Towards an Isotopic Assay for Nutrient Stress in the Oceans – Bacterial Phosphatases, Nutrient Recycling and Ocean Productivity

Jonathan Behrens

The University of Chicago
Department of Chemistry

May 13, 2016

Advisor: Albert Colman
Department of Geophysical Sciences

Abstract

Microorganisms in oceanic environments, responsible for nearly half of global primary production, play an influential role in the global cycling of nutrients – including nitrogen (N), carbon (C) and phosphorus (P). Phosphohydrolytic enzymes in marine microbes are essential for the cycling and remineralization of orthophosphate (PO_4^{3-}; P_i), the most biologically accessible form of phosphate necessary for primary producers, from organically-bound phosphate (DOP). This study provides a foundation for understanding the activity of these enzymes, especially after cell lysis and death, in the oligotrophic ocean – where low levels of P_i limit primary production. The careful growth of a model bacterium, *E. coli*, was achieved under P_i-deplete conditions in the excess of organic-bound phosphate. The subsequent activity of alkaline phosphatase, selected for during growth, was identified through fluorescence spectroscopy. Developed methods provide a groundwork for subsequent isotopic measurements of biological mechanisms of P_i remineralization – including the kinetic fractionation of P_i upon cell growth and the isotopic tracing of the hydrolysis of DOP after cell lysis catalyzed by alkaline phosphatase. The fingerprint oxygen isotope compositions of these pathways will provide an important proxy for identifying these biologically-mediated routes for P_i remineralization in aquatic environments.

1. Introduction

Microorganisms are responsible for the biochemical transformation of essential elements including carbon, nitrogen, phosphorus and sulfur (Stout 2014). These elements are the fundamental building blocks for life and the effective cycling of these nutrients in various forms through physical space and oxidation state are of particular interest for understanding constraints to biological production and ecosystem structure (Arrigo 2005). Marine microbes are responsible for approximately half of Earth's primary production and have a significant role in the global cycling of nutrients (Arrigo 2005). Limitation on these primary producers inhibits the diversity and population levels of higher trophic levels. Thus, cycling of these nutrients through biota has been investigated widely through analysis of microbial growth and enzyme activity (Stout 2014).

1.1 Marine Phosphorus Nutrient Cycle

The element phosphorus (P) is ubiquitous and necessary for sustaining life, especially in its oxidized form as orthophosphate (PO_4^{3-}; hereafter P_i). P is a major building block for cell structure (phospholipids), storage and expression of hereditary genetic information (nucleic acids) and energy transportation (adenosine triphosphate) (De Duve 1991). The supply of P to the ocean is dominated by the weathering of continental rocks carried by rivers to the ocean, tectonic and ocean circulation (upwelling), and internal recycling processes dominated by primary producers (Delaney 1998). In the open ocean, both distant from continents and characterized as oligotrophic (low nutrient concentration), biological and enzymatic activity play a crucial role in cycling P (Blake 1997). Therefore, the rigorous recycling of P in a marine biota is of germane interest for understanding the level of biological production.

The deficiency of P_i in the oligotrophic (low nutrient) ocean disrupts metabolism and growth leading to reduction in primary production, solar energy capture, carbon dioxide (CO_2)

sequestration and population density of higher trophic levels (Karl 2014). The distribution of P_i in the surface waters of the global oceans is shown in Figure 1. A large portion of the open ocean is composed of oligotrophic regions (white and light blue) where nutrient-stressed environments have the potential to stifle microbial activity.

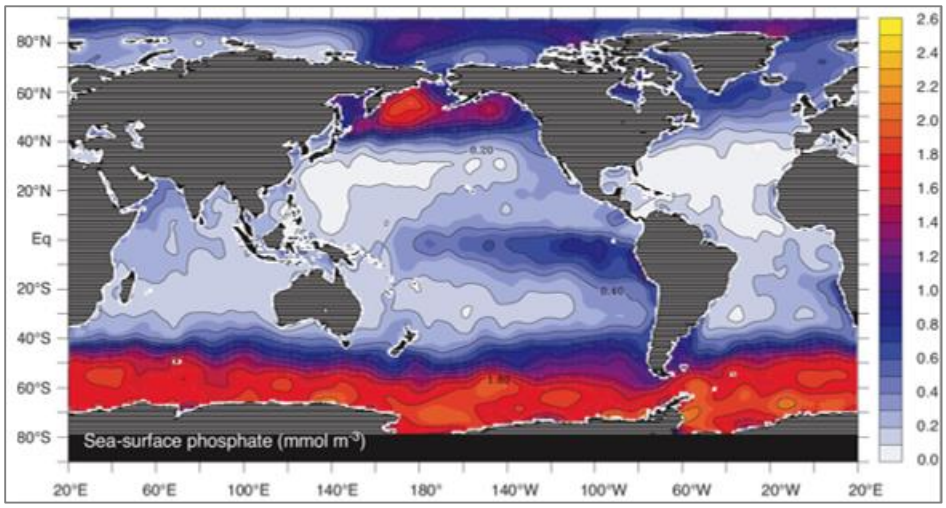

Figure 1: The concentration of P_i in surface waters in the global oceans is shown (Sarmiento and Gruber, 2006).

In extreme nutrient limited environments, only a select few microorganisms have been found to partially substitute sulfur for phosphorus *temporarily* (Van Mooey et al. 2009), indicating the requirement of P_i to sustain life (Karl 2000). In some parts of the world's oceans the scarcity of multiple resources (P, N, and metals for cofactors) are found to co-limit the growth of primary producers. Yet as a result of rigorous cycling, these regions characterized by low nutrient levels ("oligotrophic" regions) are also responsible for a large fraction of global net primary production (Bryant 2003).

1.2 Cycling of Phosphate on the Microbial Level

In marine systems phosphate and phosphate-containing molecules are classified under four main categories. The dominant form of inorganic phosphate, not bound to organic material,

is orthophosphate (PO_4^{3-}, primarily HPO_4^{2-} in the ocean) (Colman 2005). P_i is the least substituted form of phosphate that subsequently is the most biologically accessible for uptake into cells for (Karl 2007b). The remaining classes include DOP and POP which are phosphate-bound molecules (synthesized from biological processes and cell growth) that are attached by ester bonds to a variety of substituents. The nominal differentiation between DOP and POP is based on size of molecule, the former is considered dissolved in sea water and the latter is classified as too large for dissolution. Experimentally, dissolved (DOP) material is defined as any molecule that passes through a 0.2-0.7 µm pore size filter (Benitez-Nelson, 2000). The linkage of P_i monomers through phosphor-ester bonds leads to a final class of chains of phosphate molecules (PP_i).

In the oligotrophic (low nutrient) ocean, the concentration of P_i in the surface waters is significantly lower than phosphate bound to organic material (DOP), shown in Figure 2. The trend seen in the water column results from biological activity and remineralization. In the surface waters, primary producers consume the phosphate in the form of P_i and upon death or lysis of cellular material, phosphate is released as both P_i and organically-bound phosphate (DOP – such as RNA). The higher concentration of DOP relative to P_i provides a reasonable evolutionary selection for microbial primary producers with (a) high-affinity P_i transporters and uptake capacity and (b) monoesterase and diesterase enzymes capable of hydrolyzing DOP compounds (to P_i) (Dyhrman et al. 2007).

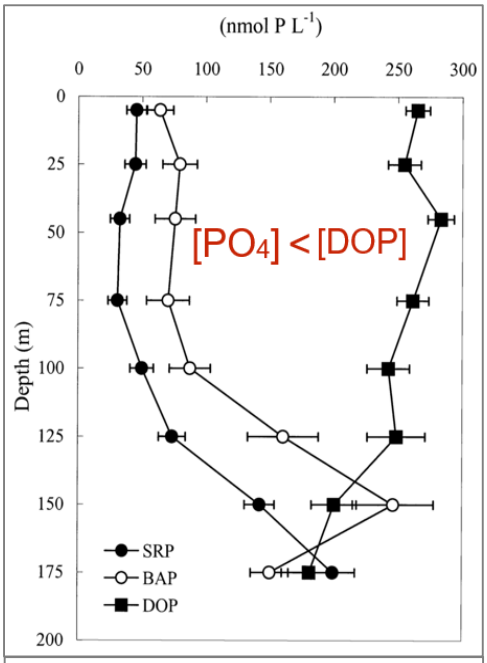

Figure 2: Depth gradient of [P_i] and [DOP] in the oligotrophic ocean (Bjorkman and Karl 2003, modified)

Phosphate undergoes rigorous cycling in marine environments as a result of dynamic biological processes occurring at the microbial level. The influence of microbial activity on the previously discussed classes of phosphate is shown in Figures 3a and 3b.

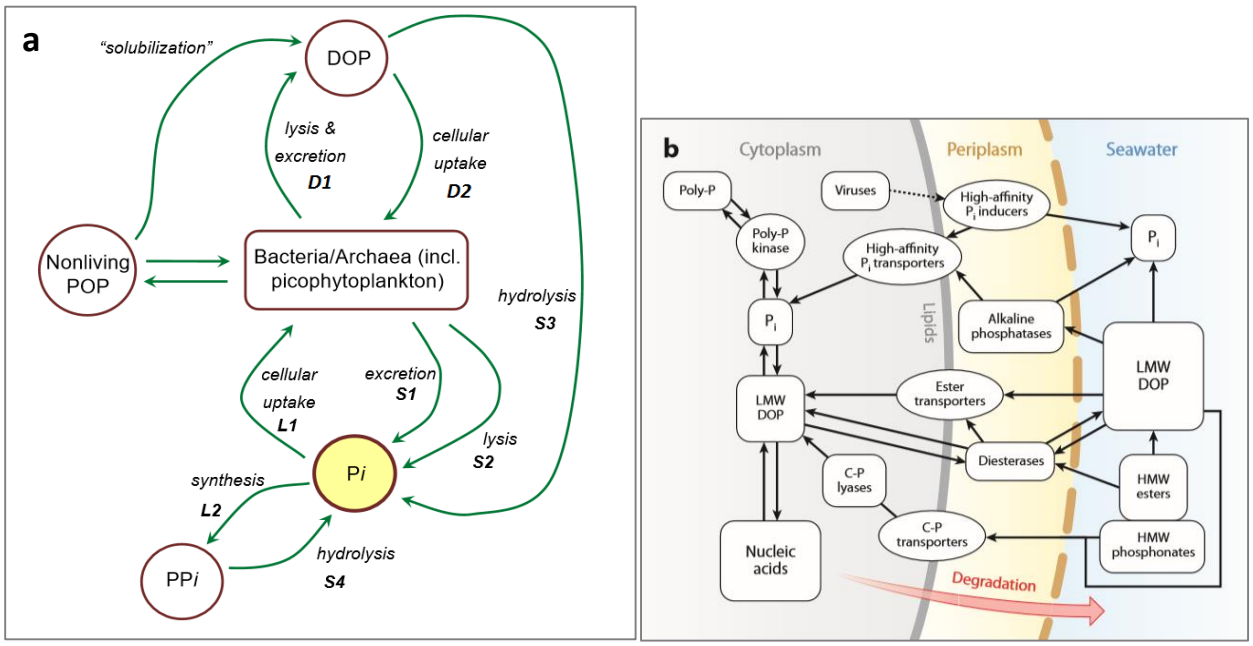

Figure 3: (a) Marine microbial mechanisms for phosphate and phosphate-bound molecules cycling (Colman 2016) and (b) predicted assimilation pathways for transit of bioavailable phosphate in bacterial cells are shown (Dyhrman et al. 2007).

A widely studied monoesterase found in all marine organisms (including bacteria in the open ocean such as Prochlorococcus and Synechococcus) is alkaline phosphatase (hereafter, APase) (Hoppe 2003). APase is found in the periplasmic space of bacteria, a region that separates the cytoplasm of cells from their environment (Figure 2b). The non-selective enzyme has been found to catalyze the hydrolysis of DOP into P_i that is subsequently transported as P_i into the cell (correlating to pathway D1 in Figure 3a). APase is crucial in oligotrophic marine environments, providing a source of P_i for cellular activity and growth. The activity of APase far outweighs the activity of diesterases (Apase has been estimated as 10^6 more efficient; O'Brien and Herschlag 2001). Therefore, the activity of APase during cell growth and after cell lysis is crucial for

linking the D1 and S3 routes for the hydrolysis of DOP into P_i in the open ocean. This study provides the foundation for bridging APase activity after cell lysis to the hydrolysis of abundant DOP into P_i in oligotrophic regions of the ocean.

1.3 Quantification of P_i as Soluble Reactive Phosphate

To quantify the concentration of P_i in the growth medium of bacterial assays, P_i is isolated from other phosphorus-bound molecules through a selective process to form a deeply blue colored metal complex. Under acidic conditions, P_i reacts with a mixture of ammonium molybdate and antimony potassium tartrate to form a heteropolymolybdate anion. The species is further reduced by ascorbic acid to produce a deeply blue colored anion "molybdenum blue." The absorbance of light at 880nm by the dissolved complex is monitored with UV spectroscopy and P_i concentration is calculated in accordance to Beer's Law and a standard curve (KH_2PO_4 in deionized water). This method has been widely used and adapted for measuring [P_i] levels below 30nM in natural and lab grown microbial cultures (Baginski et al., 1967; Alghren, 1970; Campbell and Thomas, 1970; Benitez-Nelson, 2000). The analytical method breaks down at phosphate concentrations above 30nM due an observed non-linear relationship, deviating from Beer's Law, between absorbance and nominal [P_i] at high [P_i] levels.

Experimentally, the growth matrix of bacterial assays is complicated by a variety of forms of phosphate-bound molecules in addition to P_i. P-bound species report widely in oceanographic studies are broken down into soluble reactive phosphate (SRP), total dissolved phosphate (TDP) and non-reactive phosphate (SNP) or dissolved organic phosphate (DOP). SRP encompasses all phosphate-bound molecules that are reactive to form the "molybdate blue" complex – dominated by P_i. SNP encompasses all remaining dissolved phosphate-bound molecules that do not undergo P-complexation to form "molybdate blue" due to steric and

electronic hindrance. Numerous dissolved organic phosphate (DOP) compounds are found to be unreactive and defined as SNP, leading to the estimation of SRP = P_i and SNP = DOP (Benitez-Nelson 2000).

The quantification of TDP is achieved through the oxidation of phosphate-bound DOP species (SNP) and the subsequent isolation of P_i that results from the induced hydrolysis of SNP. TDP is the summation of the two prior species (TDP=SRP+SNP) and experimentally is defined as any phosphate containing molecule that can pass through a 0.2-0.7 μm pore size filter (Benitez-Nelson, 2000). Remaining particulate matter is removed from the matrix through filtration or centrifuge. To quantify TDP, the SNP in solution is oxidized to SRP (leading to hydrolysis) by persulfate or UV oxidation (Koroleff, 1983; Ridal and Moore, 1990). The subsequent P_i (from the initial [SRP] and [SNP]) is often concentrated through a modified MagIC method (Karl and Tien, 1992); SRP is scavenged out from solution upon the precipitation of brucite ($Mg(OH)_2$) under alkaline conditions and subsequently dissolved in a minimal volume of acidic solution (Benitez-Nelson, 2000).

1.4 Probing Nutrient Cycling of Phosphate through Oxygen Isotopes

There exist three stable isotopes of oxygen found in nature. The natural abundance of the oxygen isotopes ^{16}O, ^{18}O and ^{17}O are 99.76%, 0.21% and 0.04% respectively (Rosman 1999). An isotopic ratio between the two most abundant oxygen isotopes (^{18}O and ^{16}O) can be taken for P_i. At average Earth surface temperatures, the exchange of P_i with ambient water only occurs rapidly when catalyzed by enzymatic activity (Blake 1997). In the absence of biological cycling, it would take over 6,000 years for 10% of P_i oxygen to exchange with ambient water at 10 ºC (Colman 2005). Therefore, isotopic measurements of P_i may provide fingerprint evidence for the various microbial mechanisms outlined in Figure 3a for cycling P_i through the water column.

2. Experimental Methods

A significant effort was placed into developing methods for probing the growth of *E. Coli* and the subsequent enzyme facilitated breakdown of DOP compounds after the lysis of cells. To investigate the selection of alkaline phosphatase enzymes commonly found in bacteria in the oligotrophic ocean, *Escherichia Coli* cells (K-12 MG1655) were grown under nutrient limited conditions and subsequently lysed to examine the activity of alkaline phosphatase after cell death. *E. Coli* was chosen for analysis due to the bacterium's rapid growth relative to other bacteria that dominate marine environments including *Prochlorococcus* and *Synechococcus*. All reagents were ACS grade and supplied by Fisher or Sigma-Aldrich. Experiments were completed under sterile technique in acid washed, autoclave sterilized glassware. All deionized water was sourced from a Barnstead Nanopure filtration system (Thermo Fisher).

2.1 Microbial Culture and Growth

E. coli K-12 MG1655 cells are plated for growth on a plate prepared with nutrient rich Miller's LB agar (Fisher BP 1425) and de-ionized water. After growth overnight an isolated colony is selected and incubated in 50mL of Miller's LB broth (Fisher BP 1426). The Miller's LB media is a nutrient rich medium with excess amounts of P_i and essential nutrients. The isolated colony is allowed to grow and multiply overnight to stationary growth. A small aliquot of the resulting inoculate, taken prior to the death phase in the *E. coli* growth curve, is introduced into nutrient limited media for subsequent analysis.

2.1.1 Growth under limited nutrient conditions

A small aliquot of the inoculated LB media is introduced into two separately prepared M9 media (50µL LB inoculate per 50mL of M9 medium). The composition of the media matrix

is shown in Figure 4. The inorganic phosphate replete media (M9 +) is prepared with an excess of P_i, whereas the inorganic phosphate deplete media (M9 -) is prepared with a minimal P_i concentration and an excess of glycerol phosphate (GYP), an organic bound phosphate derivative. The presence of organically bound phosphate molecules (DOP) in the form of GYP promotes the synthesis of alkaline phosphatase enzymes that catalyze the hydrolysis of organophosphorus-oxygen bonds to release P_i for cell usage (Blake 1997). The minimal P_i concentration in the P_i-deplete medium initially sustains cellular activity as alkaline phosphatase enzymes are activated. Cells are incubated under sterile conditions at 37ºC and 180rpm. Every 30min 1mL aliquots are removed for subsequent analysis.

	M9 $^{+}$ Media	M9 $^{-}$ Media
Glucose	0.4% (w/v)	0.4% (w/v)
$MgSO_4$	1.0 µM	1.0 µM
$CaCl_2$	0.10 µM	0.050 µM
NH_4Cl	17 mM	17 mM
NaCl	8.5 mM	8.5 mM
KH_2PO_4	22 mM	5.8 µM
$Na_2HPO_4*7H_2O$	48 mM	-
Glycerol phosphate	-	5.8 mM

Figure 4: The composition of the two nutrient-controlled growth mediums is shown.

The optical density (OD_{600}), a linear proxy for cell count (Sezonov 2007), is measured at 600nm for the first aliquot. This analytical method is discussed further in the following section. The cells are subsequently isolated for cell count by vacuum filtration over a 0.2µm pore-size polycarbonate membrane filter (Millipore) under pressures at or above 0.197 atm to prevent cell lysis. Filters are then stained with SYBR Gold and stored at 2ºC. A second 1mL aliquot is pelleted at 3600G for 15min. The supernatant above the pellet is isolated and frozen. The frozen aliquot is later thawed for [Pi] quantification by spectrophotometric methods and stored for later processing to isolate phosphate for $\delta^{18}O$ analysis.

2.2 Cell Lysis and Alkaline Phosphatase Activity Assay

After cells reach log-based growth in both media, cells are subsequently pelleted by centrifuge at 3600G for 15min. The supernatant is removed and the pellet is washed (3x10mL with Pi-free 50mM Tris buffer). Upon washing of cells, the wash solution and pellet are centrifuged for 10min to reduce loss of cells during each wash step. The final pellet is re-suspended in 2.0mL of 50mM Tris.

2.2.2 Lysis of cells and alkaline phosphatase activity

Re-suspended solution (1.9mL) is mixed with 100 µL of 10mg/mL lysozyme solution. The lysozyme solution is prepared by the dissolution of lysozyme isolated from egg white (ThermoFisher BP 535-1), commonly used to lyse *E. coli* for plasmid DNA (Smékal 1973), in de-ionized water. The suspension is incubated for 1hr at 37^{o}C to allow for the full lysis of cells. Upon lysis inorganic (Pi) and organic (DOP) forms of phosphate stored within cells are released.

Cells growing in the P- media, where DOP (in the form of glycerol-phosphate) is the dominant source of phosphate for cells, select for the expression of a phosphatase enzyme. The activity of this enzyme in the *E. coli* cells is measured through the introduction of the synthetic fluorinated DOP compound of 6, 8-difluoro-4-methylumbelliferyl phosphate (ThermoFisher D22065) to the lysed cell solution. The enzyme catalyzed hydrolysis of DiFMUP produces a fluorescent product (DiFMU) that can subsequently be quantified by fluorescence measurements. A five-fold dilution of the lysed cell solution is spiked with DiFMUP for a final concentration of 0.01mM DiFMUP. The concentration of the fluorescent DiFMU product was measured with a Tecan fluorimeter (Infinite M200 PRO) for 30 min.

2.3 Analytical Methods

Two aliquots of the growth medium are taken throughout the growth of E. Coli, as previously discussed, for the quantification of cell density (OD_{600}) and P_i concentration in the growth medium. The methods for these analyses are discussed in further detail for clearity.

2.3.1 Optical density as an estimate of cell density

As cells multiply, the density of cellular material increases, reducing the passage of light through the inoculated medium. Therefore, the absorbance of light at a wavelength of 600nm is measured on a Shimadzu UV-1800 UV-Vis spectrometer (Shimadzu Scientific Instruments) to provide OD_{600} measurements for isolated aliquots (1mL of inoculated medium). Measured aliquots are introduced into a 1 cm length plastic cuvette cell for analysis in the spectrometer. Aliquots of the growth media are taken at regular intervals throughout the growth curve to estimate the changing cell density in the inoculated medium as cells multiply.

After the optical density of aliquots are taken, cells are filtered as explained in the prior section. Cells are counted on a microscope and associated to the OD_{600} measurements taken prior to filtration. Complications with getting uniform cell distributions on the filters convoluted cell counting. Therefore, all results in this study are presented as optical density measurements. Prior studies indicate that OD_{600} measurements for *E. coli* have a linear relationship to cell count for the low levels reported in this study (Koch 1968). Therefore, qualitative comparison is possible.

2.3.2 Spectrophotometric quantification of Pi in solution

The concentration of P_i ([SRP]) is measured through the formation of a reduced heteropolymolybdate anion (“molybdate blue”). Colorimetric assays involving molybdate blue are also sensitive to aqueous species including silica and arsenic (Benitez-Nelson, 2000), thus the growth medium is carefully prepared with de-ionized water and ACS-certified salts to minimize

silica and arsenic contamination. Samples of inoculated medium are centrifuged to remove cell material prior to [SRP] analysis. Supernatants of the growth medium are stored frozen and thawed within a week after collection.

A mixed reagent is introduced into the sample to form the blue complex that is quantified by UV spectroscopy. The mixed reagent is prepared in a sterile Falcon tube with the sequential addition of ammonium molybdate (24 mM), sulfuric acid (5 N), ascorbic acid (0.31M) and antimony potassium tartrate (2.0 mM) in a ratio of 1.0:2.5:1.0:0.5. The samples are diluted with de-ionized water to a final concentration below 20 µM of P_i. 1mL of the diluted sample is subsequently pipetted into a 1cm path length plastic cuvette. 100µL of mixed reagent is added to each cuvette and color is allowed to develop for 30min. The absorbance of the formed molybdate blue is analyzed on a UV spectrometer (UV-1800 Shimadzu, 120V) at 880nm. The [SRP] is calculated against a standard curve created using KH_2PO_4 dissolved in de-ionized water.

2.3.2 Measuring APase activity through catalyzed hydrolysis of synthetic DOP compound

APase activity was measured after the introduction of the fluorinated synthetic DOP compound DiFMUP to the cell suspension. The fluorescence of the hydrolyzed product, DiFMU, was measured on a Tecan fluorimeter (Infinite M200 PRO). To a 200µL well plate, 20 µL of the lysate solution (and a control with cells treated without lysate solution) is diluted in 178 µL 50mM Tris buffer solution. Each diluted sample is subsequently spiked with 2 µL of 1mM DiFMUP and immediately placed in the Tecan fluorimeter for analysis. Every minute for 30 minutes the plate of samples is mixed briefly then excited at a wavelength of 358 nm (bandwidth of 9 nm). The subsequent emission is measured at 450nm (bandwidth of 20 nm) with an integration time of 20 µs.

3. Results

Methods were developed to probe (a) the growth of *E. Coli* in nutrient controlled media, (b) the uptake of P_i and breakdown of DOP during growth and (c) the breakdown of DOP to biologically accessible P_i after cell lysis due to continued APase activity. The methods and results of this work provide a foundation for the isotopic measurements of oxygen in P_i isolated from the growth medium that will follow to (d) further characterize the kinetic fractionation of P_i during cell growth and (e) provide an isotopic tracing of APase activity of lysed cell material in media spiked with a higher fraction of ^{18}O water ("heavier" water).

3.1 *E. Coli* growth in nutrient controlled media

E. Coli were grown in three distinct mediums with varying levels of accessible P_i and glycerol phosphate (model DOP compound). A collected growth curve of the *E. coli* in the LB nutrient excess medium is shown in Figure 5. The growth curves of the inoculated samples in P-deplete (M9-) and P-replete (M9+) media are shown in Figure 6 against a blank standard. Variations in the optical density of cells between triplicates taken for each medium was within 7%. The absence of growth in the M9- blank indicates effective sterile technique used throughout manipulations during the growth and analysis of the samples.

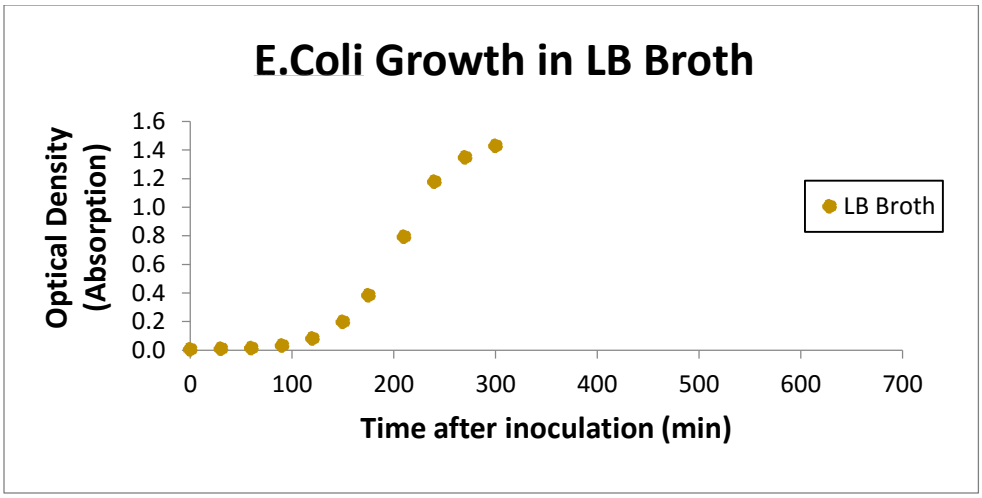

Figure 5: The growth curve for *E. coli* in a medium with an excess of nutrients (Miller's LB Broth) is shown. Cells reach log-phase growth rapidly within 3 hours.

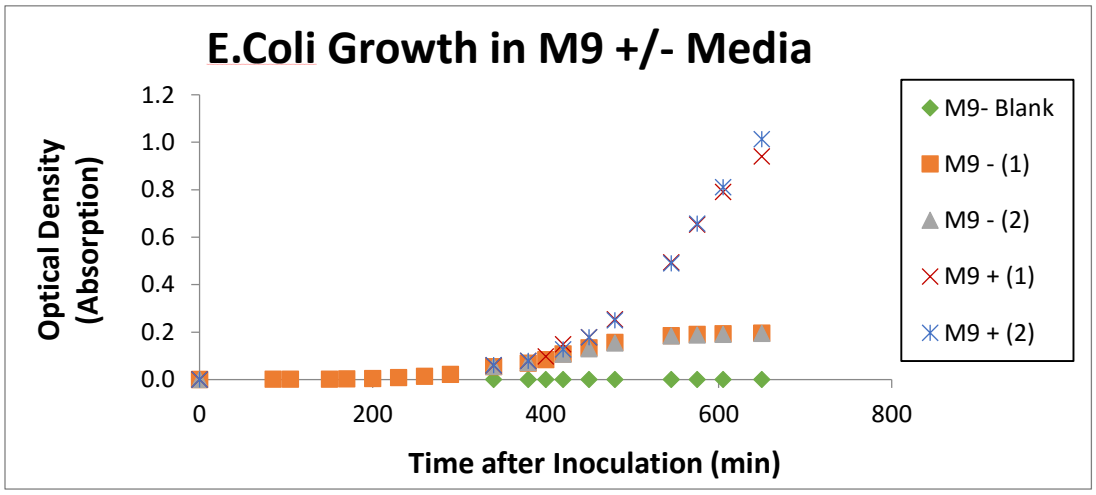

Figure 6: Growth curves for *E. coli* in regulated nutrient mediums (M9+ and M9-) are shown.

After the cells were introduced into a new medium (from LB to M9), a lag time was observed (roughly 5 hours) as cells adapted to the new nutrient conditions. Whereas log phase growth was reached in under 3 hours for the LB medium, both M9 mediums did not reach log phase growth till 5-6 hours after inoculation. Glucose levels in both mediums were in excess (source of C), whereas P_i was limited in the M9- media (with a high concentration of DOP) to simulate P-deplete conditions in the oligotrophic ocean. Upon stationary growth, the cellular density of the P-replete medium was comparable to the nutrient-excess LB broth – reaching a

cellular density only 25% smaller than the cellular density of cells grown in the LB media. However, under P-limited conditions, the cellular density upon stationary growth in the P-deplete media was only 20% of the cellular density reached in the P-replete media.

These findings indicate that under P_i–deplete conditions, cells undergo a growth curve that is dampened. Direct access to "free" inorganic phosphate (P_i) promotes higher growth, illustrated by the M9+ inoculated media. Under conditions where P_i is minimal and DOP is the main phosphate source (M9-), growth of *E. coli* is inhibited but not prohibited. Although productivity is reduced under these oligotrophic conditions where DOP dominates as a phosphate source, selection and activation of alkaline phosphatase induces an enzymatic hydrolysis of DOP (glycerol phosphate) to labilize P_i. The rate of enzymatic labilization is presumably slower than direct diffusion of P_i into the cell, indicating a rationale for reduced cellular growth for bacteria in oligotrophic conditions.

3.2 P_i in Nutrient Limited Media throughout Cell Growth

Upon cell division and growth, the measured levels of P_i indicate the cellular dependence on the two forms of phosphate present in the two M9 media. Shown in Figure 7 is a time series of the concentration of P_i in the P-replete media (M9 +). As cells grow the concentration of P_i ([SRP]) decreases as phosphate is consumed, metabolized and stored within cell material. To confirm the storage of phosphate in cells, the concentration of P_i was calculated after the lysis of cells. The release of P_i from cells was found to increase the concentration of P_i in the growth medium by 0.020% ± 0.002% – minimal relative to concentration of the medium. Therefore, P_i from the media consumed by cells, not accounted for upon cellular lysis, is presumed to be bound to cell material and other DOP materials not measured as [SRP].

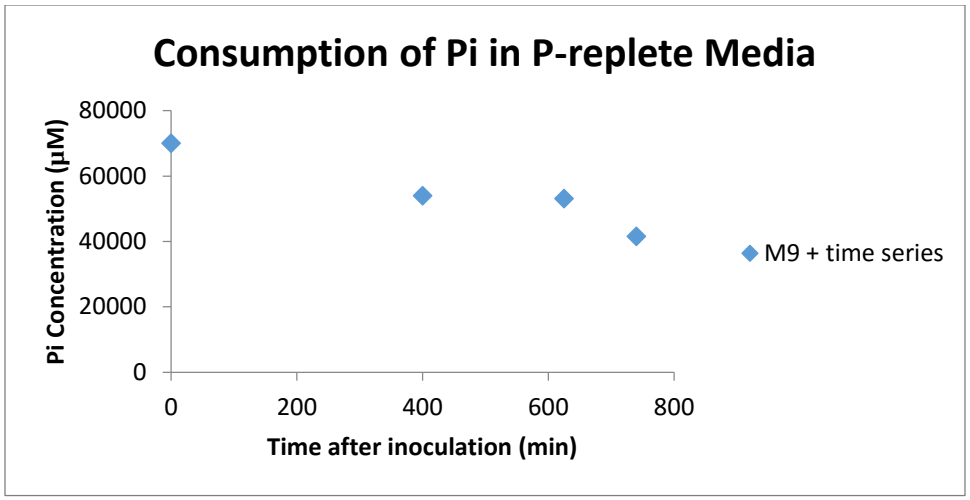

Figure 7: The concentration of inorganic phosphate in the growth medium is measured throughout the growth curve of E. Coli in P_i-replete (M9 +) media.

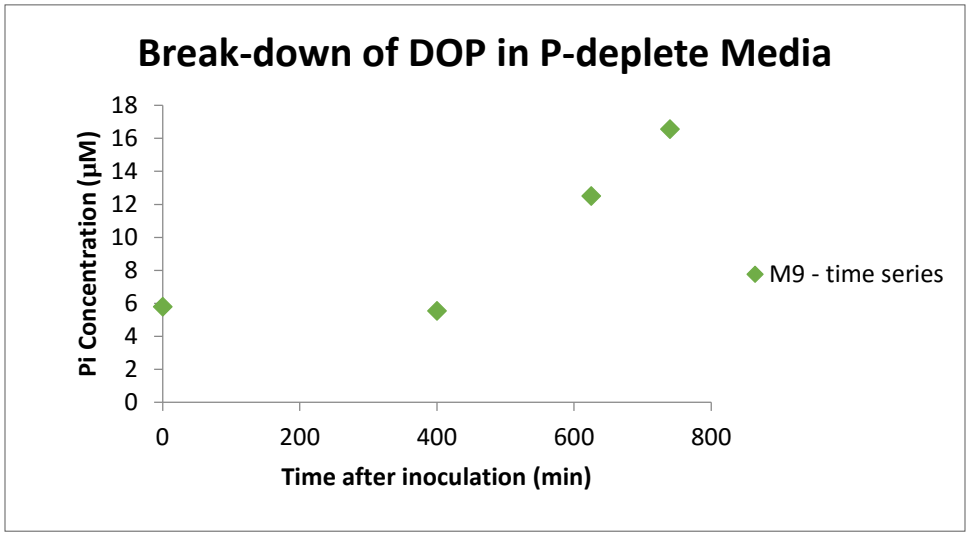

Figure 8: The concentration of inorganic phosphate in the growth medium is measured throughout the growth curve of E. Coli in Pi-deplete (M9 -) media.

In nutrient-limited media, enzymes within *E. coli* catalyze the hydrolysis of glycerol phosphate to P_i as the initial concentration of P_i alone is insufficient for growth. Shown in Figure 8 is a time series of the concentration of P_i throughout the growth phase of the cells grown under P_i-deplete conditions. Whereas the concentration of P_i was on the order of mM in the P-replete media, the concentration of P_i in the P-deplete media was on the order of µM throughout the

growth of cells. As cells grow in M9-, the P_i concentration initially is shown to decrease as cells are transitioning from P_i to DOP as a main source of phosphate for cellular function. Upon log-phase growth, the P_i concentration in the growth medium is seen to increase as monoester-phosphatase enzymes actively catalyze the hydrolysis of DOP into the biologically usable form of phosphate – P_i. Upon lysis the cells release stored P_i to the equivalent of 3.0µM – or 18% of the concentration of the growth medium prior to lysis. The release of P_i indicates the storage of P_i in cells when under nutrient stressed conditions.

The release of P_i is of particular interest when considering recycling of P_i in the open ocean where concentrations of P_i are on µM levels (Karl 2005). At the time of lysis, the density of cells in the M9+ medium was a factor of 5.0 greater than the density of cells in the M9- medium. Cells were incubated for 1.5 hours for complete lysis of cells. After 1.5 hours a P_i measurement was taken of the media – indicative of the amount of P_i released upon cell lysis and any DOP hydrolyzed by active alkaline phosphatase. The amount of P_i released upon lysis from cells grown under P-deplete conditions, when cell density is normalized, was greater by a factor of 1.2 relative to cells growth under P-replete conditions. However, prior studies have indicated that P_i storage is higher for cells in P-replete environments (Karl 2005). Therefore, the higher relative P_i concentration in the medium of lysed cells grown in P-deplete medium (which select for APase), encompasses both the P_i released upon lysis and the DOP that is successively hydrolyzed by APase for the 1.5 hours after lysis.

3.3 Cell lysis and alkaline phosphatase activity

In P_i-starved environments prior studies have indicated bacteria, including *E. coli*, select for the monoesterase, alkaline phosphatase (APase) (Hoppe 2003). This enzyme allows for cells to break down phosphate-bound DOP compounds (monoesters) to free P_i for cellular use.

Experimentally the activity of APase in living and lysed (non-living) cells was examined through the growth of cells in the P-deplete medium (P-), selecting for APase activity. Shown in Figure 9 are two assays taken for cells harvested at the end of log-phase growth. The first assay contained living cells (blue line) and was spiked with the DOP compound DiFMUP as explained in the method section. The second assay was taken 1.5 hours after the incubation of cells in the lysozyme solution (to lyse cells), and an equivalent amount of DiFMUP was added as explained in the method section. The cell density in both samples were within 1% of each other as determined by optical density measurements.

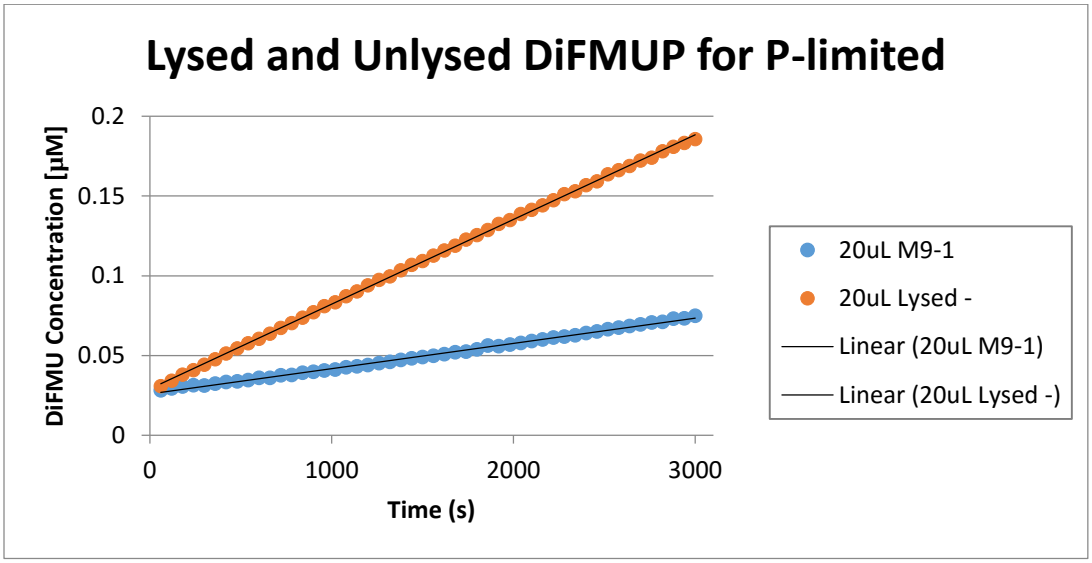

Figure 9: Hydrolysis of DiFMUP to DiFMU + P_i catalyzed by APase is shown for lysed cells. A control with non-lysed cells is shown for comparison.

As discussed in Figure 8, the release of P_i into the growth medium indicates the activity of APase and similar monoesterases in the periplasmic region of the E. coli cells. The activity of these enzymes is further supported by the observed catalyzed hydrolysis of the introduced DiFMUP as depicted by the increasing concentration of DiFMU (blue curve) in Figure 9. However, upon lysis of cells, the activity of APase continues even after the lysis (and subsequent

death) of the cell material. Therefore, these results indicate the continued activity of APase (orange curve) and the continued breakdown of DOP (including DOP released from within cells upon lysis) after cell lysis and death. The hydrolysis of DOP catalyzed by APase *after* cell lysis provides a new mechanism for understanding the remineralization of organically-bound phosphate to P_i in the surface waters of the open ocean where microbial activity dominates.

3.3.1 Processing fluorescence data for enzyme catalyzed

The data shown in Figure 9 underwent a basic linear correction. The measured intensity for a blank, composed of 198 µL of 50mM Tris and 2 µL 1mM DiFMUP, was subtracted from the measured values (at each time point) of each sample. However, the hydrolysis of DiFMUP in ambient water was found to be unmeasurable against instrument noise for the timescale of the measurement (30 min); all values were within 1% of the t=0 reading.

Furthermore, the measured emission intensity at each time step was converted into a concentration of DiFMU by comparison against a standard curve of pre-determined DiFMU concentrations ran under the same conditions as the samples. DiFMU standards were prepared in Tris buffer. The standard curve is provided with further details in the supplementary section. Overall the standard curve relating emission intensity to [DiFMU] is found to have a linear relationship, leading to the data shown in Figure 9.

4. Conclusion

Methods that have been developed in this study provide the necessary foundation for subsequent analysis that will couple isotopic measurements with kinetic fractionation of P_i during cell growth and APase enzyme activity after cell lysis for bacteria grown in nutrient limited conditions. When grown under nutrient limited conditions, the growth curves of *E. coli*

indicated a delayed division of cells as the bacteria adapted to the new media. Under P_i distressed conditions, cells selected for APase to convert DOP to P_i as seen through [P_i] measurements during growth. The subsequent activity of APase after cell lysis measured provides further indication of an alternative biologically-mediated mechanism for the hydrolysis of DOP to the biologically favored P_i form in surface waters. Characterization and subsequent modeling of isotopic fractionation of each pathway has the potential to be applied to isotopic measurements of P_i in the open ocean for quantifying the biological cycling of P_i.

Analytical methods were optimized for the measurements taken of APase activity and [SRP]. DiFMUP was chosen as a model DOP compound due to the fluorescent product, DiFMU, that resulted after the enzyme catalyzed hydrolysis of the compound. When compared against a control (unlysed cells in the same medium with the same cell density), APase activity in lysed cells were seen to continue even after cell death (through lysis). The activity of APase after *E. coli* cell lysis provides an important indicator of the continued role APase has in recycling P_i (especially in oligotrophic regions of the ocean) even after cell death. This biologically-dependent mechanism for DOP hydrolysis will serve as an important kinetic pathway in the recycling of DOP that is immediately released upon cell death.

The changing P_i concentration in both growth media during cell growth indicated the dynamic process of P_i intake by cells. Isotopically lighter P_i would be expected to diffuse into a cell at a slightly higher rate than isotopically heavier P_i (isotopic mass difference due to the different stable isotopes of oxygen). This kinetic fractionation will be further investigated to understand and probe the cycling of P_i in the open ocean. This hypothesis resulted from initial measurements of phosphate levels that were found to noticeably change upon cellular growth. The kinetic fractionation of phosphate during intake into cells will be investigated through

isotopic measurements of the oxygen isotopes of P_i. To achieve isotopic measurements, P_i will be isolated from the growth medium and subsequently analyzed on a mass spectrometer. Isolation of P_i will require a modified method that will use anion/cation exchange chemistry and MagIC to remove salts from solution prior to silver phosphate precipitation.

In conclusion, the relationship between kinetic fractionation and DOP hydrolysis from microbial activity has the potential to be modelled and applied to stable oxygen isotope measurements of natural sea water samples in the oligotrophic ocean. Conclusions drawn from the biologically-mediated pathways will quantify the dynamic micro-biological mechanisms for recycling P through biota. As human activities continue to induce warming through increased greenhouse gas emissions, the world's oceans are predicted to become more stratified (Karl 2005) – complicating layer mixing and P_i transport. An isotopic tool to track these dynamic changes to biologically mediated P_i cycling would be invaluable for monitoring the accessibility of limiting nutrients (like P_i) to some of the most biologically productive regions on the globe.

5. Acknowledgements

I would like to thank Albert Colman, Aric Mine and Gerarld Olack for their endless support and guidance throughout the process. Additionally, the support in lab from Johana Holo and feedback on figures from Miquela was greatly appreciated. This work was supported by grants from the National Science Foundation (OCE 0082416 and 0827122 to Albert S Colman).

6. References

Alghren, G., (1970). Test av analysmetoder for fosfatfosfor. *Inst. Of Limnology Univ. Uppsala*, 17 pp

Arrigo, K. R. (2005). Marine microorganisms and global nutrient cycles. *Nature*, *437*(7057), 349-355.

Baginski, E. S., Foa, P. P., & Zak, B. (1967). Determination of phosphate: study of labile organic phosphate interference. *Clinica Chimica Acta*, *15*(1), 155-158.

Benitez-Nelson, C. R. (2000). The biogeochemical cycling of phosphorus in marine systems. *Earth-Science Reviews*, *51*(1), 109-135.

Blake, R. E., O'neil, J. R., & Garcia, G. A. (1997). Oxygen isotope systematics of biologically mediated reactions of phosphate: I. Microbial degradation of organophosphorus compounds. *Geochimica et Cosmochimica Acta*, *61*(20), 4411-4422.

Blake, R. E., Alt, J. C., & Martini, A. M. (2001). Oxygen isotope ratios of PO4: an inorganic indicator of enzymatic activity and P metabolism and a new biomarker in the search for life. *Proceedings of the National Academy of Sciences*, *98*(5), 2148-2153.

Bryant, D. A. (2003). The beauty in small things revealed. *Proceedings of the National Academy of Sciences*, *100*(17), 9647-9649.

Campbell, F. R., & Thomas, R. L. (1970). Communicaton. Automated method for determining and removing silica interference in determination of soluble phosphorus in lake and stream waters. *Environmental Science & Technology*, *4*(7), 602-604.

Colman, A. S., Blake, R. E., Karl, D. M., Fogel, M. L., & Turekian, K. K. (2005). Marine phosphate oxygen isotopes and organic matter remineralization in the oceans. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(37), 13023-13028.

De Duve, C. (1991). *Blueprint for a cell: the nature and origin of life*. Burlington, NC: N. Patterson.

Delaney, M. L. (1998). Phosphorus accumulation in marine sediments and the oceanic phosphorus cycle. *Global Biogeochemical Cycles*, *12*(4), 563-572.

Dyhrman, S. T., Ammerman, J. W., & Van Mooy, B. A. (2007). Microbes and the marine phosphorus cycle.

Hoppe, H. G. (2003). Phosphatase activity in the sea. *Hydrobiologia*, *493*(1), 187-200.

Karl, D. M., & Tien, G. (1992). MAGIC: A sensitive and precise method for measuring dissolved phosphorus in aquatic environments. *Limnology and Oceanography*, *37*(1), 105-116.

Karl, D. M. (2000). Aquatic ecology: Phosphorus, the staff of life. *Nature*, *406*(6791), 31-33.

Karl, D. M. (2007). Microbial oceanography: paradigms, processes and promise. *Nature Reviews Microbiology*, *5*(10), 759-769.

Karl, D. M. (2014). Microbially mediated transformations of phosphorus in the sea: New views of an old cycle. *Annual review of marine science*, *6*, 279-337.

Koroleff, F., 1983. Determination of nutrients. In: Grasshoff, K., Ehrherd, M., Kremling, K. Eds. , Methods of Seawater Analysis. 2nd edn. Verlag Chemie, Weinheim, pp. 125–135

O'Brien, P. J., & Herschlag, D. (2001). Functional interrelationships in the alkaline phosphatase superfamily: phosphodiesterase activity of Escherichia coli alkaline phosphatase. *Biochemistry*, *40*(19), 5691-5699.

Ridal, J. J., and R. M. Moore (1990). A re-examination of the measurement of dissolved organic phosphorus in seawater. *Marine Chemistry* 29: 19-31.

K.J.R. Rosman, (1999). *P.D.P. Taylor Pure Appl. Chem*. 71, 1593-1607.

Koch, A. L. (1968). Theory of the angular dependence of light scattered by bacteria and similar-sized biological objects. *Journal of theoretical biology*, *18*(1), 133-156.

Sarmiento, J. L. (2013). *Ocean biogeochemical dynamics*. Princeton University Press.

Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007). Escherichia coli physiology in Luria-Bertani broth. *Journal of bacteriology*, *189*(23), 8746-8749.

Smékal, F. (1973). Lysis of lyophilized Escherichia coli cells with egg-white lysozyme without ethylenediaminetetraacetic acid. *Folia microbiologica*, *18*(2), 146-148.

Stout, L. M., Joshi, S. R., Kana, T. M., & Jaisi, D. P. (2014). Microbial activities and phosphorus cycling: An application of oxygen isotope ratios in phosphate. *Geochimica et Cosmochimica Acta*, *138*, 101-116.

Van Mooy, B. A., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblížek, M., ... & Rappé, M. S. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, *458*(7234), 69-72.

7. Supplementary Data

Figures 10 and 11 show the standard curves taken for [SRP] and [DiFMU] respectively. The [SRP] measurements were taken for known concentrations of KH_2PO_4 dissolved in de-ionized water (as reported on the x-axis). The [DiFMU] measurements were taken for known concentrations of DiFMU dissolved in de-ionized water (reported on x-axis). The resulting linear fits are shown for the standard curves and are numerically reported within the figures.

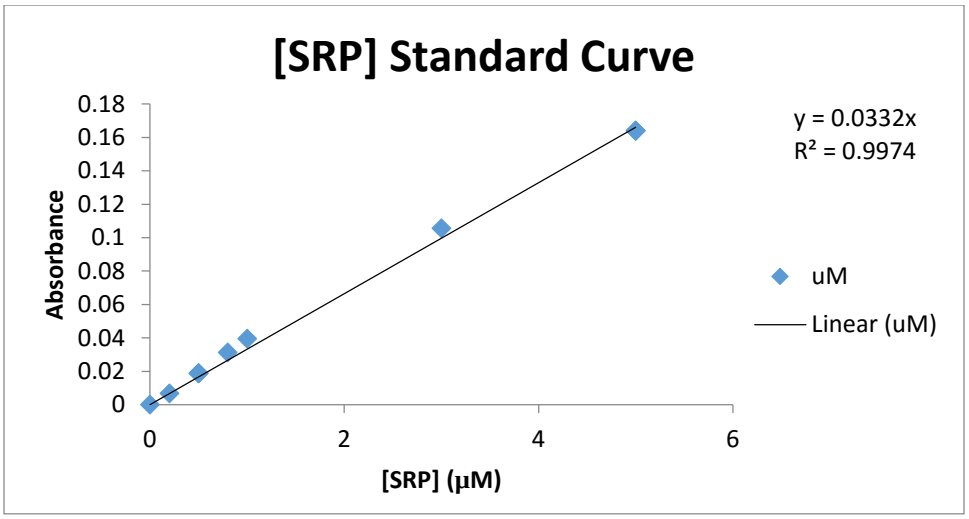

Figure 10: Standard curve used for estimating [SRP] in samples is shown.

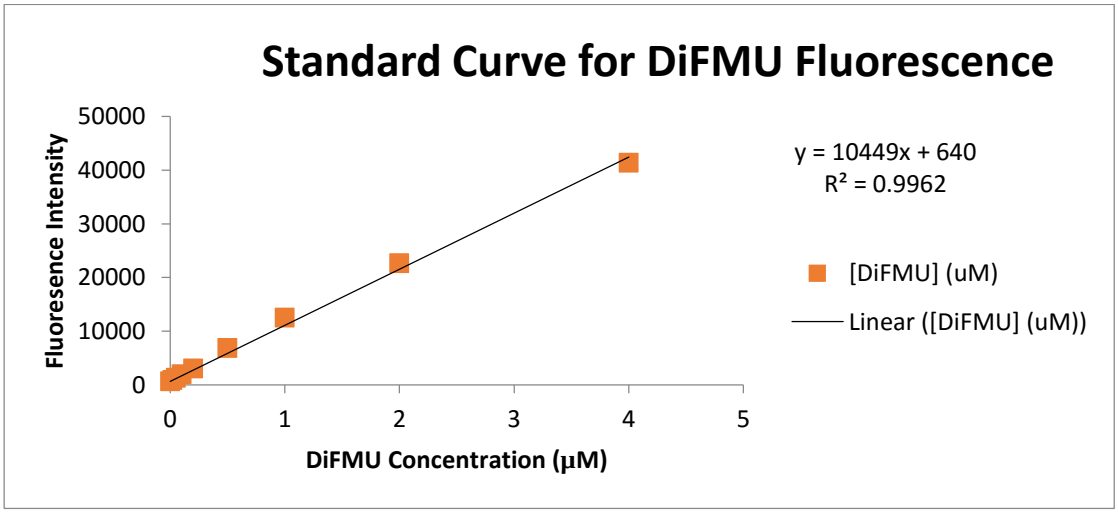

Figure 11: Standard curve used for estimating [DiFMU] in APase assays is shown.