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Preferential utilization of inorganic polyphosphate over other bioavailable phosphorus sources by the model diatoms *Thalassiosira* spp.

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Summary

Polyphosphates and phosphomonoesters are dominant components of marine dissolved organic phosphorus (DOP). Collectively, DOP represents an important nutritional phosphorus (P) source for phytoplankton growth in the ocean, but the contribution of specific DOP sources to microbial community P demand is not fully understood. In a prior study, it was reported that inorganic polyphosphate was not bioavailable to the model diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. However, in this study, we show that the previous finding was a misinterpretation based on a technical artefact of media preparation and that inorganic polyphosphate is actually widely bioavailable to *Thalassiosira* spp. In fact, orthophosphate, inorganic tripolyphosphate (3polyP), adenosine triphosphate (ATP) and adenosine monophosphate supported equivalent growth rates and final growth yields within each of four strains of *Thalassiosira* spp. However, enzyme activity assays revealed in all cultures that cell-associated hydrolysis rates of 3polyP were typically more than ~10-fold higher than degradation of ATP and the model phosphomonoester compound 4-methylumbelliferyl phosphate. These results build on prior work, which showed the preferential utilization of polyphosphates in the cell-free exudates of *Thalassiosira* spp., and suggest that inorganic polyphosphates may be a key bioavailable source of P for marine phytoplankton.

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Introduction

Phosphorus (P) is a universal nutrient required for the assembly of essential biomolecules such as DNA, phospholipids and adenosine triphosphate (ATP). In the ocean, microbes drive a dynamic short-term P cycle, in which dissolved, particulate, organic and inorganic forms of P are rapidly transformed in order to recycle and retain bioavailable P within microbial communities. Dissolved organic P (DOP) is an essential source of bioavailable P in oligotrophic systems due to a chronically short supply of the presumably most preferred P source, dissolved orthophosphate (Karl, 2014; Karl and Björkman, 2015). Yet even in P-replete coastal environments where orthophosphate is actively consumed, highly labile forms of DOP are also rapidly utilized by microorganisms (Benitez-Nelson and Buesseler, 1999; Nausch *et al*., 2018). Thus, DOP cycling is a dynamic component of the marine microbial P cycle in diverse ocean environments across extremes in trophic status.

DOP is a complex assemblage of molecules, which consists of organic, inorganic and polymeric forms of P. Much remains unknown about DOP composition, especially at the molecular level, but DOP is broadly composed of three principal bond classes: phosphoesters (P–O–C bonds), phosphoanhydrides (P–O–P) and phosphonates (P–C). The relative proportion of each of these bond classes appears to be relatively consistent across DOP samples collected from a range of ocean environments, with phosphoesters accounting for the majority (80%–85%) of DOP, and phosphoanhydrides (8%–13%) and phosphonates (5%–10%) accounting for roughly equal proportions (Young and Ingall, 2010). Microorganisms are ultimately responsible for the production of DOP, which is released from biomass into seawater through mechanisms such as viral lysis and sloppy zooplankton feeding. For example, the major DOP bond classes are present in highly labile biomolecules such as ATP (phosphoester

and phosphoanhydride bonds), its precursor adenosine monophosphate (AMP, phosphoester bond) and inorganic polyphosphate (phosphoanhydride bonds). Each of these P compounds are present in seawater and are biologically utilized (Azam and Hodson, 1977; Ammerman and Azam, 1985; Björkman and Karl, 2005; Diaz *et al*., 2008; Diaz and Ingall, 2010; Martin and Van Mooy, 2013; Martin *et al*., 2014, 2018). However, the relative contribution of specific DOP sources to community-level P demand and productivity remains poorly constrained.

The biological utilization of phosphoesters and nucleotides by marine microorganisms has long been acknowledged (Ammerman and Azam, 1985; Dyhrman and Ruttenberg, 2006; Duhamel *et al*., 2010; Karl, 2014; Mahaffey *et al*., 2014), whereas an appreciation for the role of inorganic polyphosphate as a potentially bioavailable P source has recently been growing (Björkman, 2014). Inorganic polyphosphate is an essential biopolymer of at least three and up to thousands of phosphate ions, which is likely synthesized by every organism in nature (Kornberg, 1995; Kornberg *et al*., 1999; Rao *et al*., 2009). In the Indian Ocean and chronically low P Sargasso Sea, particle-associated polyphosphates are preferentially recycled relative to other forms of P, suggesting that polyphosphates have a high degree of bioavailability (Martin *et al*., 2014, 2018). Inorganic polyphosphate is also bioavailable as a sole P source to representative strains of the cyanobacteria *Synechococcus* and *Prochlorococcus* (Moore *et al*., 2005), as well as the coccolithophore *Emiliania huxleyi* and the diatom *Thalassiosira oceanica* (Diaz *et al*., 2016). *Thalassiosira pseudonana* and *Thalassiosira weissflogii* were reportedly unable to utilize inorganic polyphosphate as a sole P source, according to one prior study (Diaz *et al*., 2016). Yet paradoxically, we recently found that inorganic polyphosphate is rapidly hydrolysed by cell-free (0.2 μm filtered) exudates of *T. pseudonana* (Diaz *et al*., 2018), leading us to question the original negative bioavailability result. In order to address this inconsistency and clarify the potential role of inorganic polyphosphate as a P source for phytoplankton growth, we assessed the ability of four strains of *Thalassiosira* spp. to hydrolyze and assimilate three representative DOP sources: inorganic tripolyphosphate (3polyP), ATP and AMP.

Results and discussion

The goal of this study was to assess the capacity of *Thalassiosira* spp. to externally degrade and assimilate P from phosphoanhydride- and phosphomonoester-containing compounds that are representative of natural marine DOP: ATP, AMP and 3polyP. DOP acquisition involves the extracellular hydrolysis or degradation of P-containing molecules via release of orthophosphate through the activity of P hydrolase enzymes such as alkaline phosphatase and 5′-nucleotidase (Ammerman and Azam, 1985; Hoppe, 2003). The enzymatically released orthophosphate is subsequently taken up via cellular transport systems (Lin *et al*., 2016) and incorporated into biomass to support growth, which is referred to as assimilation. A DOP source that can be assimilated is considered bioavailable. We define DOP utilization as DOP degradation that may or may not be followed by assimilation.

DOP bioavailability

The ability of *Thalassiosira* spp. to grow on ATP, AMP or 3polyP as the sole P source was assessed in f/2 media (Guillard and Ryther, 1962) prepared through a variety of approaches (Supporting Information Fig. S1). First, cultures were inoculated into f/2 media prepared with no P amendment (−P) in order to account for the levels of growth possible on background levels of P in the natural seawater base and any P carried over during inoculation. In all cases, growth under −P conditions was significantly less than in P-amended cultures (Figs 1–3), confirming that growth on background P sources was negligible.

The bioavailability of specific DOP sources was assessed relative to control cultures prepared with orthophosphate (PO_4) as a sole P source. Experiments initially followed the methods of Diaz *et al*. (2016), in which PO_4- and DOP-amended media were prepared by different approaches (type 1 and type 2 respectively). In type 1 media, all media ingredients, including orthophosphate, were added before autoclaving because this approach presents the lowest contamination risk by airborne bacteria and fungi, consistent with protocols followed by the National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratories, East Boothbay, Maine. As we are focused on P nutrition, we refer to type 1 media here as +PO_4(1) because orthophosphate is provided as the sole P source. In contrast, type 2 media were autoclaved without the addition of P, and sterile-filtered DOP sources [+ATP(2), +AMP(2), +3polyP(2)] were added to the autoclaved −P media in order to avoid potential degradation of DOP sources during the autoclave cycle. These experiments reproduced observations from Diaz *et al*. (2016), which showed, for *T. weissflogii* and *T. pseudonana* CCMP1335, that type 2 polyphosphate media supported significantly less growth than +PO_4(1) (Figs 1A,C,E and 2A,C,E). Diaz *et al*. (2016) interpreted this result to suggest that inorganic polyphosphate is not bioavailable to these diatoms. However, in the present study, diminished growth relative to +PO_4(1) cultures was also observed when orthophosphate was provided as a sole P source in type 2 media [+PO_4(2)] (Figs 1A,B,E and 2A,B,E). This result confirmed that the growth responses were primarily a result

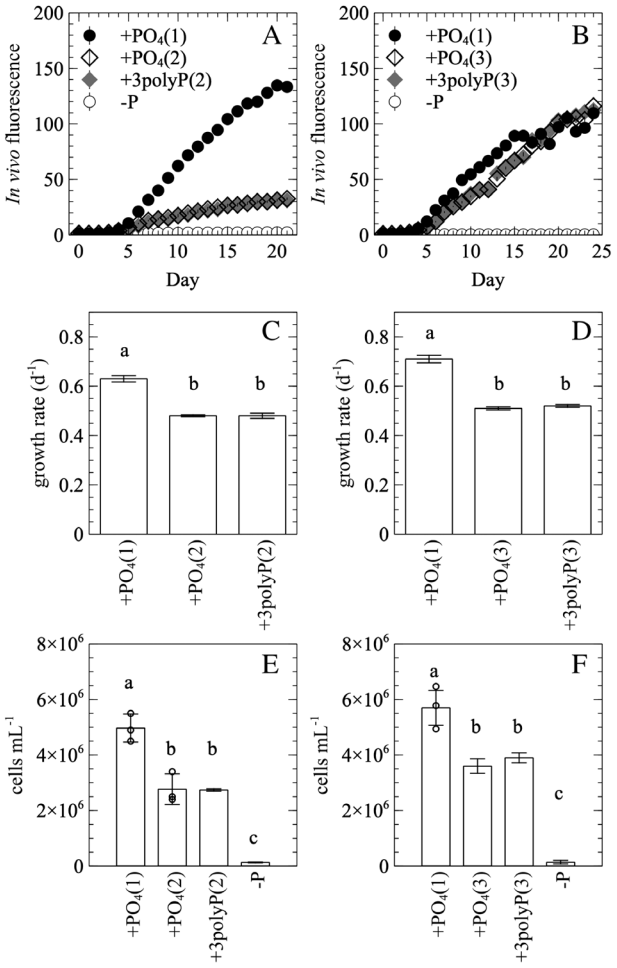

Fig. 1. Growth of *T. pseudonana* CCMP1335 on orthophosphate (PO_4) and inorganic tripolyphosphate (3polyP) as sole P sources in 25 mm tubes on three different media types (media type is indicated by the number in parentheses). Each column presents results from a separate experiment, in which the left column (A,C,E) compares growth in type 1 and type 2 media, and the right column (B, D, F) compares growth in type 1 and type 3 media. *In vivo* fluorescence (A,B), growth rates (C,D) and final growth yields (E,F) were calculated as the average of three biological replicates. Error bars represent one standard deviation of the mean. Error bars not visible are smaller than the data symbol. Averages that do not share a common letter are significantly different ($P < 0.05$, Tukey HSD), and those that do are statistically the same. Individual data points are shown for select treatments where the ratio of SD to the mean is greater than 10% (open circles).

of media type, rather than P source, which challenges the negative results for 3polyP bioavailability originally reported in Diaz *et al*. (2016).

We postulated that the suppressed growth yields in type 2 cultures could be explained either by a decreased availability of DOP due to syringe filtration or a diminished bioavailability of one or more other nutrients due to autoclaving in the absence of added phosphate. To investigate these potential explanations, type 2 cultures of *T. pseudonana* CCMP1335 were grown to stationary phase in 25 mm borosilicate glass tubes and separately amended with f/2 concentrations of each nutrient stock. Phosphate amendments did not stimulate growth (Supporting Information Fig. S2), ruling out a lack of P availability due to syringe filtration of DOP sources as the cause of the low growth yields in type 2 media. Only the addition of iron stimulated growth (Supporting Information Fig. S2), suggesting that iron availability is diminished in type 2 media due to autoclaving in the absence of added phosphate. This iron/autoclave artefact consistently resulted in diminished growth yields for *T. pseudonana* CCMP1335 and *T. weissflogii* on type 2 media in 25 mm tubes.

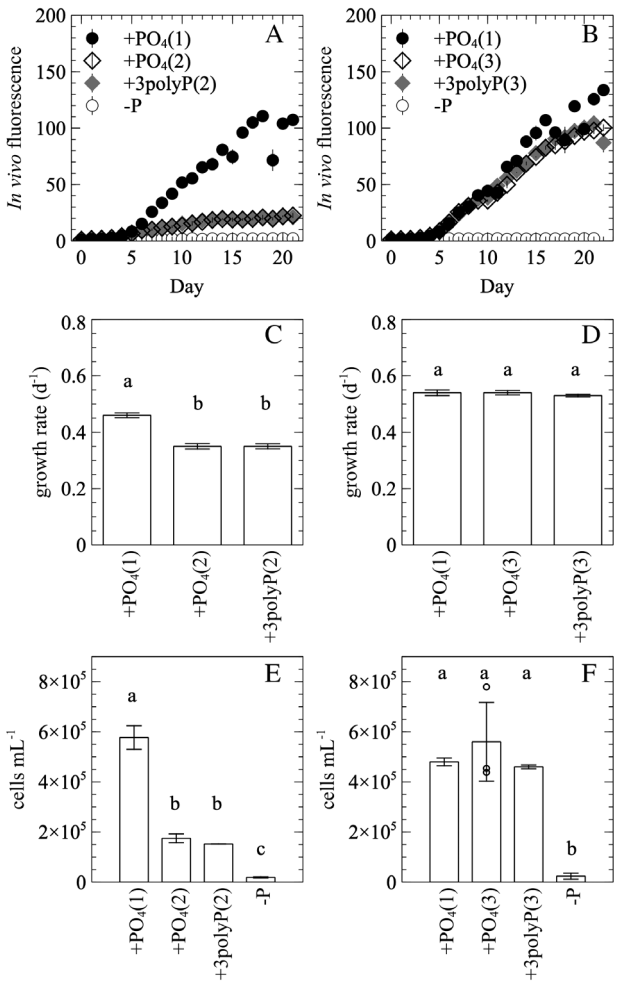

Fig. 2. Growth of *T. weissflogii* on orthophosphate (PO_4) and inorganic tripolyphosphate (3polyP) as sole P sources in 25 mm tubes on three different media types (media type is indicated by the number in parentheses). Each column presents results from a separate experiment, in which the first column (A,C,E) compares growth in type 1 and type 2 media, and the second column (B, D, F) compares growth in type 1 and type 3 media. *In vivo* fluorescence (A,B), growth rates (C,D) and final growth yields (E,F) were calculated as the average of three biological replicates. Error bars represent one standard deviation of the mean. Error bars not visible are smaller than the data symbol. Averages that do not share a common letter are significantly different ($P < 0.05$, Tukey HSD), and those that do are statistically the same. Individual data points are shown for select treatments where the ratio of SD to the mean is greater than 10% (open circles).

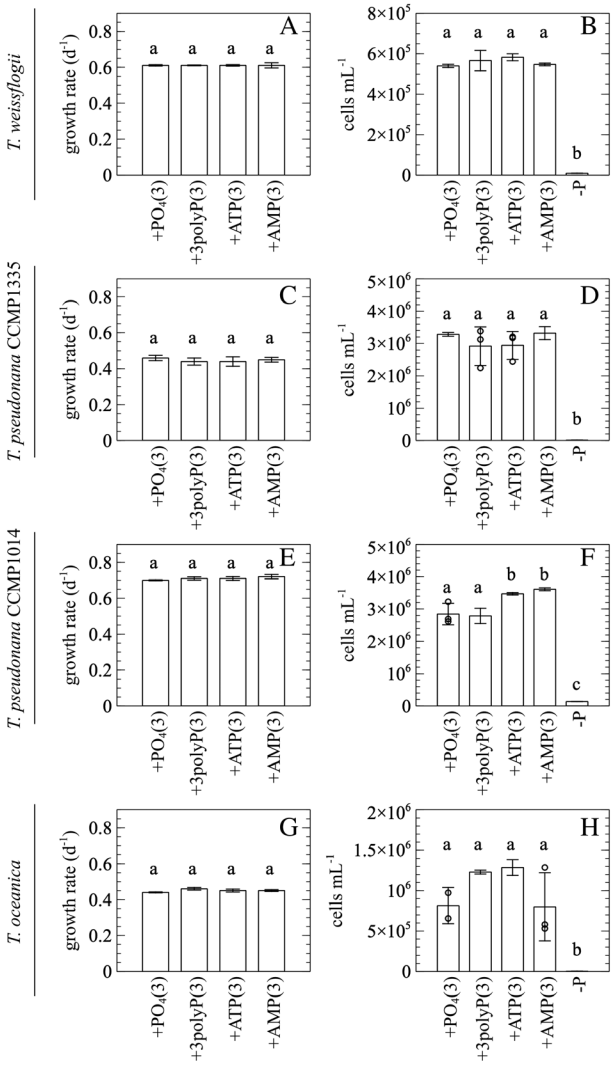

Fig. 3. Growth of *T. weissflogii* (A,B), *T. pseudonana* CCMP1335 (C,D), *T. pseudonana* CCMP1014 (E,F) and *T. oceanica* (G,H) on orthophosphate (PO4), inorganic tripolyphosphate (3polyP), ATP and AMP as sole P sources in type 3 media. Growth rates (A,C,E,G) and final growth yields (B,D,F,H) were calculated as the average of three biological replicates. Error bars represent one standard deviation of the mean. Averages that do not share a common letter are significantly different ($P < 0.05$, Tukey HSD), and those that do are statistically the same. Individual data points are shown for select treatments where the ratio of SD to the mean is greater than 10% (open circles).

Regardless of the type 2 media artefact, the identical growth yields and growth rates of $+PO_4(2)$ and + 3polyP (2) cultures of *T. pseudonana* CCMP1335 (Fig. 1A,C and E) and *T. weissflogii* (Fig. 2A,C and E) clearly suggest that 3polyP is bioavailable to these diatoms. Furthermore, *T. weissflogii* and *T. pseudonana* CCMP1335 showed identical levels of growth on orthophosphate and 3polyP in a third type of media prepared without autoclaving (Supporting Information Fig. S1), further indicating the bioavailability of 3polyP (Figs 1B,D,F and 2B,D,F).

In contrast to type 2 media, in which sterile-filtered P sources were added after autoclaving, type 3 media was prepared by sterile-filtering the finished media and omitting the autoclave step (Supporting Information Fig. S1). Type 3 media supported more growth than type 2 media overall. For example, *in vivo* fluorescence of *T. weissflogii* and *T. pseudonana* CCMP1335 was higher in type 3 media than on type 2 media (Figs 1A,B and 2A, B). In the case of *T. weissflogii*, this translated into higher growth rates (Fig. 2C and D) and growth yields (Fig. 2E and F). Therefore, type 3 media was chosen for further experiments with additional P sources and diatom strains. Within each strain of *Thalassiosira* spp., growth rates were identical in type 3 media, regardless of whether ATP, AMP, 3polyP or PO_4 was provided as the sole P source (Fig. 3A,C,E and G). Final growth yields were also identical within each strain, except in the case of *T. pseudonana* CCMP1014, in which cell counts were higher in ATP and AMP cultures than orthophosphate and 3polyP cultures (Fig. 3B,D,F and H). Similar results indicating equivalent bioavailability of diverse DOP sources, such as ATP and AMP, have been reported in other phytoplankton species such as *Synechococcus* and *T. pseudonana* (Moore *et al*., 2005; Dyhrman *et al*., 2012). The comparable levels of growth observed within each diatom across all of these P sources provide further confirmation of the bioavailability of 3polyP to these microorganisms, consistent with a number of other representative phytoplankton species that have been tested to date (Table 1). Overall, these results suggest that nutritional physiology studies in phytoplankton would benefit from the use of multiple approaches to media preparation in order to rule out potential methodological artefacts.

DOP hydrolysis

In a prior study, degradation rates of 3polyP, ATP, AMP and 4-methylumbelliferyl phosphate (MUF-P) were determined in cell-free (0.2 μm filtered) exudates of *Thalassiosira* spp (Diaz *et al*., 2018). In contrast, the aim of the present study was to determine degradation rates of the same P sources by living, intact cells. Because AMP and MUF-P were degraded at similar rates in diatom cell-free filtrates (Diaz *et al*., 2018), MUF-P was used as an analogue for AMP here. DOP hydrolysis rates were determined by adding DOP sources to a dilution series of culture samples and monitoring the degradation of each DOP source over time. MUF-P hydrolysis was determined with a standard fluorescence technique (see Experimental procedures). In the case of 3polyP and ATP, degradation rates were determined by monitoring the production of orthophosphate. The ability of phytoplankton cells to absorb and assimilate substantial quantities of orthophosphate, especially during stationary phase (Sanudo-Wilhelmy *et al*., 2004), could

Table 1. Phytoplankton capable of utilizing inorganic polyphosphate as a sole P source.

Organism	Reference	Average polyphosphate length (P atoms)
Prochlorococcus (MED4, MIT9312, MIT9313)	Moore *et al*. (2005)	3
Synechococcus WH8102	Moore *et al*. (2005)	3
E. huxleyi CCMP374	Diaz *et al*. (2016)	130
T. oceanica CCMP1005	Diaz *et al*. (2016), This study	3, 15, 45, 60, 130
T. pseudonana CCMP1335[a]	This study	3
T. pseudonana CCMP1014	This study	3
T. weissflogii CCMP1336[a]	This study	3

a. Results from this study replace prior results reported for these organisms in Diaz *et al*. (2016).

potentially interfere with this method. Therefore, 3polyP and ATP hydrolysis rates were corrected for orthophosphate uptake. Cultures took up ~10%–80% of the orthophosphate amendment over the course of 24 h, depending on the degree of sample dilution. More diluted samples took up less orthophosphate (Supporting Information Fig. S3). For example, in fivefold diluted samples, the correction for orthophosphate uptake increased 3polyP and ATP hydrolysis rates by a factor of ~2 and ~5 respectively. In 10- and 20-fold dilutions, however, the correction for orthophosphate uptake only increased hydrolysis rates by ~30%–50% and ~10% respectively (Supporting Information Fig. S4).

DOP hydrolysis rates were measured under conditions representative of cultures grown on ATP, 3polyP and AMP as sole P sources. First, hydrolysis rates were assessed in type 1 or type 3 $+PO_4$ cultures during stationary phase. In this growth phase, orthophosphate concentrations in the surrounding media would be the most depleted, which simulates early conditions in cultures that were provided with DOP as a sole P source, while also furnishing ample biomass for DOP hydrolysis measurements. Second, DOP concentrations were higher in bioavailability experiments (36 μM P) than hydrolysis assays (20 μM P), but results nevertheless indicate that DOP hydrolysis rates would be comparable at these two different concentrations. Specifically, DOP hydrolysis rates were measured over a 20-fold dilution series of culture samples, such that the concentration of DOP relative to the amount of enzymes present was higher in more diluted samples. Michelis–Menten enzyme kinetics would predict that higher DOP concentrations would result in higher DOP hydrolysis rates, unless DOP concentrations were sufficiently rate saturating to yield maximum potential DOP hydrolysis rates (V_{max}). After correcting for dilution and orthophosphate uptake, DOP hydrolysis rates typically did not increase with higher relative concentration of DOP (Supporting Information Fig. S5), confirming that DOP concentrations were rate saturating. Since the addition of more than 20 μM DOP would not increase the observed DOP hydrolysis rates, the rates measured at this concentration of DOP are representative of V_{max}, consistent with results expected in cultures amended with 36 μM DOP.

Overall, hydrolysis measurements revealed that each *Thalassiosira* strain degraded 3polyP at least 8.5-fold faster than MUF-P and ATP, except *T. oceanica*, which degraded ATP at nearly two-thirds the rate of 3polyP (Table 2, Fig. 4). These trends confirm and corroborate the preferential degradation of extracellular 3polyP relative to ATP and AMP by *Thalassiosira* spp., as previously suggested based on DOP dynamics in the cell-free exudates of these cultures (Diaz *et al*., 2018). Despite the preferential extracellular degradation of 3polyP (Fig. 4), all DOP sources supported equivalent growth of *Thalassiosira* spp. (Fig. 3). In fact, assuming typical cellular P quotas for *Thalassiosira* spp., observed rates of extracellular 3polyP, ATP and AMP hydrolysis are more than sufficient to sustain the growth of each strain on these sole P sources (Table 2).

DOP hydrolysis rates determined from the whole culture samples in this study reflect the sum of cell-associated and cell-free hydrolysis mechanisms. In a previous study by Diaz *et al*. (2018), the degradation rates of 3polyP, ATP, AMP and MUF-P were determined in filtered cell-free samples of *Thalassiosira* spp. during a similar point in stationary phase. As expected, DOP degradation rates in cell-free filtrates were relatively low, typically no more than 30% of the rates observed here in whole cultures (Diaz *et al*., 2018). This result is consistent with filtration removing most of the DOP hydrolytic activity. However, this finding does not rule out the possibility that filtration could release cell-associated enzymes into solution, thereby leading to the overestimation of DOP hydrolysis in diatom exudates. For example, this may have been the case for *T. oceanica* cell-free filtrates, which degraded DOP sources 50% faster than whole culture samples (Diaz *et al*., 2018). However, this result more likely reflects other types of natural variability, such as inconsistencies in cellular P status and viability, because the whole culture and cell-free hydrolysis rates were conducted in separate experiments.

Results showed a lack of DOP hydrolysis in filtered and boiled culture samples, indicating that the degradation of

Table 2. DOP hydrolysis rates, growth rates and proportion of growth demand.

Organism	Growth rate (day^{-1})	P quota (fmol $cell^{-1}$)	Hydrolysis rate, average(±SD) (fmol $cell^{-1}$ h^{-1})	Proportion of growth demand
T. oceanica CCMP1005	0.4	4.03[a]	3polyP: 1.8(±0.3), *n* = 9 ATP: 1.1(±0.1), *n* = 9 AMP: 0.13(±0.04), *n* = 12	3polyP: 27 ATP: 16 AMP: 1.9
T. pseudonana CCMP1335	0.5	4.03[a]	3polyP: 6.9(±2.2), *n* = 6 ATP: 0.8(±0.2), *n* = 6 AMP: 0.26(±0.08), *n* = 6	3polyP: 82 ATP: 9 AMP: 1.9
T. pseudonana CCMP1014	0.7	4.03[a]	3polyP: 17.0(±4.5), *n* = 9 ATP: 1.1(±0.6), *n* = 9 AMP: 1.8(±1.3), *n* = 12	3polyP: 145 ATP: 9 AMP: 15
T. weissflogii CCMP1336	0.6	47.3[b]	3polyP: 86.7(±25.3), *n* = 9 ATP: 6.0(±1.2), *n* = 9 AMP: 5.4(±2.5), *n* = 12	3polyP: 861 ATP: 60 AMP: 54

a. Núñez-Milland *et al*. (2010), Reinfelder (2012).
b. Laws *et al*. (2013), Reinfelder (2012) and Sugie and Yoshimura (2016).
Growth rates were determined in type 3 media and are the same as reported in Fig. 3. DOP hydrolysis is sufficient to support measured levels of growth when the proportion of growth demand is greater than or equal to one (see Experimental procedures). Cell-associated DOP hydrolysis rates are reported here, which differ from rates previously reported in cell-free (0.2 μm filtered) exudates of *Thalassiosira* spp. (Diaz *et al*., 2018).

DOP by *Thalassiosira* spp. was enzymatically driven. Alkaline phosphatases and 5′-nucleotidases (Ammerman and Azam, 1985) may be involved in these DOP dynamics. The genomes of *T. oceanica* and *T. pseudonana* CCMP1335 encode putative homologues of the major bacterial alkaline phosphatase isoforms (Diaz *et al*., 2018). Furthermore, several putative alkaline phosphatases are upregulated by *T. pseudonana* CCMP1335 under P deficiency (Dyhrman *et al*., 2012). However, it is unclear to what degree alkaline phosphatase and other potential P hydrolase enzymes are involved in the DOP transformations reported in this study. For example, some mammalian and *Escherichia coli* alkaline phosphatases also function as polyphosphatases (Lorenz and Schroder, 2001; Huang *et al*., 2018), but the substrate specificities of *Thalassiosira* spp. alkaline phosphatases are not fully characterized. Moreover, cell-free filtrate samples of *Thalassiosira* spp. were capable of hydrolyzing 3polyP, ATP and AMP, but putative bacterial alkaline phosphatase homologues were absent from these samples, indicating that other P hydrolases are important in DOP cycling by these microorganisms (Diaz *et al*., 2018).

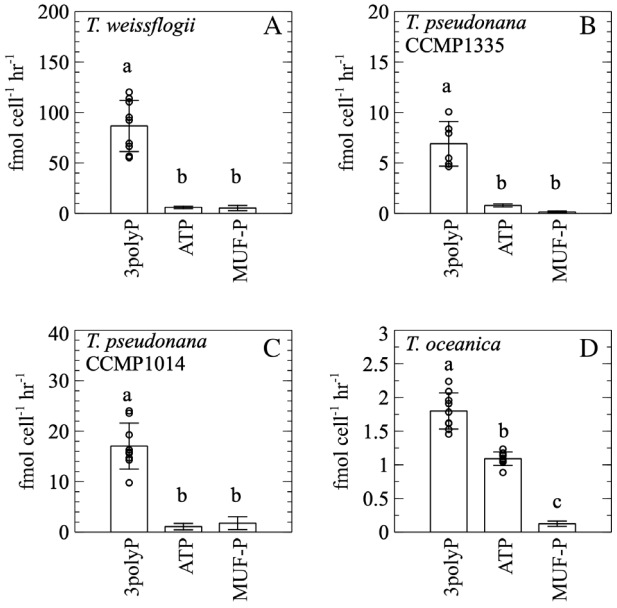

Fig. 4. Cell-associated DOP hydrolysis rates in cultures of *T. weissflogii* (A), *T. pseudonana* CCMP1335 (B), *T. pseudonana* CCMP1014 (C) and *T. oceanica* (D) were calculated as the average of at least six biological replicates, as indicated in Table 2. Error bars represent one standard deviation of the mean. Averages that do not share a common letter are significantly different ($P < 0.05$, Tukey HSD), and those that do are statistically the same. Individual data points are shown for select treatments where the ratio of SD to the mean is greater than 10% (open circles). The cell-associated DOP hydrolysis rates are reported here differ from rates previously reported in cell-free (0.2 μm filtered) exudates of *Thalassiosira* spp. (Diaz *et al*., 2018).

It is unclear why the degradation rates of ATP and AMP would be lower than 3polyP hydrolysis by *Thalassiosira* spp. Perhaps because natural inorganic polyphosphates can reach lengths of up to thousands of phosphate molecules, investment in phosphoanhydrases could potentially yield more P than phosphoesterases, which target compounds with a relatively lower P content. Indeed, inorganic polyphosphate bioavailability to *Thalassiosira* spp. is independent of chain length up to an average of 130 phosphate units in size (Diaz *et al*., 2016). Regardless of the reason for preferential 3polyP hydrolysis, however, this finding may reflect a higher abundance and/or greater activity of dissolved and/or cell surface P hydrolases that have a specific affinity for inorganic polyphosphate. Consistent with this hypothesis, we recently found that the phosphatase diversity in exudates of *Thalassiosira* spp. was dominated by putative phosphoanhydrases (Diaz *et al*., 2018).

The four strains of *Thalassiosira* showed some distinct differences in extracellular DOP hydrolysis (Supporting

Information Fig. S6). *T. weissflogii* exhibited the overall highest rates of cell-normalized DOP degradation. This result is to be expected because *T. weissflogii* is about fifteen times larger than the other strains in terms of biovolume and requires about ~10-fold higher P per cell (Reinfelder, 2012). *T. pseudonana* CCMP1014 exhibited the next highest DOP hydrolysis rates, followed by *T. pseudonana* CCMP 1335, and finally, *T. oceanica.* Unlike the other three strains, *T. oceanica* degraded ATP almost as extensively as 3polyP (Table 2, Fig. 4). In fact, ATP degradation was 61%, or nearly two-thirds, the rate of 3polyP hydrolysis by this diatom. Because initial molecular concentrations of ATP and 3polyP were the same, this result means that, on average, ATP was only about two-thirds as extensively degraded as 3polyP at any time point during the period of linear hydrolysis. ATP hydrolysis would need to be observed to completion (i.e. > 24 h) in order to confirm the final extent of ATP degradation, but this finding is consistent with the transformation of ATP to AMP via the complete hydrolysis of phosphoanhydride bonds. For instance, both ATP and 3polyP contain two phosphoanhydride bonds. The complete hydrolysis of these bonds yields three orthophosphate molecules from 3polyP and yields AMP plus two molecules of orthophosphate from ATP. Therefore, if the complete hydrolysis of phosphoanhydride bonds is sufficient to explain the degree of ATP hydrolysis by *T. oceanica*, then ATP should be approximately two-thirds as extensively degraded as 3polyP, as was observed. Indeed, the phosphoester bond of AMP was probably more resistant to degradation than the phosphoanhydride bonds of ATP, as suggested by the relatively low MUF-P hydrolysis rates in this culture (Fig. 4D). These results suggest that the extracellular phosphatases of *T. oceanica* have a broader substrate preference for phosphoanhydride bonds than the other three *Thalassiosira* strains. This enzymatic versatility, which was also evident in the cell-free filtrate of *T. oceanica*, may improve the ability of this diatom to acquire DOP (Diaz *et al*., 2018). This interpretation is consistent with the origin of *T. oceanica* from the chronically low-P Sargasso Sea and the importance of polyphosphate cycling in this environment (Martin *et al*., 2014).

Implications for DOP cycling in natural marine systems

Phosphomonoester hydrolysis rates, or alkaline phosphatase activity (APA), are commonly measured using fluorogenic probes like MUF-P in order to assess the P nutritional status of marine phytoplankton communities. Indeed, APA is upregulated by phytoplankton under low P conditions, making DOP utilization via phosphomonoester hydrolysis an important survival mechanism for phytoplankton experiencing P depletion in oligotrophic waters (Dyhrman and Ruttenberg, 2006; Duhamel *et al*., 2010; Lin *et al*., 2016). Additionally, highly labile forms of DOP are rapidly remineralized in P-replete coastal systems, although the composition of this bioavailable pool is not completely understood (Benitez-Nelson and Buesseler, 1999; Nausch *et al*., 2018). Results from this study showing the bioavailability and preferential utilization of 3polyP by *Thalassiosira* spp. suggest that, like phosphoesters, polyphosphate is a key nutritional P source for phytoplankton, which is likely included in the highly labile fraction of DOP, consistent with a growing recognition of polyphosphate in the marine P cycle (Diaz *et al*., 2008; Björkman, 2014; Martin *et al*., 2014, 2018). Indeed, natural levels of alkaline phosphatase activity tend to correlate broadly with the intensity of particulate polyphosphate recycling observed in the oceans (Martin *et al*., 2018), which is consistent with biological control over polyphosphate dynamics, although the exact enzymes responsible for the transformation of natural polyphosphate in seawater are not currently known. The identification of such enzymes is critical to understanding the biogeochemical cycling of polyphosphates, as these compounds are resistant to abiotic degradation under conditions typical of most ocean environments (Karl and Tien, 1992; Rashchi and Finch, 2000). In agreement with the biological utilization and preference for inorganic polyphosphate observed in *Thalassiosira* spp., we previously reported that maximum potential rates of enzymatic DOP degradation were significantly higher for polyphosphates than phosphomonoesters at several sites in the coastal western North Atlantic (Diaz *et al*., 2018). Purification of the putative phosphoanhydrases present in *Thalassiosira* spp. (Diaz *et al*., 2018) will reveal potential new molecular targets for understanding the basis of polyphosphate utilization and tracking polyphosphate dynamics in such environments.

The contribution of specific DOP sources to phytoplankton nutritional P demand will depend not only on the DOP substrate-specific hydrolytic capacity of the microbial community but also on the availability of different forms of DOP. For example, *in situ* DOP hydrolysis rates will likely be lower than maximum potential rates (V_{max}) because natural DOP sources are probably present at subsaturating concentrations. It is not possible to infer the importance of a nutritional P source based on its concentration alone, however. For instance, the relatively low abundance of natural polyphosphates compared to phosphoesters (Young and Ingall, 2010) could imply that polyphosphates are less important nutritional P sources in most ocean environments. Yet low polyphosphate concentrations may mask rapid and efficient utilization of polyphosphate by plankton communities. The elevated rates of 3polyP utilization in this study are suggestive of such cryptic cycling, which should be considered in future

work in order to clarify the relative contribution of specific DOP sources to overall microbial community-level P demand.

Results from this study demonstrated that *Thalassiosira* spp. degraded DOP sources in excess of growth demands. This finding may reflect the persistent activity of P hydrolase enzymes that are not tightly regulated in response to orthophosphate availability. For example, unlike alkaline phosphatases, 5′-nucleotidase activity is not inhibited by orthophosphate (Ammerman and Azam, 1985). If DOP sources are available, orthophosphate could accumulate in seawater through the persistent activity of such P hydrolases, which may have additional biogeochemical relevance. For example, other members of the plankton community, such as other phytoplankton and bacteria, could acquire this free orthophosphate without substantial physiological investment in the synthesis of endogenous P hydrolases. Yet excess DOP degradation relative to microbial growth demand has also been observed at the community level (Björkman and Karl, 1994), suggesting that some DOP recycling is not tightly coupled to microbial growth. In the absence of substantial uptake by the microbial community, we speculate that, if sufficiently rapid, orthophosphate production from the degradation of DOP could potentially favour the formation of authigenic phosphate mineral precursor phases. The ubiquitous in situ formation of dispersed, micron-scale calcium phosphates, or authigenic P, within coastal sediments is the major removal pathway for marine P, yet it is kinetically inhibited (Ruttenberg, 1993; Ruttenberg and Berner, 1993; Benitez-Nelson, 2000). One way that diatoms may play a role in this process is through the turnover of P within extensive benthic microalgal communities that exist within sediments along shallow continental shelf margins (Pinckney, 2018). Indeed, the accelerated capacity for 3polyP hydrolysis by *Thalassiosira* spp. is consistent with the proposed role of this P source in the formation of authigenic P (Diaz *et al*., 2008), suggesting one potential process that could be involved in the formation of these enigmatic minerals.

Experimental procedures

Organisms, culturing conditions and growth monitoring

Thalassiosira pseudonana CCMP1335, *Thalassiosira pseudonana* CCMP1014, *Thalassiosira weissflogii* CCMP1336 and *Thalassiosira oceanica* CCMP1005 were obtained from the National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratories, East Boothbay, ME. Diatoms were grown in batch cultures on f/2 media (Guillard and Ryther, 1962) prepared using filtered (0.2 μm) natural seawater from the South Atlantic Bight (SAB). *T. oceanica* was cultivated at 23°C, and the other strains were grown at 18°C, on a 14 h:10 h light: dark cycle (340 μmol photons m^{-2} s^{-1}). Cultures were assessed for bacterial and fungal contamination by inoculating 200 μl into 5 ml of marine broth and incubating in the dark for a period of several days to confirm a lack of heterotrophic growth.

Phytoplankton growth was monitored daily by measuring *in vivo* chlorophyll fluorescence with a 10-AU benchtop fluorometer (Turner Designs). Growth rates were calculated as the slope of the best-fit line over the natural log-linear portion ($R^2 \geq 0.98$) of the *in vivo* fluorescence growth curve, typically between days 1-7. Cell counts were conducted using a haemocytometer counting chamber (Karlson *et al*., 2010) or obtained using a Guava® easyCyte flow cytometer (Millipore Sigma). Flow cytometry samples were preserved with a final concentration of 0.5% glutaraldehyde. Instrument-specific beads were used to calibrate the cytometer, and samples were analysed at a low flow rate (0.24 μl s^{-1}) for 3 min. Diatom cell counts were gated and enumerated based on characteristic plots of forward scatter versus red fluorescence.

DOP bioavailability

ATP, AMP, 3polyP and NaH_2PO_4 were obtained from Millipore Sigma (Burlington, MA). Each P source was added to the growth media at a final concentration of 36 μM P. Negative controls were prepared without any additional P source and therefore only contained P that was present in the natural seawater base. Growth media were prepared using three different approaches (Supporting Information Fig. S1). In the first approach, orthophosphate-amended media was prepared by adding all nutrients to filtered SAB seawater in a borosilicate Erlenmeyer flask. This media was mixed and distributed (25 ml each) into 25 mm borosilicate glass tubes. Media were then autoclaved (121°C, 20 min) in these tubes and allowed to cool overnight before inoculating. This type 1 approach was modified out of concern that DOP sources may be altered during the autoclave cycle. Therefore, in type 2 media, all nutrients except P were added prior to the autoclave cycle. After cooling overnight, sterile-filtered (0.2 μm) P sources were aseptically added to each tube and subsequently inoculated. In the third media type, autoclave sterilization was omitted. All nutrients were added to SAB seawater in a borosilicate Erlenmeyer flask, mixed and filtered (0.2 μm) into a pre-sterilized borosilicate glass media jar using a disposable, sterile bottle top filter. Media were aseptically distributed (25 ml each) into pre-sterilized 25 mm borosilicate glass tubes and subsequently inoculated. In order to limit P carryover, media (25 ml) were inoculated with a minimal volume (~100–200 μl) of stationary phase cells into each of three replicate tubes. In one experiment, *T. pseudonana* CCMP1335 was grown to stationary phase in type 2 media using 3polyP as the sole P source. After

17 days, cultures were amended in triplicate with each 0.2 μm-filtered f/2 nutrient stock at final f/2 concentrations (Guillard and Ryther, 1962).

DOP hydrolysis

Cultures were grown in type 1 or type 3 media using orthophosphate as a sole P source and analysed during stationary phase. Each P source was added to culture samples (20 μM, final concentration), and the degradation of these P sources was monitored in a 96-well plate assay on a SpectraMax multimode plate reader (Molecular Devices, San Jose, CA), as described below.

The fluorogenic probe 4-methylumbelliferyl phosphate (MUF-P, Millipore Sigma) was added to undiluted cultures and cultures that were diluted 5-, 10- and 20-fold with 0.2 μm-filtered SAB seawater in a black 96-well plate. Degradation of MUF-P was analysed by a standard fluorescence technique (Duhamel *et al*., 2011). Briefly, hydrolysis of MUF-P to 4-methylumbelliferone (MUF) was measured (excitation: 359 nm, emission: 449 nm) every 2.5 min for 30 min. Samples were corrected for background fluorescence present in unamended controls and calibrated with a multipoint standard curve of MUF (10–500 nmol L^{-1}) prepared in 0.2 μm-filtered SAB seawater. MUF-P hydrolysis rates were calculated as the slope of the best fit line ($R^2 \geq 0.98$) of phosphate concentration versus time and were corrected for dilution. To assess MUF-P autohydrolysis, culture samples were 0.2 μm-filtered and boiled. Hydrolysis in these controls was negligible. Reported rates of MUF-P hydrolysis were averaged across all dilution levels.

Degradation of ATP and 3polyP was quantified as the production of orthophosphate over time. Orthophosphate was determined as soluble reactive P (SRP) according to a standard colorimetric protocol (Hansen and Koroleff, 1999). Cultures were diluted 5-, 10- and 20-fold with 0.2 μm-filtered SAB seawater in a clear 96-well plate and amended with 3polyP, ATP, or orthophosphate. Samples were reacted at four or more specific time points up to 24 h, and absorbance was read at 880 nm. Absorbance was calibrated with a standard curve of NaH_2PO_4 (Millipore Sigma) prepared in 0.2 μm-filtered SAB seawater. The detection limit of SRP using this method, defined as three times the standard deviation of replicate blank measurements, was 800 nmol l^{-1}. Background concentrations of SRP in unamended samples were below the detection limit and therefore neglected. Concentrations of orthophosphate released through the hydrolysis of ATP and 3polyP were corrected for orthophosphate uptake using the following equation:

$$[\mathrm{SRP}]_{t_n}^{\mathrm{DOP}^*} = [\mathrm{SRP}]_{t_n}^{\mathrm{DOP}} \times \frac{[\mathrm{SRP}]_{t_0}^{\mathrm{PO_4}}}{[\mathrm{SRP}]_{t_n}^{\mathrm{PO_4}}} \quad (1)$$

where $[\mathrm{SRP}]_{t_n}^{\mathrm{DOP}^*}$ and $[\mathrm{SRP}]_{t_n}^{\mathrm{DOP}}$ are the corrected and measured concentrations of SRP, respectively, in 3polyP- or ATP-amended samples at time point '*n*', while $[\mathrm{SRP}]_{t_n}^{\mathrm{PO_4}}$ and $[\mathrm{SRP}]_{t_0}^{\mathrm{PO_4}}$ are the measured concentrations of SRP in orthophosphate-amended samples at time point '*n*' and time zero respectively. Hydrolysis rates were determined as the slope of the best-fit line ($R^2 \geq 0.98$) of the corrected SRP concentration versus time and were corrected for dilution. To assess 3polyP and ATP autohydrolysis, culture samples were 0.2 μm-filtered and boiled. Hydrolysis in these controls was negligible. Reported rates of 3polyP and ATP hydrolysis were averaged across all dilution levels.

Growth demand calculations

DOP hydrolysis rates (H, fmol P $cell^{-1}$ day^{-1}) were related to phytoplankton growth rates (g, day^{-1}) by the following equation:

$$H = Q \times g \quad (2)$$

where Q is the cellular P quota (fmol P $cell^{-1}$) obtained from the literature. The P quotas of *T. oceanica* and *T. pseudonana* CCMP1014 were assumed to be the same as *T. pseudonana* CCMP1335, which was calculated as the average of values reported by Núñez-Milland *et al*. (2010) and Reinfelder (2012). The P quota of *T. weissflogii* was calculated as the average of values reported by Laws *et al*. (2013), Reinfelder (2012) and Sugie and Yoshimura (2016). Hypothetical values of g were calculated for each measured rate of DOP hydrolysis and expressed as a proportion of the actual measured growth rates. Ratios greater or equal to 1 reflect that DOP hydrolysis is able to provide P at a sufficient rate to satisfy growth demand.

Statistical analysis

Growth rates, growth yields and DOP hydrolysis rates were compared using Tukey's Honest significant difference (HSD) test in JMP® Pro (13.0). Pairwise comparisons yielding P values <0.05 were considered significantly different.

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Biological and Chemical Oceanography Data Management Office (http://bco-dmo.org) under project number 747715.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Workflow for the preparation of three different f/2 media types. N, nitrate; Si, silicic acid; Fe, iron; EDTA, ethylenediaminetetraacetic acid; TM, trace metals; Vit, vitamins.

Fig. S2. Growth response of *T. pseudonana* CCMP1335 grown in 25 mm tubes on type 2 media after the introduction of additional f/2 nutrients in stationary phase. (A) in vivo fluorescence of cultures amended on day 17 (indicated by the arrow) with no additional nutrients (no addition), nitrate (+N), phosphate (+PO_4), silicic acid (+Si), iron (+Fe), other trace metals (+TM) and vitamins (+Vit). (B) Final cell yields were determined on day 32 in cultures amended with no additional nutrients (no addition), phosphate (+PO_4), or iron (+Fe). Data not connected by the same letter are significantly different ($P < 0.05$, Tukey HSD). In both panels, data are plotted as the mean ± SD of three biological replicates. Error bars that are not visible are smaller than the data symbol.

Fig. S3. Orthophosphate uptake by 5-, 10- and 20-fold diluted cultures of (A) *T. weissflogii*, (B) *T. pseudonana* CCMP1335, (C) *T. pseudonana* CCMP1014 and (D) *T. oceanica*. Data symbols represent the mean ± SD of biological replicates ($n = 3–6$). Error bars that are not visible are smaller than the data symbol.

Fig. S4. Rates of 3polyP and ATP hydrolysis with and without correction for orthophosphate uptake in 5-, 10- and 20-fold dilutions of (A) *T. weissflogii*, (B) *T. pseudonana* CCMP1335, (C) *T. pseudonana* CCMP1014, and (D) *T. oceanica*. Bars represent the mean ± SD of biological replicates ($n = 3$).

Fig. S5. DOP hydrolysis rates corrected for dilution and orthophosphate uptake as a function of relative DOP concentration. Cultures of (A) *T. weissflogii*, (B) *T. pseudonana* CCMP1335, (C) *T. pseudonana* CCMP1014, and (D) *T. oceanica* were undiluted, or diluted 5-, 10- or 20-fold, and amended with 20 μM P of each DOP source. This approach yielded relative concentrations of DOP that were 5-, 10- and 20-fold higher, respectively, than the undiluted case (relative DOP concentration = 1). Data symbols represent individual biological replicates.

Fig. S6. Cell-associated hydrolysis rates of 3polyP (A), ATP (B), and MUF-P (C) in cultures of *T. weissflogii* CCMP1336, *T. pseudonana* CCMP1335, *T. pseudonana* CCMP1014 and *T. oceanica* CCMP1005 were calculated as the average of at least six biological replicates, as indicated in Table 2. Error bars represent one standard deviation of the mean. Data points that do not share a common letter are significantly different ($P < 0.05$, Tukey HSD), and those that do are statistically the same. The cell-associated DOP hydrolysis rates reported here differ from rates previously reported in cell-free (0.2 μm filtered) exudates of *Thalassiosira* spp. (Diaz *et al*., 2018).