplies, a shift in phytoplankton community composition, and possibly an increase in ocean regimes.

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Conflict of Interests

The authors declare no competing financial interests.

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