

Accurate Measurements and Analysis of Polyphosphates by Enzymatic Digestion in  
Phytoplankton

By

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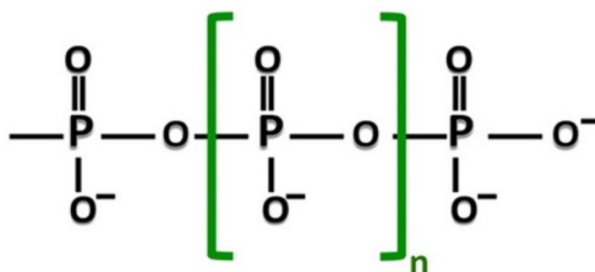
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## Abstract

Polyphosphate (polyP) is a biological inorganic polymer of orthophosphate ( $\text{PO}_4^{3-}$ ) that is found in all forms of life. Although polyP provides storage and homeostasis for cellular phosphorus (P) and several other critical functions, it is understudied due to the limitations of past quantification methods. A better understanding of cellular polyP content is particularly needed for phytoplankton since their key roles in biogeochemical cycles and ocean food webs are greatly affected by their highly variable total P content. The first aim of this study was to test if a novel enzymatic method originally designed for measuring polyP in *Saccharomyces cerevisiae* (yeast) could be used for the accurate analysis of polyP in phytoplankton. The second aim was to examine the role of polyP in overall phytoplankton P content by comparing polyP content across taxa, cell sizes, and in response to P starvation. The phytoplankton species studied includes the globally abundant picocyanobacterium *Prochlorococcus marinus*, the model diatom species *Thalassiosira weissflogii*, and two additional species of diatoms in order to examine a ~100000-fold range in cell volume. In P-replete cultures, the % polyP (of total cellular P) was similar across all species (20.4 – 29.9%) and similar after P-starvation in *Prochlorococcus marinus* (15.4%) and in *Thalassiosira weissflogii* (13.7%). These similarities were unexpected considering the wide range cell volumes and P storage capacity these species represent. While these measurements show a large contribution polyP to total phytoplankton P, this contribution is smaller than expected, which may point to a large portion of cellular P storage being in the form of dissolved orthophosphate rather than exclusively as polyP.

## Introduction

Inorganic polyphosphate (polyP) is a polymer that consists of orthophosphate residues ( $P_i$ ) that are linked together by high-energy phosphoanhydride bonds like those found in adenosine triphosphate (ATP) (Kornberg and Rao, 1999; Rao et al., 2009). The length of the chain of this polymer can vary from about 3 to 1000 monomers, depending on the location it is found in the cells and its metabolic state (Rao et al., 2009). This polymer can either exist in a linear, cyclic or branch form (Christ et al., 2020). Linear inorganic polyP (Figure 1) is of major interest because it is found in all forms of life and living cells including phytoplankton, algae, bacteria, fungi, plants and animals (Christ et al., 2020). Since the prebiotic times, microbes have used polyP as a major source of energy and phosphorous (P) (Achbergerová and Nahálka, 2011). Polyphosphate (polyP) ALSO serves protective functions; past literature highlights that microbes that lacked the enzyme that synthesizes polyP, polyphosphate kinase 1 (*ppk1*), were more susceptible to thermal, osmotic, and oxidative stress (Achbergerová and Nahálka, 2011). Hence, it protects microorganisms from extreme conditions such as high temperature, pressure, pH, stress, salinity, osmolality, desiccation and ultraviolet radiation (Achbergerová and Nahálka, 2011). Aside from these important functions, polyP may also represent a large portion of the total cellular P, particularly in microbes, making it a major part of the biogeochemical cycles of P (Martin et al. 2014; Dyhrman et al. 2016). Despite this importance, the cellular functions of polyP are not well understood and measurements of it in cells or in the environment are extremely rare due to a lack of reliable quantitative methods.

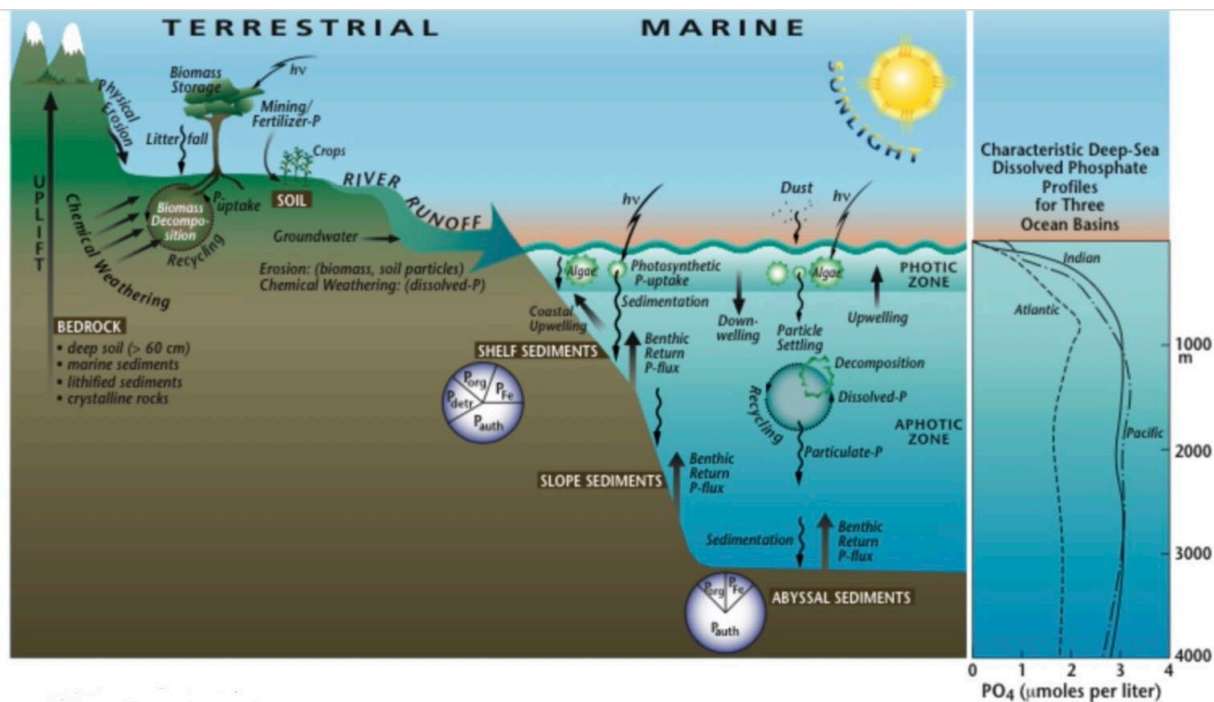


**Figure 1. The molecular structure of linear polyphosphate.** PolyP is a repeated subunit of at least two orthophosphate residues.

### **Phosphorous, An Essential Macronutrient for Life.**

The importance of polyP stems from the importance of phosphorous (P) itself, which is one of three major nutrient elements of life along with carbon (C) and nitrogen (N) (Elser, 2012; Solovchenko et al., 2016). Phosphorus is an essential element for life because it is a part of essential biomolecules such as nucleic acids (RNA and DNA), phospholipids, polyP, and ATP (Elser, 2012; Solovchenko et al., 2016). This P requirement makes it important to understand how P is cycled through the environment and made available to organisms. It is also important to understand the content of P-containing molecules in organisms and how it changes because these features determine how much P organisms require from their environment and what materials they contribute to the P cycle of their environment.

The four major components that constitute the cycling of P in the environment are the tectonic uplift rocks and exposure of phosphate rocks, the physical erosion and chemical weathering of phosphate rocks, the riverine transport of phosphorous to water bodies and the sedimentation of organic phosphorous in the ocean (Ruttenberg, 2003). Long term overgrazing also affects the C:N:P stoichiometry of the ecosystem as it renders vegetative pastures more susceptible to erosion (He et al., 2020; Smil, 2000). The global average of wind and water erosion has increased to at least 20 t/ha with a yearly loss of 10kg P/ha and 15MtP/ year from the world's crop field. During heavy rainfalls, some of these important macronutrients are lost through leaching and are washed away into water bodies that leads to eutrophication (Smil, 2000). River runoff is the major source of P to the ocean (Baturin, 2003) and is transported there in both dissolved and particulate forms (Paytan and McLaughlin, 2007). Atmospheric deposition through volcanic ash, aerosols, and mineral dust provide smaller P inputs to the ocean (Paytan and McLaughlin, 2007). Figure 2 provides an illustration of the global P cycle.



**Figure 2. The global phosphorous cycle.** The pie diagrams represent the distribution of chemical and mineral forms of in marine sediments where  $P_{org}$ ,  $P_{Fe}$ ,  $P_{det}$  and  $P_{auth}$  represents organic phosphorous, iron-bound phosphorous, detrital apatite and authigenic apatite (Ruttenberg, 2003).

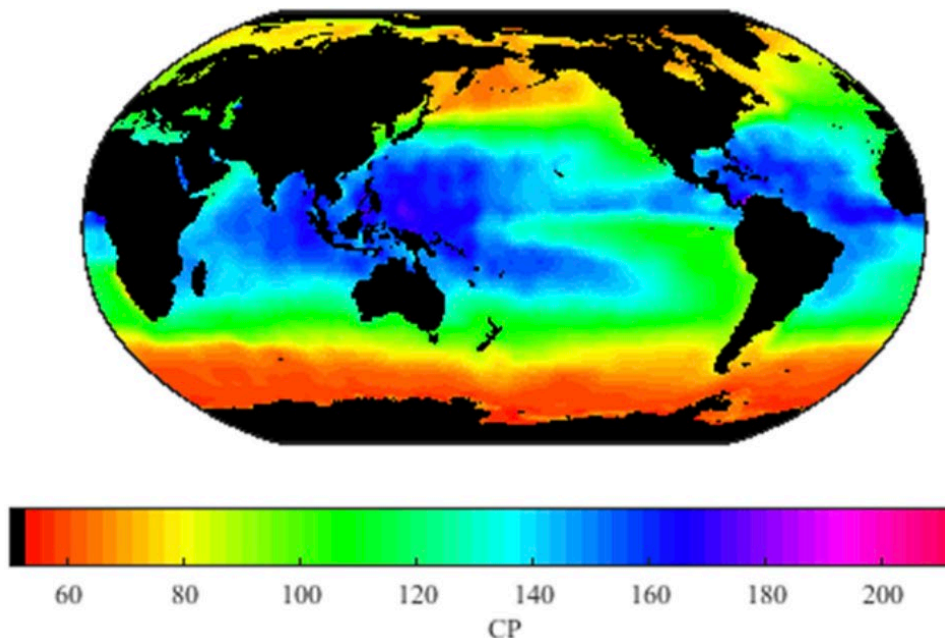
Within the ocean, the cycling of P is closely linked to the cycles of the other major biological elements, C and N, and largely controlled by biological processes. The flows of C, N, and P from atmospheric or terrestrial sources, to the ocean and its biota, and eventually to burial at the seafloor are mediated by phytoplankton, the photosynthetic microbes that are responsible for ~50% of the global primary production and form the base of marine food webs (Field et al., 1998; Winder and Sommer, 2012). Phytoplankton fix C through photosynthesis, and absorb N and P from the waters around them and the relative composition of their C-, N-, and P-containing biomolecules determines the elemental stoichiometry (C:N:P) of their cells and most other particulate matter in ocean surface; this proportion of P relative to C and N at the base of marine food webs is a central factor in how the ocean functions. The C:N:P of phytoplankton determines the food quality for marine consumers (Sterner and Elser, 2002). When live or dead phytoplankton or waste from their consumers sinks to the deep ocean, the elemental stoichiometry of phytoplankton, particularly its C:P, affects the flow of these elements from the atmosphere or ocean surface to the deep ocean where they may be sequestered. Over long time

periods, the amount of C in the atmosphere controls Earth's climate and the scarcity of P in the ocean surface limits the growth of phytoplankton, which are the ocean's main C fixers (Field et al. 1998; Tyrell 1999); as a result, the C:P of phytoplankton sinking to the sea floor is thought to be a key predictor of Earth's climate (Volk and Hofert, 1985; Galbraith et al. 2015).

### **Phytoplankton P Content and The Role of Polyphosphate**

Historically, the C:N:P of marine phytoplankton is assumed to near a mean value of 106:16:1, the "Redfield ratio" (Redfield, 1958), which defines the links between the cycles of these elements and phytoplankton in relation to their aquatic environment (Geider and La Roche, 2002). Yet recent studies show more flexibility in phytoplankton C:N:P and large deviations from the Redfield ratio among different ocean regions (Matsumoto et al., 2020). For example, phytoplankton C:N:P is higher (195:28:1) than Redfield values in oligotrophic subtropical gyres and lower (78:13:1) than Redfield values in eutrophic polar waters (Matsumoto et al., 2020).

These patterns in phytoplankton C:N:P are mainly due to high variation in phytoplankton P content and a more stable C and N content (Martiny et al. 2013; Galbraith et al. 2015) with C:P being the most variable part of this ratio (see Figure 3). This makes the composition of P-containing molecules in phytoplankton a feature at the cellular scale that may explain global patterns in C:N:P and the important processes it controls. However, the allocation of P inside phytoplankton cells is the most poorly characterized part of their elemental composition, so how this allocation is changes and what causes this change is not understood.

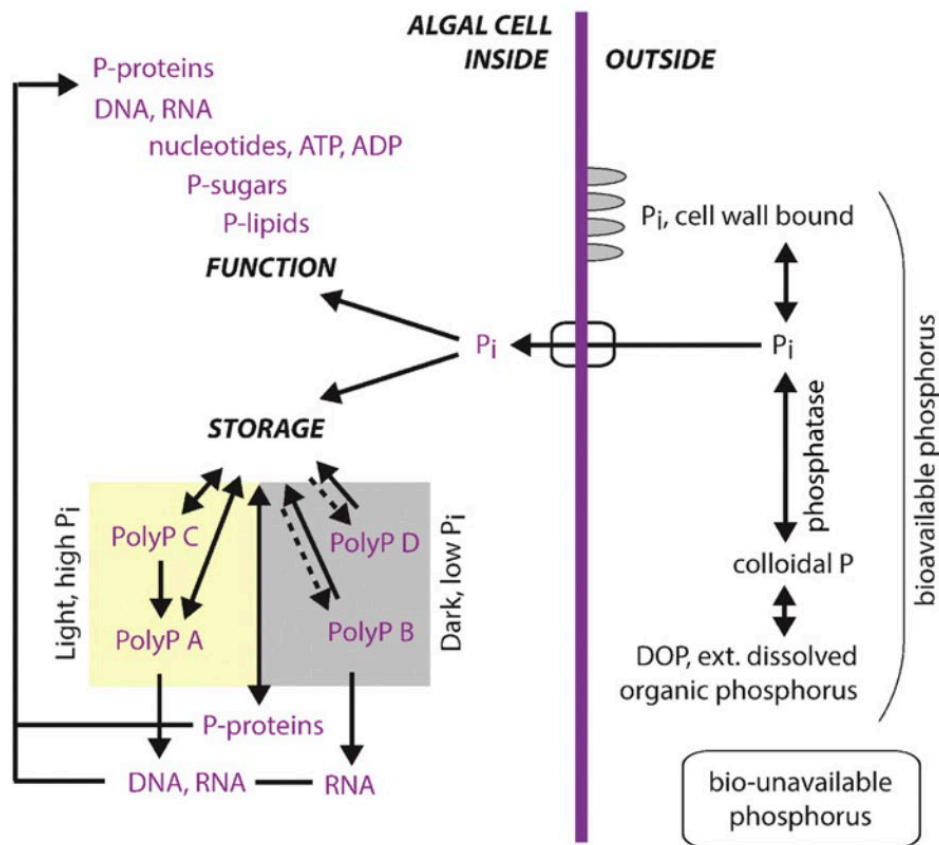


**Figure 3. The C:P ratio across the ocean in different climatic regions.** The colour gradient represents the C:P ratio in ocean phytoplankton under current conditions based on model estimates. The warm colors (red, yellow, green) represent low C:P values and are found in subtropical and upwelling regions and the cold colors (blue, violet) represent high C:P values that are found in subtropical regions (Moreno et al., 2018).

Among the features of phytoplankton cell composition, changes in P storage molecules such as polyP may provide the best explanation for high variability in phytoplankton P and the important global patterns in C:P this variability causes. Although absolute measurements of polyP in phytoplankton are lacking, recent studies using relative measurements of polyP show that it may be make up the largest portion of phytoplankton P and total particulate P in some parts of the ocean (Orchard et al. 2010; Martin et al. 2014; Diaz et al. 2016; Martin et al. 2018) and the most variable portion of phytoplankton P in laboratory cultures (Li et al. 2019). These observations of phytoplankton polyP might be due to the luxury uptake mechanisms for phosphorous that many phytoplankton have, possibly as an adaptation to the high variability of available dissolved P in water bodies. These mechanisms involve a rapid uptake of dissolved P when it is present beyond a phytoplankton cell's needs and its conversion to polyP (Powell et al. 2009). This accumulated polyP is then used as a phosphorous storage and in times of nutrient starvation when external phosphorous concentration in the ocean is low, it is metabolized to produce P-containing cell components like DNA, RNA, phospholipids or for functions like regenerating ATP or phosphorylating proteins (Kornberg, 1999; Powell et al. 2009). A schematic

diagram of this uptake and allocation of P in an algal cell is shown in (Figure 4) below. While these processes polyP have been observed in many studies, the P absorbed during luxury uptake can also be stored as dissolved orthophosphate in the cytosol (Miyata et al. 1986; Grover 1991). Therefore, P storage could be in the form of dissolved intracellular P or polyP, but there no studies that quantify how P storage is distributed between these two pools.

The accumulation of polyP beyond a cell's growth needs may explain the much lower C:P of phytoplankton found in subpolar areas, equatorial, and coastal areas where upwelling of nutrient-rich deep water is common (Martiny et al. 2013, see Figure 3). These more nutrient-rich areas of the ocean also tend to support larger phytoplankton species like diatoms that are assumed to have more cellular space for nutrient storage and expected to have a larger portion of their total cell P in storage compared to smaller taxa (Sicko-Goad, 1981; Grover, 1991). Additionally, the other cell components that account for most of a phytoplankton P, which are DNA and RNA, have restrictions on their cellular amounts that do not apply to P storage. In eukaryotes, the amount of DNA is closely related to genome size (Von Dassow et al. 2008), which cannot vary, while that amount RNA is closely related to growth rate as cells adjust their ribosome content to their current growth conditions (Sterner and Elser, 2002). In contrast, P storage components like polyP are mainly limited by cell volume and can be accumulated beyond a cell's needs (Powell et al. 2009).



**Figure 4. Schematic diagram of the different phosphorous pools and fluxes inside and out of an algal cell.** Inorganic phosphorous ( $P_i$ ) is either bound to the cell wall or dissolved in water.  $P_i$  is absorbed and readily available to the cell to carry out cellular function which include protein, DNA, phospholipids, nucleotide synthesis or be stored as polyP (Solovchenko et al., 2016).

### Problems Associated with Past Methods of Quantifying Polyphosphate

The huge knowledge gap in the study of polyP is due to the lack of accurate methods that successfully isolate and quantify polyP (Christ et al., 2020). Many studies of cellular polyP have examined specific features such as its molecular structure, chain length distribution, the cations associated with it, and its cellular localization, but the methods used in these cases have features that make them poor for accurately measuring the total amount polyP in biological samples (Christ et al., 2020). Older purification techniques lose a great amount of polyP during extraction due to the use of strong acids or alkali treatments and may also change the chemical structure or chain length of the extracted polyP (Kulakova et al., 2011). Quantification of polyP has also been attempted using electron microscopy in combination with energy disperse x-ray analysis (EM-EDX), which can quantify the mass of localized elements in individual cells, but a large amount

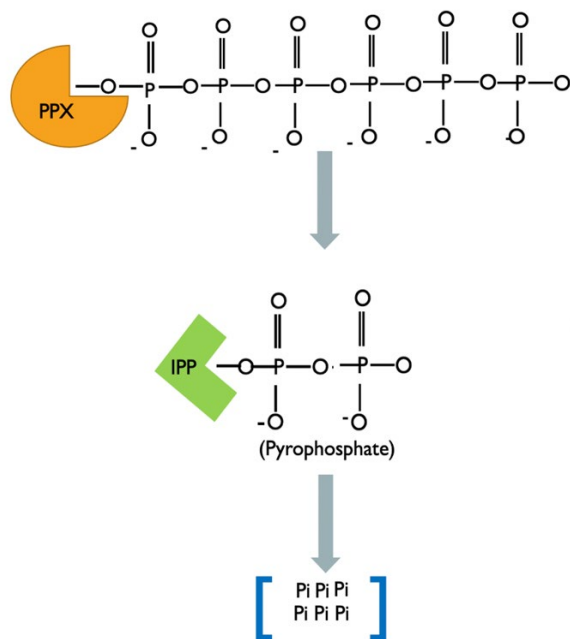
polyP seems to be lost during the intensive preparation techniques used in the x-ray analysis (Hupfer et al., 2008). With the various kinds of chromatography, including the two-dimensional thin layer chromatography (2D-TLC) and the ion exchange chromatography, there is a size restriction where only polyP chain lengths of less than 50 subunits can be measured (Holland et al., 2014; Ohtomo et al., 2008). Gel electrophoresis techniques such as polyacrylamide gel electrophoresis (PAGE) have been used to characterize the variety of polyP chain lengths in samples but are very time-consuming and their reproducibility is too poor for total polyP quantification, although capillary gel electrophoresis techniques may reduce these problems (Lee and Whitesides, 2010).

Some more recent methods have shown greater success in measuring the total amount of polyP in samples, but still have major limitations. For instance, Raman micro spectroscopy has shown great potential to accurately quantify polyP in analyses of single phytoplankton cells (Moudříková et al., 2017). However, there is no accurate and reliable biochemical reference standard that can be used with these Raman techniques (Moudříková et al., 2017), in addition to the analyses being limited to one cell at a time and requiring expenses and rare equipment. <sup>31</sup>P nuclear magnetic resonance (PNMR) is one of the most commonly used analytical methods in quantifying polyP (Bru et al., 2017; Christ et al., 2020). The main disadvantages of this method are its lack of sensitivity and its inability to distinguish between polyP and different phosphorous compounds that also contain phosphoanhydride bonds. As a result, these compounds are analyzed together, which then leads to a false positive result where there is more polyP measured than is actually present (Bru et al., 2017). Another recently developed approach is based on the fluorometric quantification of polyP in unpurified aqueous extracts of phytoplankton after staining with 4',6-diamidino-2-phenylindole (DAPI) (Martin and Van Mooy, 2013). While this technique appears to accurately measure relative changes in polyP in similar samples and roughly estimate the contribution of polyP in a sample to its total P (Martin et al. 2014; Diaz et al. 2016, Li et al. 2019), it cannot provide absolute measurements of polyP and interference from nucleic acids (which are also stained by DAPI) and the sample matrix effects lowers its precision.

## Enzymatic Quantification of Polyphosphate

In our current study, we adopted the method of Christ and Blank (2018a) which is meant to be a simpler and faster version of the experimental procedure of Bru et al (2016). The Christ and Blank procedure was optimized to have a higher extraction yield, shortens the extraction time of the Bru et al. (2016) method from 5.5 hours to about 30 minutes, and simplify the work flow. (Christ and Blank, 2018a). The number of reaction tubes per sample was reduced from five in the former to only one which in turn helps to simplify the work flow (Christ and Blank, 2018a).

In the method proposed by Christ and Blank for the quantification of polyP, two enzymes were used for the successive hydrolysis of the polymer (Christ and Blank, 2018b). As seen in (Figure 5) below *Saccharomyces cerevisiae* exopolyphosphatase 1 (PPX) hydrolyzes polyP to orthophosphate and pyrophosphate by cleaving the terminal phosphate at the end of the molecule (Christ and Blank, 2018b). The second enzyme, *S. cerevisiae* inorganic pyrophosphatase (IPP) hydrolyses pyrophosphate into inorganic phosphate, the only form that the assay can measure as seen in (Figure 5) below.



**Figure 5. Schematic diagram highlighting the enzymatic activity of exopolyphosphatase (PPX) and pyrophosphatase (IPP).** A six-chain length polyphosphate polymer is converted to pyrophosphate by PPX then IPP converts the pyrophosphate to inorganic phosphate.

In the molybdenum assay, one inorganic phosphate forms a complex with 12 molybdate ions that causes a blue colour change which marks the end of the procedure before the solution is placed in a plate reader to be measured (Christ and Blank, 2018b). Christ and Blank proposed this new gold standard method for the extraction and quantification of polyP in *Saccharomyces cerevisiae* that has been yet to be tested in phytoplankton to check its accuracy and verify that it completes the phosphorous cell budget in relation to all other phosphorous containing biomolecules in the organisms.

### **Research Objectives**

The four main goals of this study were to determine the best extraction and detection method for polyP by comparing the novel enzymatic crude extraction method to the purified method, measure and confirm if polyP fitted into the total phosphorus budget, analyze how polyP is utilized by phytoplankton across various conditions (P-replete vs P-starved) and to investigate the differences in how polyP is utilized across taxa and cell sizes. Being able to successfully measure polyP in phytoplankton will help to characterize the highly variable P content of phytoplankton, which plays a large role in their biogeochemical impacts, and help improve the limited understanding of the functions of polyP.

### **Materials and Methods**

#### ***Prochlorococcus marinus* Culture and Cell Conditions**

Cultures of the marine cyanobacterium *Prochlorococcus marinus* (strain MED4) were obtained from the National Center for Marine Algae and Microbiota (NCMA). Phosphorus-replete batch cultures of *P. marinus* were grown in the artificial sea water-based medium AMP 1, which was modified to have lower concentrations (100 $\mu$ m, 10 $\mu$ m and 10% of AMPI concentrations) of ammonium, phosphate and trace metals respectively (Moore et al., 2007). The artificial sea water base of this medium was prepared in ultrapure Milli-Q water, filtered with an acid-washed vacuum filtration system through a glass fibre filter (GF/F) filter and a 0.2 $\mu$ m polycarbonate (PC) membrane filter, and then autoclaved in acid-washed polycarbonate bottles. Concentrated stocks of the macronutrients, trace metals and vitamins were all made with the highest-grade chemicals (Sigma-Aldrich) and filter-sterilized through a 0.2 $\mu$ m PC membrane syringe filters before being added to the sterilized artificial sea water. The cultures were grown

under an irradiance of  $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  on a 12:12 light:dark cycle provided by white fluorescent lights at  $21^\circ\text{C}$  in triplicate 4 liter polycarbonate bottles. The growth of the cell, abundance and purity was monitored by fluorometric detection using a flow cytometer according to Marie et al. (2005). Phosphorous replete cultures were maintained in exponential growth by dilutions with fresh media every 2-3 days and were sampled for elemental and macromolecular composition once they were considered to be in fully acclimated exponential growth after 10 generations with less than 15% variation in growth rate. The remaining culture after this sampling was diluted with P-free modified AMP1 media (prepared the same as described above, but with no phosphorus added) and then sampled again for elemental and macromolecular composition after 8 days when they were in P-starved stationary phase.

### **Diatom Culture and Cell Conditions**

Cultures of the diatom *Thalassiosira weissflogii* (CCMP 1336) were obtained from the National Center for Marine Algae and Microbiota (NCMA). The cultures were grown in artificial seawater prepared according to Berges et al. (2001) and f/2 nutrient conditions (Guillard 1975) that were modified to contain half of the f/2 concentrations of macronutrients, trace metals, and vitamins and the addition of 2mmol sodium bicarbonate to prevent cultures from becoming carbon limited. The artificial seawater and nutrient amendments were filtered and sterilized as described above for AMP1 media. The cultures were grown in triplicate 1 liter glass bottles under an irradiance of  $60 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  on a 12:12 light: dark at  $18^\circ\text{C}$ . The cultures were monitored daily by tracking chlorophyll a (chl a) in-vivo fluorescence with a Turner Trilogy Fluorometer. As with the *P. marinus* cultures, *T. weissflogii* cultures were sampled for elemental and macromolecular composition when in fully acclimated exponential growth, diluted with a P-free version of their growth medium, and then sampled again for elemental and macromolecular composition after 8 days when they were P-starved stationary phase.

Cultures of two additional diatom species, *Thalassiosira pseudonana* and *Odontella mobiliensis* were also grown in addition to *T. weissflogii* in order to study a wide range of cell sizes among similar species. The cell volumes of these cultures of *T. pseudonana*, *T. weissflogii*, and *O. mobiliensis* were measured as  $63 \mu\text{m}^3$ ,  $814 \mu\text{m}^3$ , and  $34050 \mu\text{m}^3$ , respectively, in a previous study (Pollack, 2021) and sampled in fully acclimated exponential growth. Cultures of

*Thalassiosira pseudonana* (CCMP 1335) and *Odontella mobiliensis* (CCMP 597) were obtained from the National Center for Marine Algae and Microbiota (NCMA) and maintained in the same media and conditions as described for *T. weissflogii* with the exception that *O. mobiliensis* was grown at a lower irradiance ( $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and its media used the L1 recipe for trace metals (Guillard and Hargraves 1993) instead of the f/2 recipe.

### **Sample collection**

Portions of each culture were poured off into an acid-washed container in a sterile flow hood and samples were collected by low pressure (<18 kPa) vacuum filtration. Samples for total cellular phosphorus were collected on 25mm pre-combusted (3 hours at 450°C) glass-fiber filters (Whatman, GF/F grade), placed in acid-washed, pre-combusted glass vials and stored in a -20°C freezer. Samples for RNA, DNA, polyphosphate (polyP), were collected on 25mm polycarbonate membrane filters (Whatman Nucleopore), placed in polypropylene cryo-vials, flash frozen in liquid nitrogen, and stored at -80°C. For collection of RNA, DNA, and polyP samples from *P. marinus* cultures, membrane filters with a 0.2 $\mu\text{m}$  pore size were used while filters with 0.8 $\mu\text{m}$  pore size were used for all diatom cultures.

### **Total Phosphorus Extraction and Analysis**

Samples for total particulate phosphorus were dried and extracted according to Solórzano and Sharp (1980). Samples were dried at 90°C after addition of 2ml 0.017M  $\text{MgSO}_4$  for 2 days and then combusted at 500°C for three hours. After cooling to room temperature, the samples were extracted in 0.2M HCl at 90 °C for three hours, mixed by vortexing for one minute, transferred to clean microtubes, and centrifuged at 13000x g and 20°C for 5 minutes. The total phosphorus in each extract was then measured as orthophosphate using a phospho-molybdate assay (Chen et al., 1956) that was modified to be used in a microplate format. 150 $\mu\text{l}$  of each sample was added to a 96-well microplate and working reagent (ultrapure water, 6M  $\text{H}_2\text{SO}_4$ , 2.5% (w/v) ammonium molybdate and 10% (v/v) ascorbic acid) was prepared. The working reagent (150 $\mu\text{l}$ ) was then added to the extracts in the plate, which was sealed after with parafilm, covered with foil paper to protect from light. The plate was then placed on an orbital shaker at 70RPM for five minutes and incubated at 37°C for one hour. Orthophosphate was then measured

by absorbance at 820nm using a SpectraMax M3 microplate reader (Molecular Devices). Potassium phosphate monobasic (Sigma, trace-metal grade) was used as a reference standard.

### **Crude Extraction of Polyphosphate**

Polyphosphate (polyP) was extracted from phytoplankton using the protocol suggested by Christ and Blank (2018a). Frozen samples were transferred into a clean nuclease-free polypropylene microtube with sterile forceps that were cleaned with 70% alcohol and Kim Wipes. Two sample blanks were also prepared and were treated identically to the filtered samples from this point. Samples were initially extracted in a MOPS-EDTA (ME) buffer, which contains 25mM MOPS (Sigma #M1254), 2.5mM EDTA (Bioshop, #EDT001) and was adjusted to pH 7.0 and filtered through 0.2 $\mu$ m PES filter. The ME buffer was added to each sample and then mixed by vortexing for 30 seconds. The samples were then centrifuged for one minute at 1000x g at 20°C. Tris-EDTA (TE) saturated phenol (Bioshop, #PHE510) was added to the samples in the fume hood and then mixed again by vortexing for 30 seconds. The samples were heated for ten minutes at 45°C in a dry bath (BOEKEL Scientific) and briefly cooled in an ice bath for two minutes. Chloroform (Sigma, HPLC-grade) was added to each sample and was mixed by vortexing and centrifuged as described above. Approximately 200mg autoclaved low-density phase lock gel (Dow Corning high-vacuum grease) was dispensed into the inner lids of the tubes with an autoclaved syringe and the extracts were centrifuged for five minutes at 12000x g at 20°C to allow the phase lock gel to separate the aqueous and organic phases of the extracts. The aqueous upper phase of the extract containing polyP was transferred into a clean microtube and the exact volume was noted (450 - 500 $\mu$ l). The polyP extracts were stored at -80°C until quantification and analysis.

### **Purified Extraction Method**

This purified method of extracting polyP from phytoplankton was performed as suggested in the protocol by Bru et al. (2017). The frozen samples were transferred to 2ml bead tubes (MP Biomedicals, lysing matrix D ceramic beads) with sterile forceps that were cleaned with 70% alcohol and Kim Wipes. Two sample blanks were also prepared and were treated identically to the filtered samples from this point. Samples were initially extracted in an Acetate-EDTA (AE) buffer, which contains 50mM sodium acetate (Sigma, #S8750), 10mM EDTA. The

AE buffer was added to the bead tubes and mixed by vortexing for 30 seconds. TE-saturated Phenol and 10% (w/v) SDS (Fisher BioReagents) were then added to the tubes and samples were disrupted in a bead mill (MP Biomedicals) in three rounds of 30 seconds at a speed of  $6.5\text{m s}^{-1}$ . The samples were cooled for one minute in an ice bath after each round. The samples were then placed in a dry bath at  $65^{\circ}\text{C}$  for five minutes and cooled in an ice bath for one minute. Chloroform was then added to each sample and mixed by vortexing for a minute. Samples were centrifuged at  $20^{\circ}\text{C}$  for two minutes at  $13,000\times g$ . The aqueous upper phase of the extract containing polyP was transferred to new tubes containing chloroform, vortexed for one minute, and centrifuged at  $20^{\circ}\text{C}$  for two minutes at  $13,000\times g$  to be partitioned again. The upper aqueous phase was then transferred into a clean microtube where  $2\mu\text{l}$  of RNase A (Sigma, #R1653) solution ( $10\text{mg/ml}$ ) and  $4\mu\text{l}$  of DNase (Bioshop, #DRB002) solution ( $5\text{mg/ml}$ ) were added to each tube. The tubes containing the samples was placed in a dry bath at  $37^{\circ}\text{C}$  for one hour and then cooled on an ice bath for one minute. Sodium acetate ( $3\text{M}$ ) and 100% ethanol (Commercial Alcohols) were added to each sample, which were then vortexed briefly placed in a  $-20^{\circ}\text{C}$  freezer overnight to allow polyP to precipitate out of solution. Working in a sterile flow hood, the supernatant was gently removed from each sample to prevent disrupting of the white translucent polyP pellet. The sample pellets were rinse two times by adding  $1\text{ml}$  of 70% ethanol followed by centrifugation at  $4^{\circ}\text{C}$  for five minutes at  $13000\times g$  and removal of the supernatant. The pellets were then allowed to dry in the sterile flow hood for 2-3 hours. The polyP pellets were then dissolved in ultrapure Milli-Q water and stored at  $-20^{\circ}\text{C}$  until analysis.

### **Enzymes digestion of Polyphosphate**

Digestion was the same of for both extraction methods. Purified exopolyphosphatase (PPX) and inorganic pyrophosphatase (IPP) were prepared previously (McKay-Barr, 2021) by expressing these enzymes from recombinant plastids according to Christ and Blank (2018b). The recombinant plastids for this work were kindly provided by Jonas Christ and Lars Blank. PPX and IPP in storage buffer (50% glycerol,  $20\text{mM}$  Tris,  $50\text{mM}$  KCl) were thawed in an ice bath and diluted in an enzyme reaction buffer that consisted of  $15\text{mM}$  magnesium acetate,  $60\text{mM}$  Tris, and  $150\text{mM}$  ammonium acetate. Three enzyme reaction solutions were prepared fresh before the analyses: a “No Enzyme” solution (1:24 mix of storage buffer and reaction buffer), a

“PPX only” solution (1:1:48 mix of PPX enzyme, storage buffer, and reaction buffer), and a “PPX + IPP only” solution (1:1:48 mix of PPX enzyme, IPP enzyme, and reaction buffer). polyP extracts were then added to three wells in a microplate and one of the three enzyme reactions solutions was added to each of the wells. The plate was then incubated for one hour at 37°C to allow digestion. The samples that received the “No Enzyme” solution represents background orthophosphate that is assumed to come from dissolved intracellular phosphate or breakdown of cellular organic phosphorus. Orthophosphate in wells that received “PPX + IPP only” solution represent background orthophosphate and all orthophosphate released from the complete digestion of polyP. Orthophosphate in each well were then measured by a modified phospho-molybdate assay (Christ and Blank, 2018b) as described in the next section. PPX only solution was initially used since the average chain length of polyP in a sample can be estimated if both the amount of polyP that can be digested by PPX (chains digested to pyrophosphate) and the amount that can be digested by PPX and IPP combined are measured (Christ and Blank, 2018b). However, previous work indicated that the batch of PPX enzyme available was contaminated with IPP.

### **Detection of Orthophosphate from Polyphosphate Using the Phospho-Molybdate Assay**

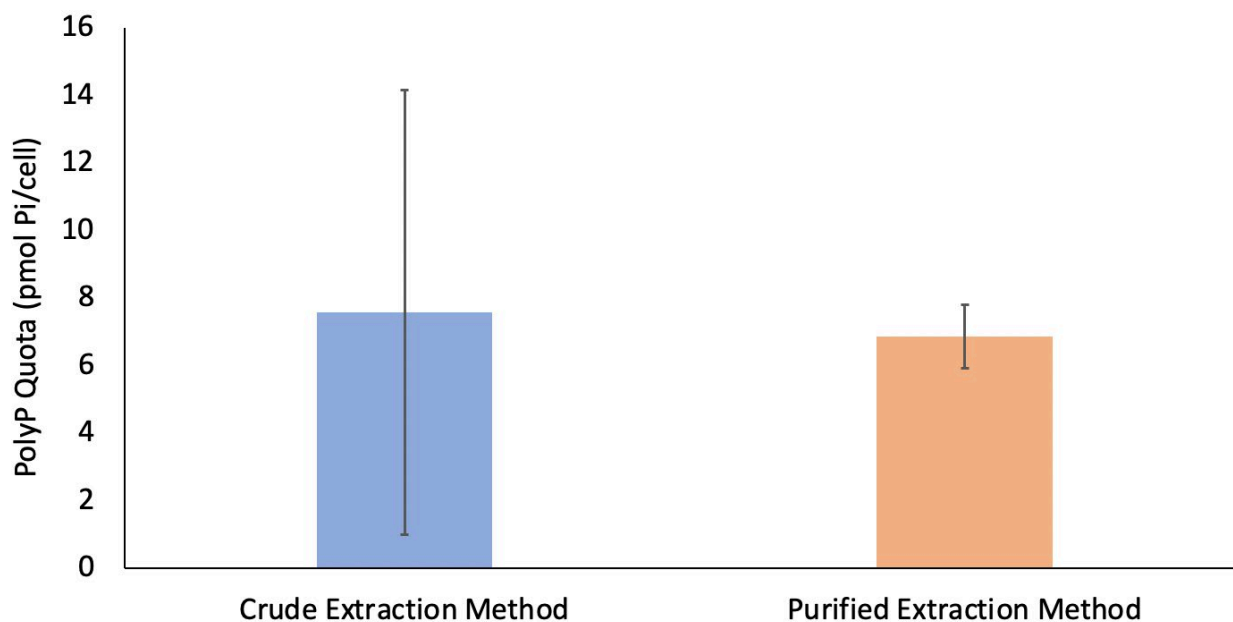
Two different methods of detecting orthophosphate after the enzyme digestion treatments described above was performed. These methods included the one proposed by Christ and Blank (2018b) (the “Fast” method) and the method of Chen et al. (1956) (the “Slow” method) described above for detecting orthophosphate after total phosphorus extraction, with 150µl of enzyme-digested polyP extract being measured instead of total phosphorus extract. Both polyP detection methods made use of the phospho-molybdate assay, with the major differences being the concentration of the working reagents, time of colour development and incubation period which are discussed below. Potassium phosphate monobasic (Sigma, trace-metal grade) was used as a reference standard for both methods. The “Fast” detection method was performed as suggested by (Christ and Blank, 2018b). Solution A (0.6mM antimony potassium tartrate, 600mM H<sub>2</sub>SO<sub>4</sub>, and 2.4mM ammonium heptamolybdate) were prepared by dissolving individual constituents in Milli-Q water in the direct order as listed above and can be stored protected from light at 4°C for a month. Solution B, the detection reagent had to be prepared freshly on the day of use by mixing 1M ascorbic acid to solution A. Once solution B (100µl) is added to the 96 well plate containing

the enzyme-digested polyP extract, it was left to develop for two minutes before absorbance was read at 882nm by SpectraMax M3 microplate reader (Molecular Devices).

## Results

### Comparison of Extraction Methods

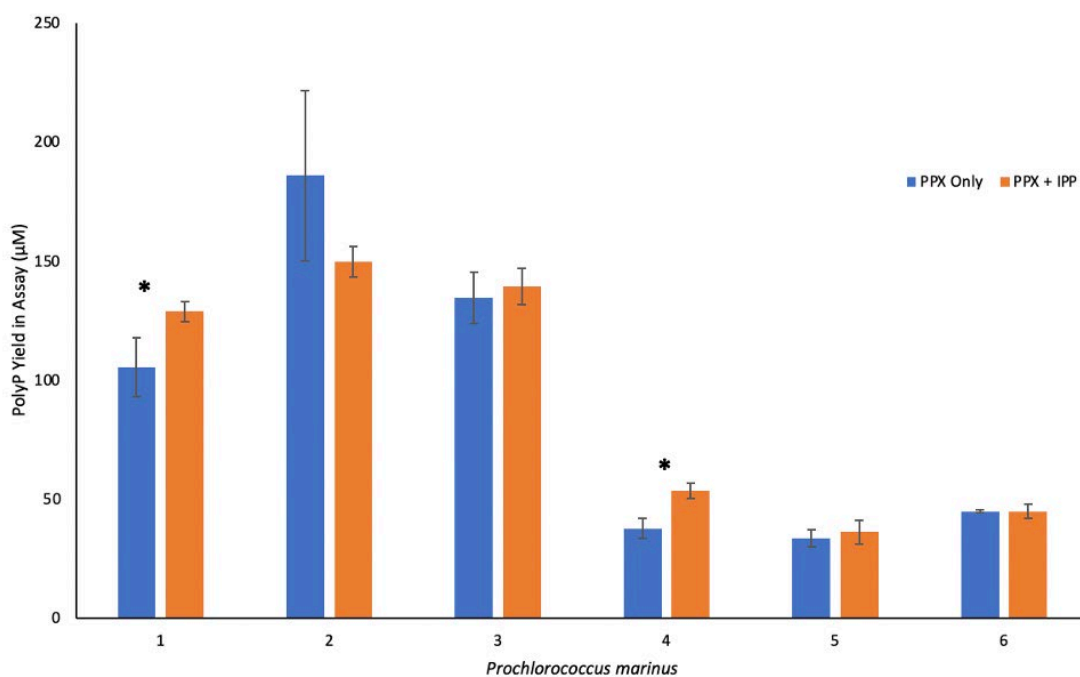
The two different extraction methods, crude and purified, proposed by Christ and Blank (2018a) and Bru et al., (2017) respectively, were used to extract polyP from various *P. marinus* samples and the amounts of polyP obtained were compared in terms of polyP cell quota (pmol Pi/cell). The total polyP quota obtained from the crude and purified extraction method were  $7.58 \pm 6.57$  and  $6.86 \pm 0.93$  pmol Pi/cell respectively. and not significantly different (Student's T-test). However, the values obtained from the crude extraction showed far more variability (86.6%) those obtained from the purified method (13.6%).



**Figure 6. Total amount of polyphosphates extracted using two different methods.** Two distinct polyP quota (pmol Pi/cell) were obtained using both the crude (blue) and purification (orange) extraction method. Error bars indicates the standard deviation between the two different extraction method.

### Enzymatic Treatment Condition

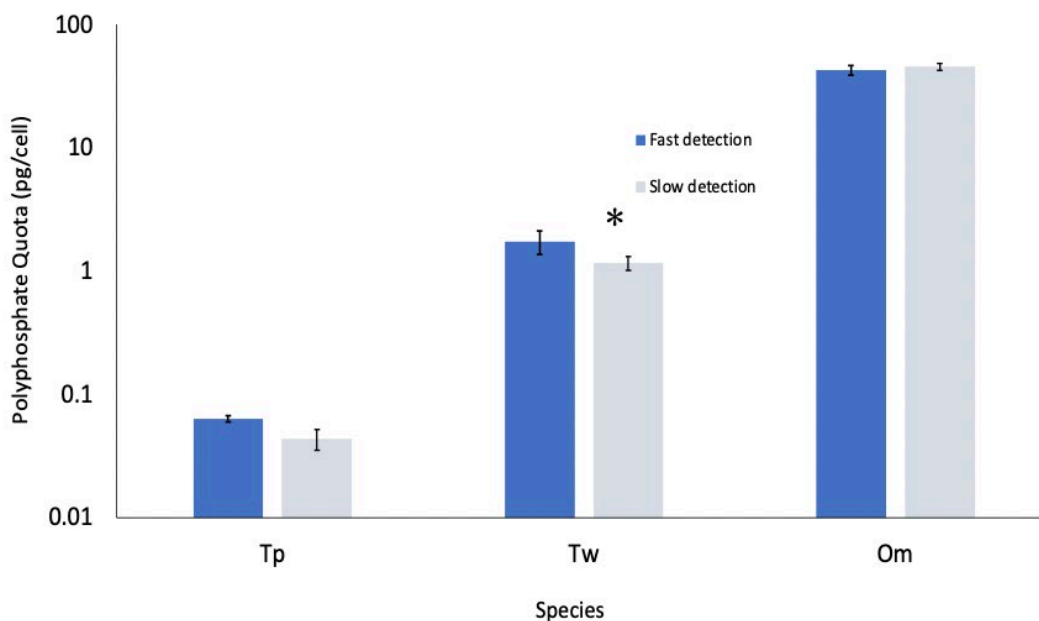
To tests for the contamination of the PPX enzyme supply and the possibility of measuring average polyP chain length in our samples, the orthophosphate released from the “PPX only” and “PPX + IPP” enzyme reaction treatments were compared between duplicates of six samples of *P. marinus* culture (three from P-replete cultures and three from P-starved). If the “PPX only” treatments were not contaminated with IPP than the digestion process in these treatments should leave behind an undigested molecule of pyrophosphate for every polyP chain that is digested and thus yield a lower response in the assay than the “PPX + IPP” treatment, where polyP would be completely digested to orthophosphate. Among the 6 replicates compared only two showed a significantly higher yield for the “PPX + IPP” treatment and in some cases the “PPX only” treatment yielded higher (though not significantly so) results for “PPX only” (Figure 7).



**Figure 7. PolyP yield in assay with the two enzyme treatments (PPX) in blue and (IPP +PPX treatment) (orange).** The asterisks indicate duplicate samples that were significantly different (Student’s T-test,  $p < 0.05$ ). Error bars indicate one standard deviation.

## Comparison of Detection Methods

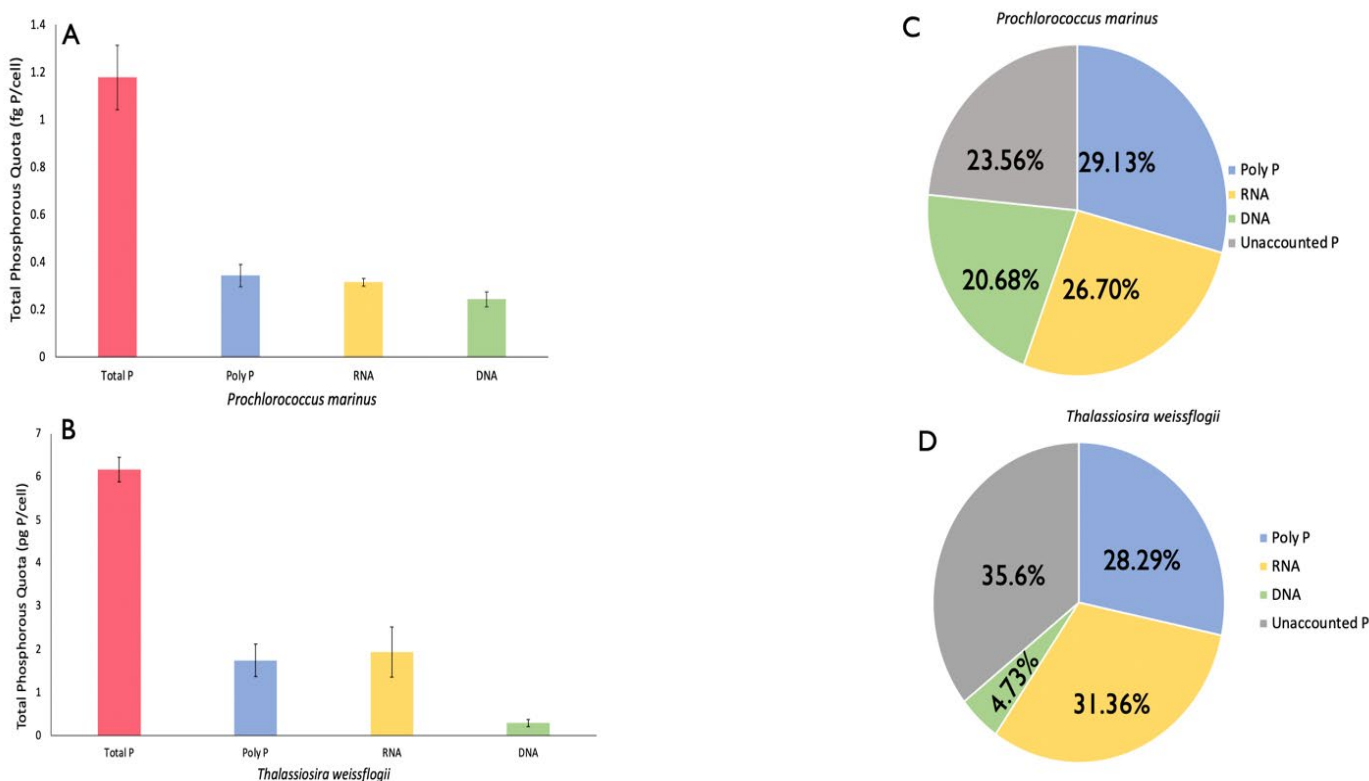
The quantification of polyP as orthophosphate after enzymatic digestion was compared between two phosphomolybdate assays, the “fast” method of Christ and Blank (2018a) and the “slow” method of Chen et al. (1956), across three diatom species, namely *T. pseudonana*, *T. weissflogii*, and *O. mobiliensis*. These species represent a large size range (63 – 34050  $\mu\text{m}^3$  cell volume) as reflected in their measured polyP quotas, which ranged from 0.044 – 45.5  $\text{pg P cell}^{-1}$  (Figure 8). Overall, there was no clear difference between the two detection methods for *T. pseudonana* and *O. mobiliensis*, however the “fast” method produced a significantly higher polyP quota (Student’s T-test,  $p < 0.05$ ) for *T. weissflogii* ( $1.74 \pm 0.4$ ) in comparison to the “slow” method ( $1.16 \pm 0.1$ ) (Figure 8). It should be noted that the higher measurement for *T. weissflogii* using the “fast” method also showed much higher variability (23%) than the other measurements.



**Figure 8.** Comparison of the the “fast” (dark blue) method of Christ and Blank (2018a) and the “slow” (light blue) method of Chen et al. (1956) in estimating the amount of PolyP (pg/cell) in three different species of diatoms: *Thalassiosira pseudonana* (Tp), *Thalassiosira weissflogii* (Tw), *Odontella mobiliensis* (Om). The asterisks indicate a significant difference between the compared methods for each species (Student’s T-test,  $p < 0.05$ ). Error bars indicate one standard deviation.

## Total Phosphorus Cellular Budget and Comparison of Phosphorous Content Across Taxa

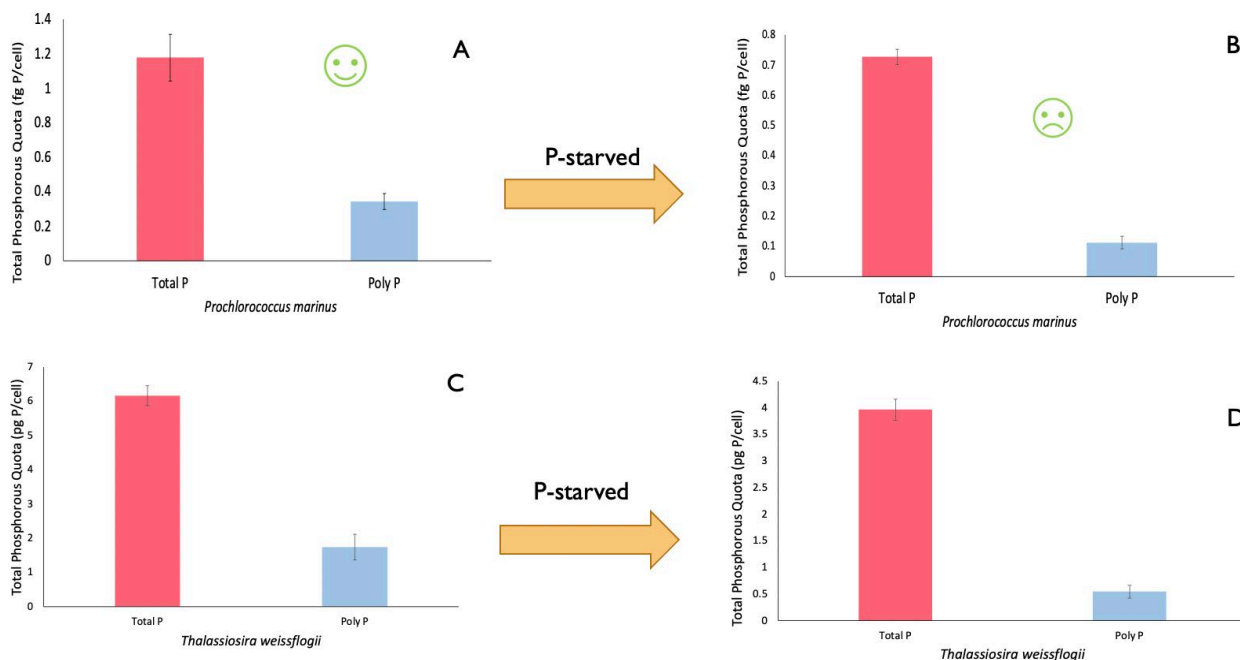
The different macromolecular contents, including polyP, RNA and DNA, that contribute to the overall phosphorus budget of phytoplankton were measured in both *P. marinus* and *T. weissflogii*. Despite the overall differences in their phosphorus cellular budget, there was a similar percentage of polyP contribution between *P. marinus* (29.1%) and *T. weissflogii* (28.3%). There was also a large amount of unaccounted phosphorus in both species (Figure 9). Across Taxa, the different phosphorous containing macromolecules and their percent constituent to the total phosphorus of the cell was measured and analyzed in *P. marinus* and *T. weissflogii* as shown below in (Figure 9). The percentage of phosphorous that was contributed by DNA was higher in *P. marinus* (20.68%) in comparison *T. weissflogii* (4.73%) to while RNA was similar as seen below.



**Figure 9. Cellular quota of total phosphorus and P-rich macromolecules across taxa in (A) *Prochlorococcus marinus* (in units of fg P/cell) and (B) *Thalassiosira weissflogii* (in units of pg P/cell) as well as the relative distribution of phosphorous (% of total cellular phosphorus) among P-rich macromolecules of (C) *P. marinus* and (D) *T. weissflogii*.**

### Utilization of Polyphosphate Content Across Taxa During P Starvation

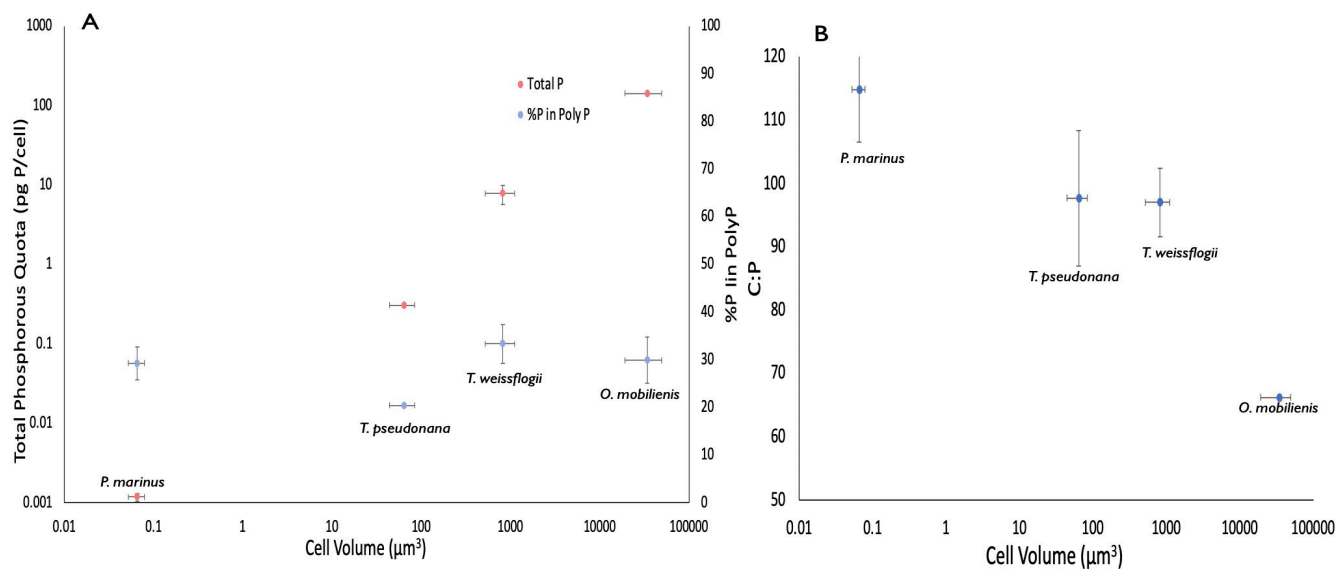
After *P. marinus* and *T. weissflogii* cultures were grown fully to acclimated exponential growth in a P-replete environment, they were diluted in P-starved media and left to grow for eight days. There was a similar use of polyP across taxa as seen in (Figure 10) below. In *Prochlorococcus marinus*, the total phosphorus and polyP quota (fg P/cell) decreased from  $(1.1780 \pm 0.1354 \text{ fg P/cell})$  to  $(0.7275 \pm 0.0254 \text{ fg P/cell})$  and  $(0.343 \pm 0.0465 \text{ fg P/cell})$  to  $(0.1126 \pm 0.0202 \text{ fg P/cell})$  respectively after cell cultures were P-starved. There was a similar trend noticed in *Thalassiosira weissflogii*, the total phosphorus and polyP quota (fg P/cell) also went down from  $(6.1680 \pm 0.2868 \text{ pg P/cell})$  to  $(3.9700 \pm 0.0200 \text{ pg P/cell})$  and  $(1.7451 \pm 0.3174 \text{ pg P/cell})$  to  $(0.5454 \pm 0.1204 \text{ pg P/cell})$  respectively after cell cultures were P starved as shown in (Figure 10) below.



**Figure 10. Total phosphorus and polyphosphate quota of *P. marinus* and *T. weissflogii* before and after phosphorus starvation. (A) *Prochlorococcus marinus* (in units of fg P/cell) and (C) *Thalassiosira weissflogii* (in units of pg P/cell) before phosphorous starvation and (B) *P. marinus* and (D) *T. weissflogii* after phosphorous starvation. Error bars indicates standard error measurements.**

### Total Polyphosphate and C:P Comparison Across Size

Four different phytoplankton namely *P. marinus*, *T. pseudonana*, *T. weissflogii*, and *O. mobiliensis* representing a large size range were used in the analysis of how total phosphorous and polyP quota change with cell size as seen in (Figure 11) below. Some noticeable trends that are shown below are the total phosphorous is proportionate to cell size but the %polyP is similar across cell sizes. Also, relative content of C and P (C:P) has a negative relationship with cell size.



**Figure 11. Total phosphorous, %polyP and C:P ratio across phytoplankton sizes. (A)** Total phosphorous and %P in polyP varying with size. **(B)** C:P ratio varying with cell size. Error bars represents standard deviation.

## Discussion

### Preferred Extraction Method

Although there were no significant differences between the two extraction methods (Figure 8) in terms of total polyP extraction yield, the purified method proposed by Bru et al., (2017) would be the preferred method going forward. Christ and Blank (2018a) highlight that their new optimized method extracted 40% more polyP in comparison to the Bru et al. 2017 method, but we saw not clear difference in the efficiency of extraction when comparing the two methods. The Bru et al. 2017 method represents a trade-off between precision and time as the purified extraction method proposed by Bru et al., (2017) produced more measurements with less variability but required more steps over a longer time period (approximately two days) to successfully extract polyP. In comparison to the crude extraction method by Christ and Blank (2018a) took approximately five hours but had high variability in measurements in *P. marinus*. There are several possible causes for the higher variability observed with Christ and Blank (2018a) method due to its use of unpurified extracts. Despite the aqueous-organic partitioning of the extract in both methods, the upper phase of the extract in both methods contains small concentrations of phenol and chloroform which could interfere with the enzymatic digestion of polyP or the colorimetric detection of orthophosphate produced by this digestion. Christ and Blank (2018a) suggested a minimum 1:10 dilution of the upper phase to avoid this inference from the extraction solvents, but this caused too much of a drop in sensitivity for the amount of biomass in our samples and collecting sufficient biomass to be able to dilute these extracts by 1:10 before analysis would very challenging due to the low densities that fully acclimated phytoplankton cultures must be grown at. Additionally, previous tests with this extraction method showed that there was no clear effect on colorimetric detection added orthophosphate standards if the extracts were diluted by at least 1:4 (MacKay-Barr, 2022), which was the level of dilution used in this study. The variability we observed with the Christ and Blank (2018a) extraction method may indicate that a 1:4 dilution is not sufficient when working with natural samples.

### Change in Enzymatic Treatments

Initially, polyP samples in this study were meant to treated with the no enzyme treatment, PPX only treatment, and the IPP + PPX treatment, but the PPX only treatment was dropped as

there was inconsistency seen in the amount of phosphorus it yielded, which is likely due to the contamination of the PPX enzyme with the IPP enzyme. Normally, less inorganic phosphate should be measured with the PPX treatment as pyrophosphate would not be broken down, but Figure 7 shows that there was a greater (though not significantly different) digestion of polyP with the PPX treatment in comparison to the PPX +IPP treatment in two of our pair-wise comparisons while the PPX + IPP treatment provided significantly higher digestion in only two of the six pair-wise comparisons. The overall interpretation of these results is no clear difference between these enzyme treatments, which indicates that the PPX enzyme stock is contaminated with IPP. Further studies might be carried out to express PPX protein again from recombinant plasmids in order to examine polyP chain length as well as polyP concentration with these methods.

### **Apparent Accuracy of Polyphosphate Budget**

One of the main goals of our study was to verify if measured polyP fit into the total phosphorus budget of phytoplankton cell by accounting for the portion of total cellular P that is not in RNA and DNA. The absolute accuracy for measurements of polyP in the cell cannot be determined as there is no “optimized gold-standard method” that can be used in the extraction and analysis of polyP. Also, a spike recovery test cannot be used as much of added known polyP spike is lost during the initial extraction step, which might be due to the differences in the structure between synthetic and natural polyP (MacKay-Barr 2022).

Most preceding studies assume that RNA makes the largest contribution to total cellular P (Sterner and Elser, 2002). Yet the results in this study (Figure 9A-9D) suggests something different as RNA contributed to about 26.7% of cellular P in *P.marinus* and 31.36% in *T. weissflogii*. We assumed that the remaining phosphorus was going to be stored in polyP and got a lower percentage budget of the polyP (Figure 9C-9D) than we expected; some of the unaccounted P could be in the form of dissolved orthophosphate in the cell or small metabolites in the cell that contains phosphorus like ATP (Geider and La Roche, 2002).

### Macromolecular and Experimental Comparison Across Taxa

There were similar contribution from polyP to total cellular P in *P. marinus* and in *T. weissflogii* which was surprising as very distinct species from different domains of life that originate from very different habitats (Donald et al., 1997; Dufresne et al., 2003). *P. marinus* grows slowly and is usually found in the open ocean with low nutrient while *T. weissflogii* has a faster growth rate and is found in the coastal regions where there is higher amount of nutrient supply (Donald et al., 1997; Dufresne et al., 2003). The results obtained here agree with the past literature in the sense that %RNA was lower *P. marinus* (26.70%) in comparison to *T. weissflogii* (31.36%) as slower growing *P. marinus* is expected to have less RNA relative to other cell parts since growth depends of RNA for ribosome (Sterner and Elser, 2002). In Figure 9, the unaccounted P was 33.76% greater in *T. weissflogii* in comparison to *P. marinus* which might be due to the differences in their cell size as diatoms are assumed to have a larger storage and can store this dissolved orthophosphate in their central vacuole (Dortch et al., 1984; Grover, 1991).

### Similar Response in Polyphosphate utilization during P Starvation Across Taxa

Cultures of *P. marinus* and *T. weissflogii* were grown in P-replete media and transferred to P-starved media to investigate how these different kinds of phytoplankton used their cellular phosphorous and their polyphosphate storage. There was a similar trend in how each species used their total phosphorus and polyP when starved of phosphorus as seen in Figure 10. *P. marinus* used up 35.6% of their total phosphorus and 68.6% of their polyP while *T. weissflogii* used 38.2% of their total phosphorus and 67.1% of their polyP when starved. Field studies performed using semi-quantitative methods highlights that cyanobacteria occupy parts of the ocean that are extremely P starved and that there are still some polyP found in the cell during P starvation which agrees with our result for *P. marinus* (Martin et al., 2016; Li, 2019). On the other hand, diatoms have shown a large decline in their phosphorus content when they are P-starved in previous studies (Leonardos and Geider, 2004), which was not the here Figure 10.

### Comparison Across Diatom Sizes

Three diatoms species and *P. marinus*, together representing a size range spanning six orders of magnitude, were used to measure how total cellular phosphorus and polyP content varied with cell size. Total cellular phosphorus increased in proportion to cell size Figure 11A as

would be expected. On the other hand, the C:P ratio had an inverse relationship with cell size, indicating that larger diatoms are more P-rich on a relative basis. Other essential cell components like proteins and lipids have been shown to increase less than would be expected in proportion to cell size, which would suggest that larger cells use a lower proportion of their cell volume for these essential components (Finkel et al. 2010). We assumed that the proportion of polyP would increase with cell size as larger cell size have larger vacuoles and more cellular space to be used for P storage. This was not the case as shown in Figure 11 since there was no clear trend in the % polyP with cell size.

## **Conclusion and Future Directions**

This new enzymatic method shows that polyP is a large fraction of cellular phosphorous as was expected which is promising. It also highlights that polyP constitutes to a large fraction of phosphorous across taxa, cell sizes and the different growth conditions, confirming that this polymer plays a major part in the role that phytoplankton plays in the oceans' phosphorous dynamics. There was also a large amount of unaccounted phosphorous than expected which indicates that the phosphorous storage might be in the form of dissolved orthophosphate within the cells rather than in a packed storage form like polyP. As of now there is no “gold standard method” in the extraction and analysis of polyP that has been established yet. Future studies will look at optimizing existing protocols by using different buffers other than the ones we used can be tested and other sample disruption methods aside vortexing and bead milling. Lastly, a new method can be developed to accurately measure the dissolved phosphorous in the cell to help close the budget and confirm the accuracy of our polyP measurement which in turn will help better understanding in what causes the variation phytoplankton's phosphorous across the ocean regions.

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