SYNTHESIS AND DEGRADATION OF POLYPHOSPHATE: SCALING-UP OF MOLECULAR REACTIONS TO UNDERSTAND PHOSPHORUS REMOVAL IN A WASTEWATER TREATMENT PLANT

by

Yuge Bai

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Approved: _____
Deb P. Jaisi, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
D. Janine Sherrier, Ph.D.
Chair of the Department of Plant and Soil Sciences

Approved: _____
Mark Rieger, Ph.D.
Dean of the College of Agricultural and Natural Resources

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

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ABSTRACT

Polyphosphate (poly-P) is a ubiquitous long-chain phosphorous (P) compound that has many biological functions and plays an important role in the environmental P cycling. Poly-P responds strongly to oxic-anoxic conditions and P availability, however its synthesis and degradation mechanisms remain largely understudied. This research investigated the mechanisms of poly-P cycling at different scales from enzyme- substrate reaction to bacterial cell culture and to field study in a wastewater treatment plant (WWTP). It included measurements of P speciation [orthophosphate (PO_4) and poly-P], enzyme activity, microbial gene expression, and phosphate oxygen isotope ratios ($\delta^{18}O_P$). Enzyme reaction results show that both acidic phosphatase and alkaline phosphatase enzymes are capable of catalyzing poly-P degradation, with contrasting efficiency of > 70% and < 18%, respectively. Isotope fractionation factors during enzymatic degradation of poly-P varied from +1.63‰ to +4.39‰, a positive fractionation factor, which is uncommon and hence distinct from degradation of many other organic P compounds. Results from bacterial incubations (*Escherichia coli* JM103 and *Pseudomonas putida* KT2440) suggest that poly-P synthesis and degradation is strongly associated with cell growth stage: poly-P is synthesized during exponential growth, causing an apparent isotope fractionation (~+5‰) in the residual PO_4 in *E. coli* incubation. Degradation of poly-P in late stationary phase, however,

leads to a lighter $\delta^{18}O_P$ values. Poly-P cycling in WWTP responded strongly to variations in dissolved oxygen concentrations: under oxic condition high amount of poly-P suggest poly-P synthesis, while the lighter $\delta^{18}O_P$ values suggest rapid microbial P cycling potential via organic matter degradation. Under anoxic condition, degradation of poly-P was accompanied by continued assimilation of PO_4, suggested by decrease in its concentration and heavier $\delta^{18}O_P$ values. Overall these findings indicate towards alternative poly-P cycling mechanism in the WWTP in this study that some microorganisms may continue to take up PO_4 under anoxic condition even though degradation of poly-P and release of PO_4 are otherwise common processes under this condition.

Chapter 1

INTRODUCTION

1.1 Polyphosphate (poly-P) and Its Cycling

Polyphosphate (poly-P) is a long-chain linear polymer containing three to several hundred of orthophosphate (PO_4) structure units linked by sharing oxygen atoms via high-energy phosphoanhydride bond (Figure 1). It was first isolated from yeast (Lieberman, 1890) and later detected by staining with basic blue dye under a microscope (Meyer et al., 1904). However, the stained granules had been mistaken for nucleic acids for many years until the advent of electron microscopy. Under an electron beam, poly-P granules were seen to be highly refractive but they disappeared quickly. They were then named as poly-P (Wiame, 1947). Since its identification, poly-P has been found ubiquitously, in almost all living organisms—bacteria, archaea, fungi, protozoa, plants, and animals (Kulave, 1979). It has been found to be closely associated with many biological functions including metal chelation, buffering against alkaline pH and more importantly, as a reservoir of energy and phosphorus (P) (Kornberg, 1995).

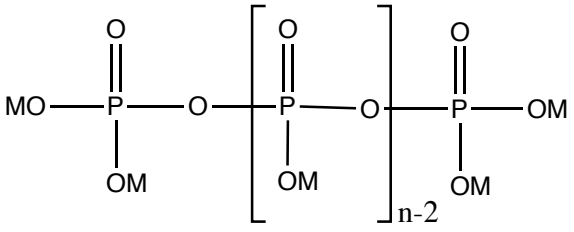

Figure 1 Structure of poly-P (of chain length *n*). M represents a hydrogen or a monovalent metal cation.

Microorganisms form poly-P as a source of P and energy under favorable conditions, such as P sufficient and oxic conditions. Poly-P can be degraded to release PO_4 or to form ATP at any time, but particularly during P starvation or oxygen deficit conditions (Mudd at al., 1958; Kulaev and Vagabov, 1983; Kornberg, 1995). The P-storage strategy is evidenced by the observation of the strong dependence of poly-P content inside microorganisms on the PO_4 amount in cell culture (Nesmeyanova et al., 1974). It has been suggested that poly-P is an ideal and convenient compound for a large amount of PO_4 and energy storage in a cell, because the accumulation of poly-P has little effect on the osmotic pressure within the cells (Harold, 1966). On the other hand, poly-P helps maintaining the concentration of free PO_4 in cells at a relatively stable level, which is critical to many cell biochemical processes. On the other hand, it is also increasingly realized that poly-P plays a significant role in biogeochemical cycling of P in aquatic ecosystems (e.g., Benitez-Nelson, 2000; Hupfer et al., 2004). For example, poly-P synthesis and degradation by microorganisms under different conditions maintains an appreciable proportion of bioavailable P for primary production, especially for low-P aquatic environments (Martin et al., 2014).

1.2 Enzymatic Degradation of Poly-P

Mechanisms of poly-P metabolism in microorganisms have been investigated for past several decades given its crucial role in cell functioning and importance in P cycling in the environment (Kornberg, 1957a,b; Kulaev and Bobyk, 1971). Biochemical reactions involving poly-P are predominately catalyzed by enzymes. Therefore the enzymes associated with poly-P synthesis and degradation have received scientific attention in the past. Many enzymes have been shown to respond to variations in concentrations of poly-P and PO_4 (Nemeyanova et al., 1974a). For

example, in an *E. coli* study (Figure 2), the activity of alkaline phosphatase (APase, EC 3.1.3.1) increases with the increasing concentration of intracellular PO_4, indicating its ability to break down poly-P. Alkaline phosphatase is one of the most common enzymes in natural environment and is capable of catalyzing degradation of many phosphomonoester compounds (Torriani-Gorini et al., 1994). However, its capability for degrading poly-P and mechanism of degradation is not well known. Acidic phosphatase (EC 3.1.3.2), another important extracellular enzyme, is well known for its ability to hydrolyze the P-O bond of phosphomonoesters as well (Shaw, 1966; Eivazi and Tabatabai, 1977), but is not known for its capability and mechanisms in poly-P degradation.

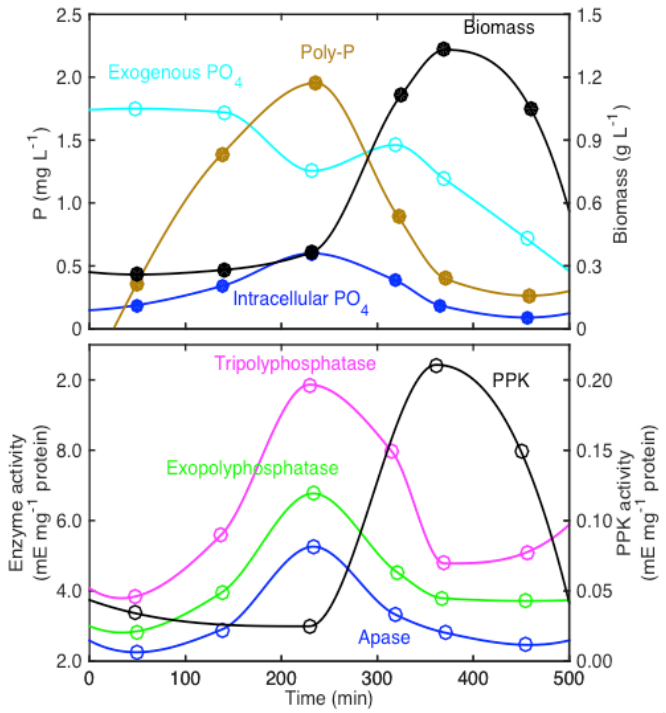

Figure 2 Poly-P concentration and activities of poly-P-metabolizing enzymes [exopolyphosphatase, tripolyphosphatase, alkaline phosphatase (APase), and polyphosphate kinase (PPK)] during growth of *E. coli* K-12 (re-ploted from Nesmeyanova et al., 1973).

1.3 Mechanism of Microbial Poly-P Synthesis and Degradation in A Wastewater Treatment Plant (WWTP)

Microbial poly-P synthesis and degradation have been leveraged in wastewater treatment plants (WWTP) for P removal to achieve nutrient loads reduction specified by environmental/regulatory standards in the effluent water (Levin et al., 1965). Activated sludge in WWTPs is designed to support the growth of poly-P accumulating organisms (PAO), either engineered or native, to remove P. Metabolic cycling of poly-P in PAOs is induced by alternating the incubation conditions of the bioreactor between carbon-rich, strictly anoxic and carbon-poor, oxic conditions, as shown in Figure 3.

Under anaerobic conditions, poly-P accumulated by PAOs is broken down to provide energy required to deplete organic matter from the wastewater and the energy is stored in biopolymers, mainly poly-β-hydroxyalkanoates (PHA) and glycogen (Figure 3; Deinema et al., 1980). When the bioreactor is changed to oxic, PHA serves as energy and carbon sources for PAOs to assimilation large amounts of orthophosphate and synthesize poly-P. This reaction results in lowering PO_4 concentrations in the effluent wastewater. This alternating oxic and anoxic cycling is commonly known as the enhanced biological P removal (EBPR). Over the years, several research and development efforts have been directed towards identifying (micro)organisms or microcosms that are more efficient for EBPR process as well as other abiotic and structural (design) conditions conducive for higher P removal (Mino et al., 1998). Many studies have focused on pairing microbial communities and their occurrence and behaviors in an EBPR system; other studies have focused on improving the engineering designs of the EBPR system, particularly on varying dissolved oxygen concentrations (Wilen and Balmer, 1998; DeMoyer et al., 2003).

However, systematic studies on mechanistic aspect of poly-P formation and cycling processes in EBPR systems are still limited. Moreover, understanding of processes and mechanisms of poly-P synthesis and degradation in WWTP could also provide useful insights to the roles of poly-P in overall P cycling in the natural environment.

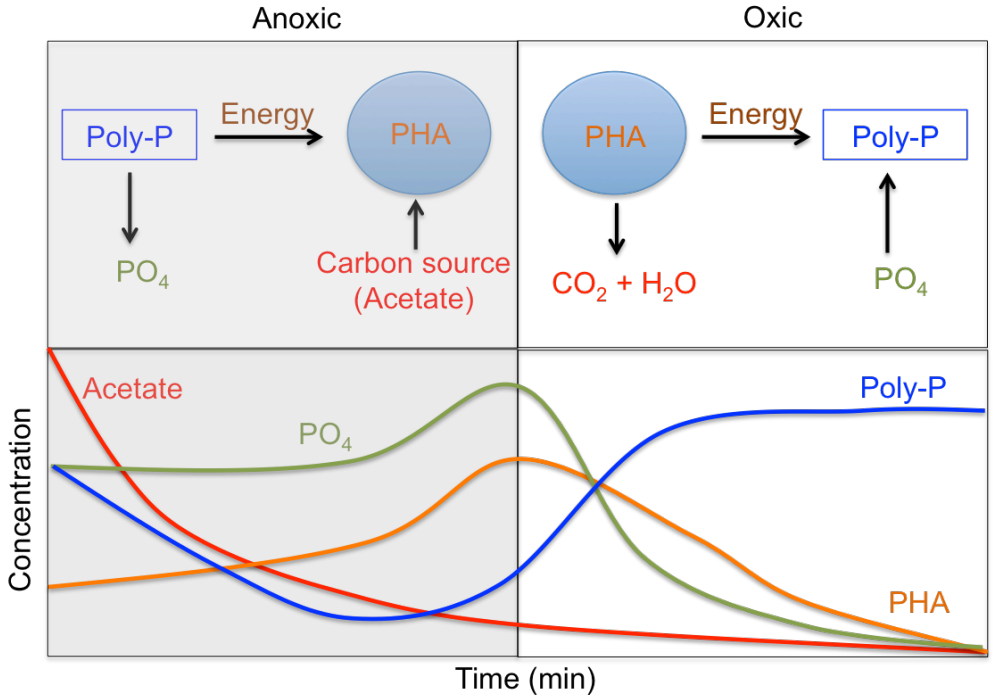

Figure 3 Trends of PHA, PO_4, poly-P, and acetate concentrations during alternating anoxic and oxic cycles in EBPR process (re-drawn from Forbes et al., 2009).

1.4 Phosphate Oxygen Isotope Ratios as A Tracer for P Cycling

P is strongly bound to oxygen and present as orthophosphate (PO_4) in most environments. The four oxygen atoms surrounding the central P atom in PO_4 have three stable isotopes, namely ^{16}O, ^{17}O, and ^{18}O. The distribution of two oxygen

isotopes (^{16}O and ^{18}O) in PO_4, i.e. phosphate oxygen isotopic ratios ($\delta^{18}O_P$), provides a potential means to trace P cycling in the natural environment (Jaisi and Blake 2014; Paytan and McLaughlin, 2011). Application of phosphate oxygen isotope ratios as a P tracer is preferred because of the specific properties of this isotopes and distinct fractionations caused during different processes and reactions. The P-O bond in PO_4 is very strong and remains intact during abiotic processes and reactions under normal environmental temperature (< 70 °C), pH, and pressure conditions (O'Neil et al., 2003). Therefore phosphate carries isotope signatures of its sources even after having undergone various abiotic reactions such as sorption, precipitation, and transport (Liang and Blake, 2006; Jaisi et al., 2010; Jaisi, 2013). This enables the use of $\delta^{18}O_P$ as a tracer of P sources in the environment (Blake et al., 2005; Jaisi et al., 2010, 2014; Jaisi, 2013).

Biological processes have large effects on $\delta^{18}O_P$ values. Isotopic fractionation in biological reactions occurs due to the enzyme-catalyzed oxygen isotope exchange between phosphate and water (Blake et al., 1997). Among the many phosphohydrolase enzymes that catalyze isotope exchange, pyrophosphatase (PPase) behaves differently: inside a cell, PPase catalyzes the reversible oxygen isotope exchange between PO_4 and water, which promotes wholesale isotope exchange and thus results in equilibrium isotope fractionation. The temperature-dependent equilibrium isotope fractionation was empirically established by Longinelli and Nuti (1973) from bioapatite minerals. Most recently, following a series of research on pure enzyme and isotope exchange reaction (e.g., Blake et al., 1998, 2005) a revised equilibrium equation was developed by Chang and Blake (2015) for better description of isotopic fractionation between water and dissolved PO_4. Equilibrium fractionation has been observed in many

environments, including tissues of many multicellular organisms, bacteria, and algae (Kolodny et al., 1983; Blake et al. 1997, 2005; Paytan et al., 2002; Paytan and McLaughlin, 2011). To the other hand, many extracellular enzymes, such as alkaline phosphatase (APase), catalyze unidirectional reaction (break down of P compounds) and cause kinetic isotope effects (Blake et al., 2005; Jaisi and Blake, 2011). APase catalyzed hydrolysis of phosphomonoesters, for example, is described in Figure 4: the PO_4 released from degradation of a phosphomonoester has one oxygen atom incorporated from water. Since the isotopically lighter water is preferentially incorporated, it introduces isotope fractionation. Still three oxygen atoms are inherited from the original phosphomonoester compound. Therefore the isotope composition ($\delta^{18}O_P$) of released PO_4 can be expressed as

$$\delta^{18}O_P = 0.75\ \delta^{18}O_{PO4\text{-}R} + 0.25\ (\delta^{18}O_W + F) \qquad \text{Eq. 1}$$

where $\delta^{18}O_P$, $\delta^{18}O_{PO4\text{-}R}$, and $\delta^{18}O_W$ are oxygen isotope values of released PO_4, original phosphomonoester, and water, respectively; and F is the fractionation factor.

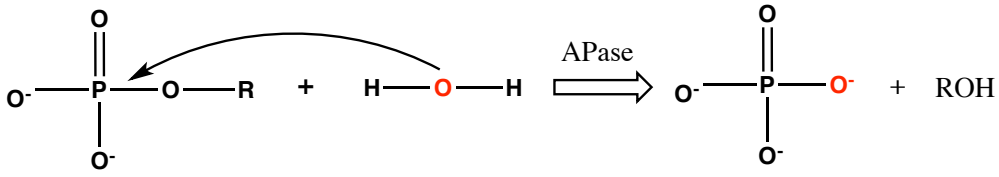

Figure 4 Mechanism of phosphomonoester hydrolysis catalyzed by alkaline phosphatase (Liang and Blake, 2009).

Isotopic fractionation factors are enzyme- and substrate- specific (Liang and Blake 2006a, 2009). So far, isotopic fractionations for organic P compounds (P_o) degradation have been studied on several P_o compounds including P-diesters (RNA

and DNA) and monoesters (glucose-1 phosphate, glycerol-phosphate, phytate, and 5'-nucleotide) (Blake et al., 1997, 1998; Liang and Blake, 2006a,b, 2009; von Sperber et al., 2014; Wu et al., 2015). These distinct fractionation factors have provided basis for interpretation of isotope data obtained from natural environments (Colman et al., 2005; Paytan and McLaughlin, 2011; Joshi et al., 2015). This study will determine the isotopic fractionation for enzymatic degradations of poly-P, and apply phosphate oxygen isotopes to understand poly-P cycling mechanisms in WWTP.

1.5 Study Site: Kent County Regional Wastewater Treatment Facility

Kent County Regional Wastewater Treatment Facility (KCRWTF), located in Milford, Delaware, was chosen for this research. This facility has been operating since 1973, with several modification since then particularly on the enhanced biologically phosphate removal (EBPR). At the current capacity, KCRWTF has an average daily flow of 12.0 million gallon and serves a population of about 130,000 in Kent County. It is equipped with two 10 million gallon Parkson Biolac® activated sludge in the aeration basins (Fig. 5) with a depth of 16 feet to remove P in wastewater. Airflow distribution through the oscillating aeration bars in this basin is automatically controlled and this movement is designated to create moving waves of multiple oxic and anoxic zones (Demoyer et al., 2003). The aeration condition in this basin is often changed in every 2.5 hours, but this timing may change depending on the season and wastewater load.

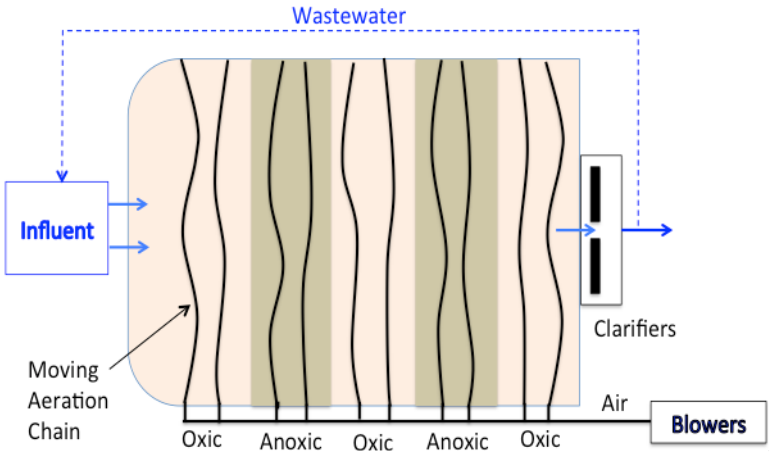

Figure 5 Diagram of Parkson Biolac® aeration basin in KCRWTF.

Research Objectives

This research was organized in an increasing scale from molecular reactions (enzyme- substrate experiments) to cell scale processes (cell- substrate experiments), and finally to ecosystem scale in a wastewater treatment plant. The major research objectives are summarized as follow:

i) To determine the phosphate oxygen isotope fractionation during enzymatic degradation of poly-P.

ii) To investigate the feasibility of using $\delta^{18}O_P$ as a tracer to identify poly-P cycling, specifically the synthesis and degradation of poly-P by microorganisms in environmentally relevant conditions.

iii) Understand mechanisms of poly-P synthesis and degradation under different redox conditions in activated sludge and wastewater from Kent County Regional Wastewater Treatment Facility (KCRWTF).

Chapter 2

MATERIAL AND METHODS

2.1 Determination of Oxygen Isotopic Fractionation During Enzymatic Degradation of Poly-P

To determine oxygen isotopic fractionation during degradation of poly-P, sodium polyphosphate (with an average chain length of 5) from Sigma Aldrich was incubated with acidic phosphatase (extracted from wheat and potato) and alkaline phosphatase (extracted from *E.coli*) enzymes. Experiments were conducted in a series of ^{18}O labeled waters to identify oxygen incorporation during the degradation. Isotopic composition of PO_4 released from poly-P degradation was measured to calculate isotope fractionation factors. The details of experimental and analytical methods are described below.

2.1.1 Enzymatic Degradation of Poly-P

Poly-P was incubated separately with acidic and alkaline phosphatase enzymes. The enzyme assays for two acidic phosphatase enzymes (from wheat and potato) and one alkaline phosphatase enzyme (from *E.coli*) consisted of 1.5 mM of poly-P and 2 UN enzyme (1 UN represents the activity required to generate 1 μmol of PO_4 per minute). Incubations were conducted in 15 mL centrifuge tubes with a total volume of 10 mL. For acidic phosphatase enzymes (from wheat and potato), incubations were conducted in citrate buffer (pH 4.8) with 0.9 mM $MgCl_2$ used as a metal cofactor, following the manufacturer's protocol. Similar assays with alkaline

phosphatase from *E.coli* were conducted in glycine buffer at pH 10.4, using Zn and Mg (0.97 mM $ZnCl_2$ and 0.90 mM $MgCl_2$) as metal cofactors (Liang and Blake, 2006; von Sperber et al., 2013). All reagents were prepared in double deionized (DDI) water.

Degradation experiments were carried out in a temperature-controlled incubator (at 4, 21, and 37 ºC) with ^{18}O labeled waters of different $\delta^{18}O_W$ values (-7.0 to 15.0‰ VSMOW). The concentrations of released PO_4 in all treatments were measured using colorimetric (phosphomolybdate blue) method (Murphy and Riley, 1962). Experiments were continued until PO_4 concentration became constant. Experiment was considered to be completed when spiking fresh enzymes did not cause additional increase in poly-P degradation. Reactions in each subsample taken at different time points were terminated by adjusting the pH lower than 5 using 1.0 M hydrochloric acid (HCl) (Liang and Blake, 2006). After centrifugation (to remove enzyme residues), PO_4 generated was quantified (as above) and samples were further processed for isotope analyses.

2.1.2 Processing and Purification of Samples Prior to Isotopic Measurements

To purify samples collected from degradation experiments to measure $\delta^{18}O_P$ values, all other compounds/ions containing oxygen, besides dissolved PO_4, have to be removed. Residual poly-P was removed by an anion resin exchange, following the method described in Jolley et al. (1998). Briefly, PO_4 and poly-P were separated by anion exchange chromatography using Dowex 1-X8 resin (100–200 mesh, Cl^- form). Fifty mL 0.2 M KCl/acetate buffer (pH 4.5) solution was eluted for every 100 μmol PO_4. Recoveries of the separation process ranged 86 to 99% (average ~ 93%). After removal of poly-P, samples were further purified using methods adapted from Liang and Blake (2006). In brief, a series of precipitation and dissolution steps were run to

remove residual enzymes and associated contaminants. First, precipitation and separation of APM (by 0.1 μm polysulfone filter, Pall Scientific) was used to remove ions and contaminants that are soluble at low pH. APM was rinsed (with 5 % ammonium nitrate) and dissolved in citrate solution before MAP precipitation, which removes ions soluble at high pH. The separated MAP precipitates (by filtration as above) was rinsed (5 % ammonium hydroxide) and adjusted to neutral pH before passing through a cation exchange column (packed with AG50W-X8 resin, BioRad) for removing residual cations (primarily Mg^{2+} and NH_4^+). Finally, silver amine solution was added in the concentrated PO_4 solution to precipitate silver phosphate. The precipitate was separated and dried (at 110 ^oC for an overnight) before measuring $\delta^{18}O_P$ values.

2.1.3 Measurement of Phosphate Oxygen Isotope Ratios

Silver phosphate powder (250–300 μg) was packed in silver capsules for the determination of $\delta^{18}O_P$ values using a Thermo-Chemolysis Elemental Analyzer (TC/EA) coupled to a Delta V continuous flow isotope ratio monitoring mass spectrometer (IRMS, Thermo-Finnigan; precision of 0.3 ‰) at the University of Delaware. For each sample, three replicates were analyzed. Measured isotope values were calibrated against two internal isotope standards, YR-1a and YR-3-2 with $\delta^{18}O_P$ values of −5.49 and +33.63‰, respectively. All measured oxygen isotope compositions are reported relative to the Vienna standard mean oceanic water (VSMOW) standard in the unit of per mil (‰).

2.1.4 Water Oxygen Isotope Ratios and Isotopic Equilibrium Values

Water oxygen isotope ratios ($\delta^{18}O_w$) were measured using a Finnigan™ GasBench II coupled with IRMS. About 200 μL of water samples were injected into pre-flushed (with 300 PPM of CO_2) Labco Exetainer vials (12 mL) to allow CO_2–H_2O equilibrium for >24 hours at 26 °C. After complete equilibrium, small amount of CO_2 in the headspace of the sample vials was introduced into IRMS and measured for its $^{18}O/^{16}O$ ratios. The $\delta^{18}O_w$ values were calibrated against two USGS standards [W67400 (-1.97‰) and W32615 (-9.25 ‰)]. Typical precision for replicate standards was less than 0.06 ‰. Measured $\delta^{18}O_w$ values are reported following standard delta notation relative to VSMOW, as in PO_4.

The isotopic equilibrium values ($\delta^{18}O_{Eq}$) expected for wholesale exchange of oxygen between phosphate and ambient water, catalyzed by pyrophosphates can be determined using phosphate- water fractionation equations. In this study we used both the commonly used equation derived from observed O-isotope fractionations between PO_4 and water in biogenic phosphate minerals (Longinelli and Nuti, 1973)

$$T\ (°C) = 111.4 - 4.3\ (\delta^{18}O_{Eq} - \delta^{18}O_w) \qquad \text{Eq. 2}$$

and the equation for equilibrium O-isotope fractionations between dissolved PO_4 and water (fractionation factor $\alpha_{(PO_4 - H_2O)}$) (Chang and Blake, 2015):

$$1000 \ln \alpha_{(PO_4 - H_2O)} = 14.43 \bullet 1000 / T\ (K) - 26.54 \qquad \text{Eq. 3}$$

2.1.5 Calculations of Isotopic Fractionation Factors

Isotopic fractionation factor (F) was calculated using the equation developed by Liang and Blake (2006) from the measured $\delta^{18}O_P$ values of PO_4 released from enzymatic degradations of poly-P as:

$$\delta^{18}O_P = a\ (\delta^{18}O_{water} + F) + (1-a)\ (\delta^{18}O_{Poly\text{-}P}) \qquad \text{Eq. 4}$$

where $\delta^{18}O_w$, and $\delta^{18}O_{Poly\text{-}P}$ are the oxygen isotope values of labeled water and poly-P respectively; *a* is the fraction of O incorporated from water and (1-*a*) is the fraction of O inherited from poly-P. A linear relationship is expected between $\delta^{18}O_P$ and $\delta^{18}O_w$. The slope and intercept of the fitted line allow calculating fractionation factor (F) from the predetermined values of $\delta^{18}O_{poly\text{-}P}$, which was measured directly in IRMS after pyrolyzed at TC/EA.

2.2 Application of Phosphate Oxygen Isotope Composition to Examine Poly-P Synthesis and Degradation in Bacterial Culture

Poly-P metabolism in microorganisms is complex because of the variable synthesis, transformation, and degradation of poly-P catalyzed by a multitude of enzymes (Kortstee et al., 2000). As a result, pathways of poly-P synthesis and degradation in bacteria are still uncertain. In this study, poly-P synthesis and degradation experiments were conducted using two bacteria, *Escherichia coli* JM103 (*E. coli* JM103) and *Pseudomonas putida* KT2440 (*P. putida* KT2440) under aerobic conditions. Both bacteria are known to accumulate intracellular poly-P. Cell growth and PO_4 concentrations in the culture media, poly-P concentrations both inside and outside of cells, activities of alkaline and acidic phosphatase enzymes, and water and $\delta^{18}O_P$ values in the media were measured during incubation. Details of the experimental set up and measurements are described as below:

2.2.1 Bacteria Cultures

E. coli JM103 and *P. putida* KT2440 were grown aerobically at 30 °C in the mineral salt media which contained ferric ammonium citrate, KCl, $CaCl_2$, NH_4Cl, KH_2PO_4, vitamins, and glucose as a carbon source (Kulakova et al., 2011). Cell growth was monitored using turbidity at 440 nm (Stout et al., 2014). Water oxygen

isotope ratios ($\delta^{18}O_w$) of the media were also monitored to track changes during incubation due to water-O incorporation into released PO_4 from poly-P.

2.2.2 Poly-P Quantification

Quantification of poly-P was conducted by using the DAPI (4'6-diamidino-2-phenylindole) fluorescence method (Aschar-Sobbi et al., 2008; Diaz and Ingall, 2010). The binding of poly-P to DAPI shifts its emission wavelength peak from 475 nm to 525 nm and the fluorescence intensity at this shifted wavelength is proportional to the concentration of polyphosphate (Tijssen et al., 1982). DAPI stock was prepared in double deionized water at a stock concentration of 1 mM and stored frozen (in 1 mL aliquots) in dark to minimize photodegradation. At the time of measurement, DAPI reagent was diluted to 100 μM and samples from experiments were incubated with DAPI in 20 mM HEPES (4-(2-hydroxyethyl)-1- piperazineethane-sulfonic acid) buffer under ambient light for 7-8 min to obtain a stable fluorescence (Aschar-Sobbi et al., 2008). To ensure thorough mixing of samples with DAPI reagent and homogenous fluorescence color development, the vials were vortexed three times during incubation. After the completion of reaction, an aliquot of sample was transferred to black 96-well plate for fluorescence measurement in Spectramax M2 spectrophotometer. The fluorescence was then measured at 550 nm illumination wavelength and 415 nm excitation wavelength following the standard procedure (Aschar-Sobbi et al., 2008).

2.2.3 Poly-P Extraction

Poly-P was extracted and purified from *E. coli* JM103 and *P. putida* KT2440 cultures during their different growth stages using the extraction protocol developed

by Martin et al. (2012). Briefly 1.5 mM Tris buffer was added to 5 mL of samples in 15 mL centrifuge tubes. After vortexing followed by 15 s sonication, the centrifuge tubes were immersed into boiling water for 5 min. Twenty mg/mL proteinase K was added to a concentration of 0.1 g/L. The samples were then incubated at 37 ºC for 30 min with constant mixing at 300 rpm and vortexing 2 to 3 times during the incubation to achieve complete cell lysis. To separate extracted poly-P, cell residues were separated by centrifugation at 5000 × g for 10 min (poly-P remains in the supernatant). The entire reaction with proteinase K and separation was repeated 3 times on each sample to enhance extraction of poly-P (as the repeated extraction is reported to increase the recovery of poly-P as high as ~99%; Martin et al., 2012).

2.2.4 Measurement of Phosphohydrolase Enzyme Activities

Activity of alkaline phosphatase (APase) was determined using a modified method of Adams et al. (2008). Samples were incubated at 37 °C at pH 8.5 (Tris-HCl buffer) using *p*-nitrophenyl phosphate (*p*NPP) as a substrate. The hydrolyzed product *p*-nitrophenol (*p*NP) were then measured by adsorption at 405 nm. APase activity was calculated as μmol *p*NPP hydrolyzed per hour per liter of solution (μmol h^{-1} L^{-1}). Acidic phosphatase activity was measured analogously using citrate (pH 6.5) buffer.

2.3 Poly-P Cycling Dynamics in The Aeration Basin in The Kent County Regional Wastewater Treatment Facility

In this study, $\delta^{18}O_P$ values were used as a tool to trace poly-P cycling in the wastewater and activated sludge from KCRWTF. Samples (wastewater and suspended activated sludge) were taken every 20 min during the oxic– anoxic cycle in December 2015 and March 2016. Dissolved oxygen (DO) concentration and temperature were monitored on site. Wastewater samples collected were analyzed for PO_4 and poly-P

immediately upon arrival to laboratory at the University of Delaware (see methods described above). A fraction of each sample saved for further processing and analyses was stored at -18 ºC to minimize microbial activity during storage.

Activated sludge in the wastewater was separated by centrifugation at 10,000 RCF for 30 min. After separation, sludge samples (~0.80 g for each sample) was extracted for DNA and analyzed for poly-P accumulating genes at the University of Houston. The rest of the sludge samples were freeze-dried, extracted and analyzed for poly-P (see methods described above). Concentration of PO_4 in these samples were quantified after 0.5 M $NaHCO_3$ extraction for 15 h, followed by washing with double deionized water (2 times) (Hedley et al., 1982). Wastewater samples (supernatants after centrifugation) were processed to remove poly-P using the anion resin method described above and then to reduce volume by evaporating at 60 ^{o}C for two days. Remaining PO_4 in supernatant was further processed for isotope analyses (see methods above). Alkaline and acidic phosphatase enzyme activities were analyzed in both activated sludge and wastewater (supernatant) samples using the methods described above.

2.3.1 Extraction and Quantification of Poly-P Accumulating DNA

Microbial DNA in the activated sludge was extracted using PowerWater DNA Isolation kit (Mo Bio) following the manufacturer's protocol. Concentration of extracted DNA was analyzed using Spectrometer (NanoDrop ND-1000) . Extrated DNA was stored at -20 ^{o}C before genetic analysis. SYBR Green PCR Master Mix was used as primers for the polymerase chain reaction (PCR).

Chapter 3

RESULTS

3.1 Kinetics of Poly-P Degradation With Acidic and Alkaline Phosphatase Enzymes

Results of acidic phosphatase-poly-P incubation experiments performed at 4, 21, and 37 ^{o}C show that the released PO_4 reached its maximum concentration in ~ 2880 min (~2 d) and changed very slightly thereafter (Figures 6 and 7). The efficiency of degradation (ratio of released PO_4 to total P) was > 70% in all experiments. Control experiments did not show significant change in PO_4 concentration. On the other hand, efficiency of PO_4 released by alkaline phosphatase during the degradation of poly-P was low (< 18%; Figure 8). A notable difference in this set of incubation, however, was the high PO_4 at the start of the experiment.

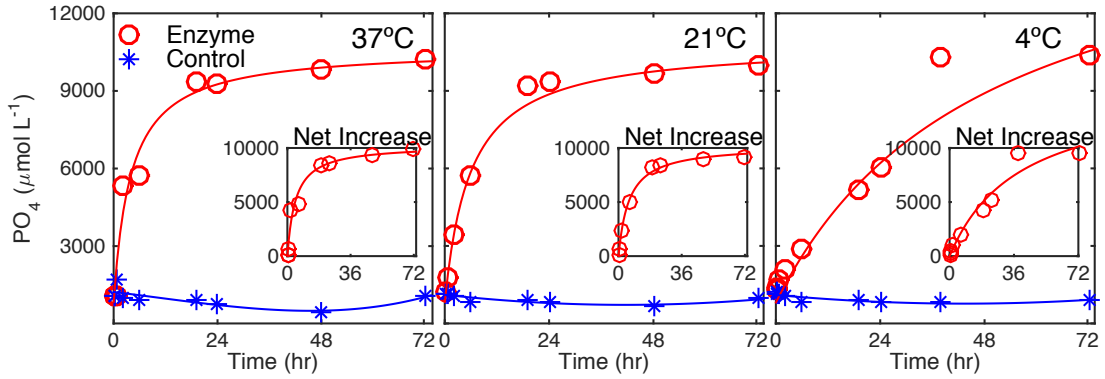

Figure 6 Phosphate released from poly-P degradation by acidic phosphatase enzyme from wheat. Poly-P was incubated under three different temperatures (37 °C, 21 °C, and 4 °C).

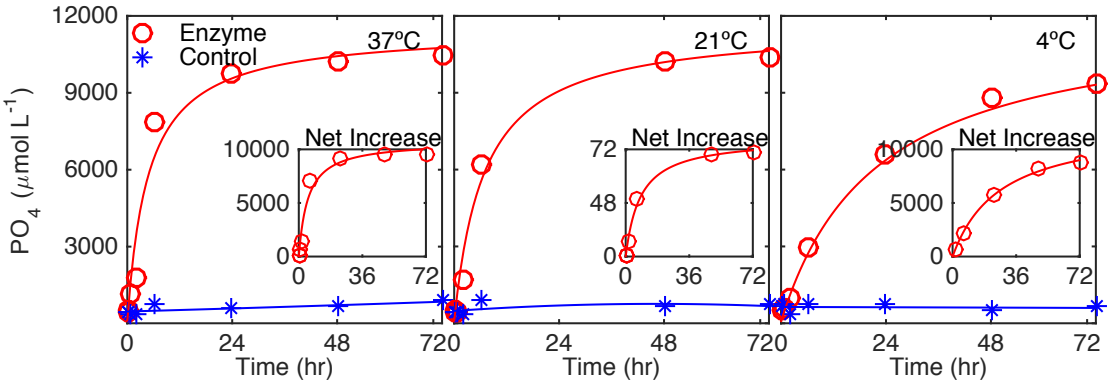

Figure 7 Phosphate released from poly-P degradation by acidic phosphatase enzyme from potato. Poly-P was incubated under three different temperatures (37 °C, 21 °C, and 4 °C).

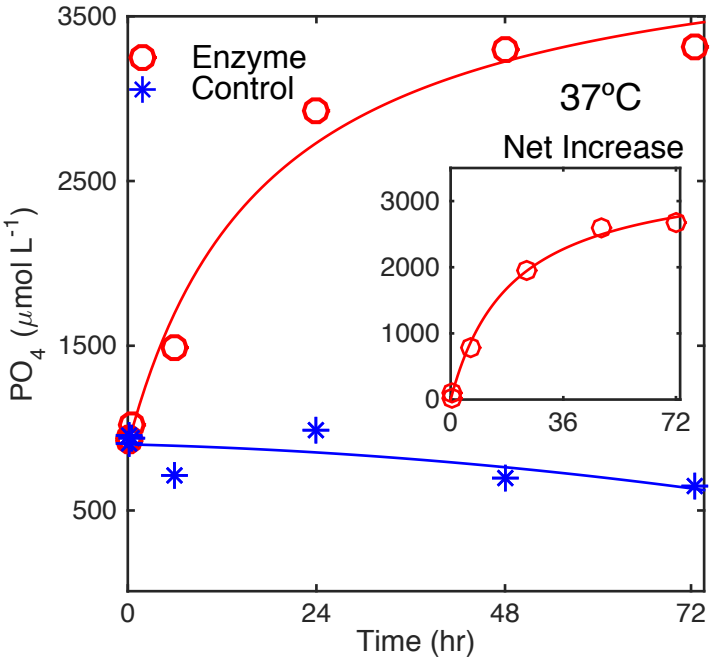

Figure 8 Phosphate released from poly-P degradation by alkaline phosphatase enzyme (from *E. coli*).

3.2 Isotope Fractionation During Enzymatic Degradation of Poly-P

The oxygen isotope ratios of water and PO_4 in the enzyme-substrate experiments are shown in Table 1. As shown, the fractionation factors vary highly in different incubations. Strong linear relationship between the $\delta^{18}O$ values of water and released PO_4 in all experiments (Figure 9), however, indicates that oxygen atom from water was incorporated into the released PO_4. The calculated isotope fractionation factors of poly-P with three enzymes, using equation 4, under different temperatures are shown in Table 1.

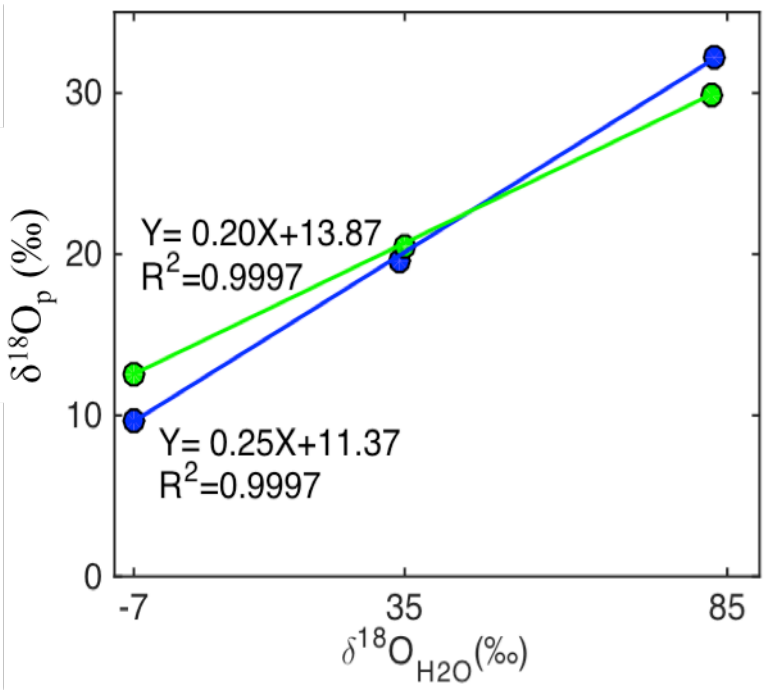

Figure 9 PO_4-H_2O exchange catalyzed by cell-free acidic phosphatase enzyme from wheat (green) and alkaline phosphatase enzyme from *E. coli* (blue) at 37 ^{o}C. The slopes of the linear regression of acidic and alkaline phosphatase enzymes are 0.20 and 0.25, respectively.

Table 1 Isotopic compositions of water ($\delta^{18}O_w$), dissolved phosphate ($\delta^{18}O_P$), substrate poly-P ($\delta^{18}O_{poly\text{-}P}$), and the calculated fractionation factor (F).

Enzyme	T (°C)	$\delta^{18}O_w$ (‰)	$\delta^{18}O_P$ (‰)	$\delta^{18}O_{Poly\text{-}P}$ (‰)	F (‰)	Mean F (‰)
Acidic phosphatase (wheat)	37	-7.02	11.32	16.65	-2.92	
	37	34.94	20.84		7.11	5.01
	37	82.60	31.4		2.92	
	21	-7.00	12.17		1.27	
	21	34.00	18.57		-7.77	-9.62
	21	83.00	25.45		-22.36	
	4	-7.00	12.30		1.89	
	4	34.00	19.90		-1.10	-2.97
	4	83.00	27.98		-9.72	

Acidic phosphatase (potato)	37	-7.02	12.60	16.65	3.40	
	37	34.94	20.46		0.85	1.63
	37	82.46	29.94		0.65	
	21	-7.00	13.31		6.97	
	21	34.00	20.10		-0.10	4.39
	21	83.00	31.18		6.29	
	4	-7.00	12.86		4.68	
	4	34.00	20.71		2.97	2.49
	4	83.00	29.89		-0.17	
Alkaline phosphatase (*E coli*)	37	-7.00	9.73	16.65	2.95	
	37	34.00	19.66		1.65	2.49
	37	83.00	32.21		2.86	

3.3 Poly-P Cycling in *E. coli* JM103 and *P. putida* KT2440

Cell growth, PO_4 concentrations, poly-P, activities of alkaline phosphatase and acidic phosphatase enzymes, and $\delta^{18}O_P$ values of PO_4 in the *E. coli* and *P. putida* culture media are shown in Figures 10 and 11. For *E. coli* JM103, during the first set of experiments, cells reached stationary growth phase after 20 h of incubation and the cells concentration started to decrease after 24 h (Figures 10A and 11A). However, *E. coli* JM103 grew much slower during the second experiment, the cell growth reached stationary phase after 30 h of incubation. Corresponding to the cell growth of *E. coli* JM103, PO_4 concentration decreased from 570 μM to 510 μM and 800 μM to 350 μM respectively (Figures 10C and 11C). However, during the incubation of second set of experiment, PO_4 concentration recovered ~200 μM after 24 h. In both experiment, poly-P concentration in *E. coli* JM103 cells increased the first 10 h, but when cell growth pass the stationary phase, poly-P concentrations in both cells and medium appeared to decrease (Figures 10C and 11C). The activity of alkaline phosphatase was low at the beginning of *E. coli* JM103 growth, but started to increase after 10 h of incubation when poly-P concentration in medium started to decrease (after stationary

phase; Figure 10E). Acidic phosphatase activity, however, remained low during the 2 d incubation.

For *P. putida* KT2440, cells growth reached stationary phase after 10 and 20 h of incubation, respectively in two experiments, and remained similar until after 30 h (Figures 10B and 11B). With the growth of cells, PO_4 concentrations in media decreased by~ 100 μM in the first 12 h (during exponential growth) in both case, but appeared to increase slightly (by ~15 μM) in the last 24 h of incubation during the first set of experiments (Figure 10D). Concentrations of poly-P in *P. putida* KT2440 cells changed differently in two experiments. In the first set of experiments, it decreased continuously during the 48 h incubation from ~16 μM to 10 μM at the end of the incubation (Figure 10D). Accordingly, poly-P concentration in the media decreased in the first ~24 h of the incubation (exponential growth) but appeared to increase thereafter (Figure 10D). However, during the second set of experiments, poly-P concentration increased in the first 12 h of incubation and then decreased from ~25 μM to less than 5 μM in the last 24 h (Figure 11D). Alkaline phosphatase activity in *P. putida* KT2440 cell culture was generally low compared to that of *E. coli* JM103 (Figure 10F), and but the acidic phosphatase activity was similar to that of *E. coli* JM103 (Figure 10F).

Figures 10G and 10H show $\delta^{18}O_P$ values of dissolved PO_4 in the media. For *E. coli* JM103, isotope values were within the equilibrium in 4 h of incubation, but became heavier dramatically at 8 h of incubation. It was slightly lighter during 8 to 24 h before becoming similar to 4 h at the later growth stage (Figure 10G). However, for *P. putida* KT2440, the $\delta^{18}O_P$ became steadily lighter with increasing incubation time (Figure 10H).

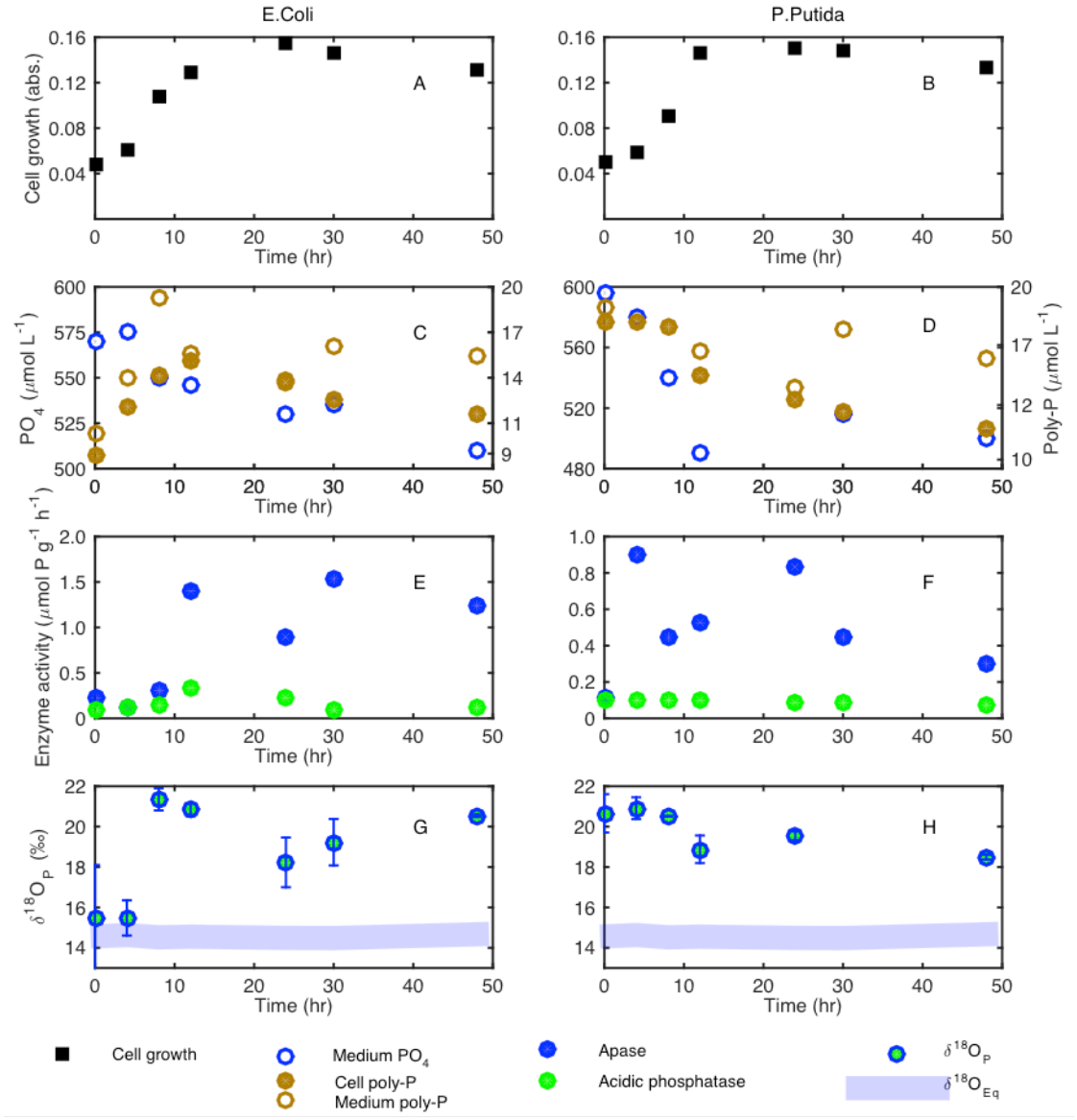

Figure 10 Cell growth of *E. coli* JM103 (A) and *P. putida* KT2440 (B) in mineral salt medium at 37ºC aerobically. Poly-P and PO_4 concentrations in *E. coli* JM103 culture (C) and *P. putida* KT2440 culture (D). Activities of alkaline phosphatase and acidic phosphatase enzymes of *E. coli* JM103 culture (E) and *P. putida* KT2440 culture (F). Phosphate oxygen isotope ratios in *E. coli* JM103 (G) and *P. putida* KT2440 (H) cultures.

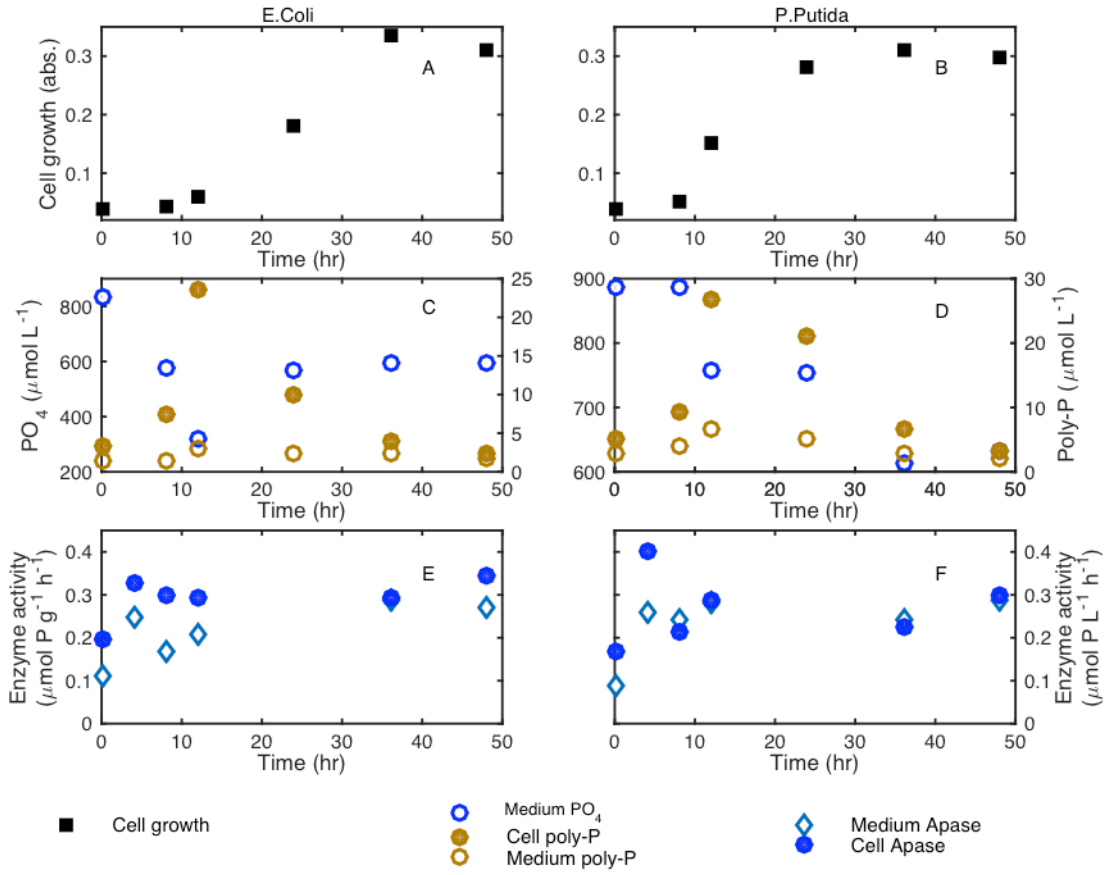

Figure 11 Cell growth of *E. coli* JM103 (A) and *P. putida* KT2440 (B) in mineral salt medium at 30 ºC aerobically. Poly-P and PO_4 concentrations in *E. coli* JM103 culture (C) and *P. putida* KT2440 culture (D). Activities of alkaline phosphatase in medium and cells of *E. coli* JM103 culture (E) and *P. putida* KT2440 culture (F).

3.4 Temperature, pH, and Dissolved Oxygen Dynamics in Wastewater Treatment Plant

Average temperature and pH measured in KCRWTF during the sampling period (from oxic, <150 min to anoxic, > 150 min) are shown in Table 2. Both

temperature and pH did not vary significantly during sampling time. However, there were strong fluctuations in dissolved oxygen (DO) concentrations in the aeration basin (Figure 12). In the first 150 min (aerobic period), the average concentration of DO was 0.85 (±0.63) mg/L between 150 and 300 min (anoxic period), the average DO concentration was 0.096 (±0.11) mg/L.

Table 2 Average temperature, pH, and DO concentration in KCRWTF aeration basin under oxic-anoxic conditions

Date	Aeration Period	**T(°C)**	**pH**	**O_2 (mg/L)**
March 2016	Oxic	17.15	7.09±0.08	0.85±0.63
	Anoxic	17.35	6.94±0.14	0.096±0.11

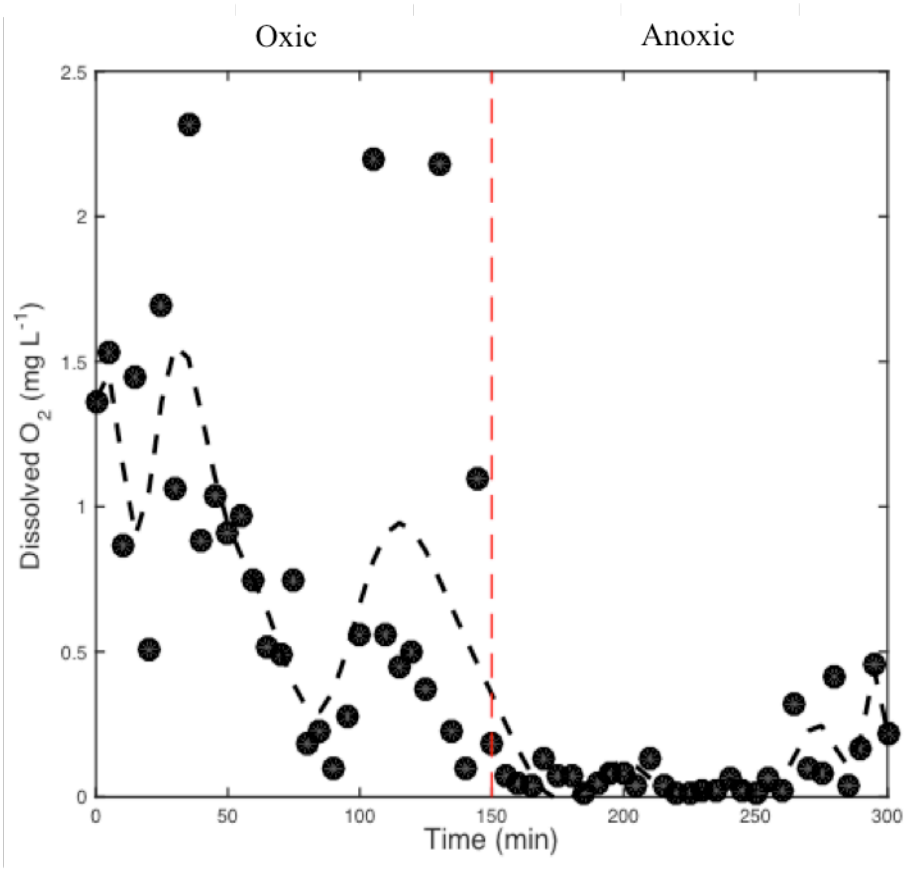

Figure 12 Dissolved oxygen concentration in KCRWTF aeration basin within 300 min (one oxic-anoxic cycle). The pumping of air was stopped at ~150 min (data from March 2016).

3.5 Dissolved PO_4 and Poly-P Concentrations in Wastewater and Sludge From KCRWTF

Concentrations of PO_4 and poly-P responded strongly to the fluctuation of DO concentration (Figure 13). For example, the concentration of poly-P in sludge decreased from ~9 μmol/g to almost zero with the decrease of DO concentration (Figure 13). In contrast, PO_4 concentration increased from ~20 μmol/g to more than 80 μmol/g with the decrease of poly-P concentration (Figure 13). However, in the supernatant water, with the decrease of DO concentration, PO_4 concentration

decreased from more than 1,000 μmol/g to less than 400 μmol/g (Figure 12). Poly-P concentration remained relatively low (< 5 μmol/g) in supernatant water throughout the entire redox oscillation in the aeration basin (Figure 13).

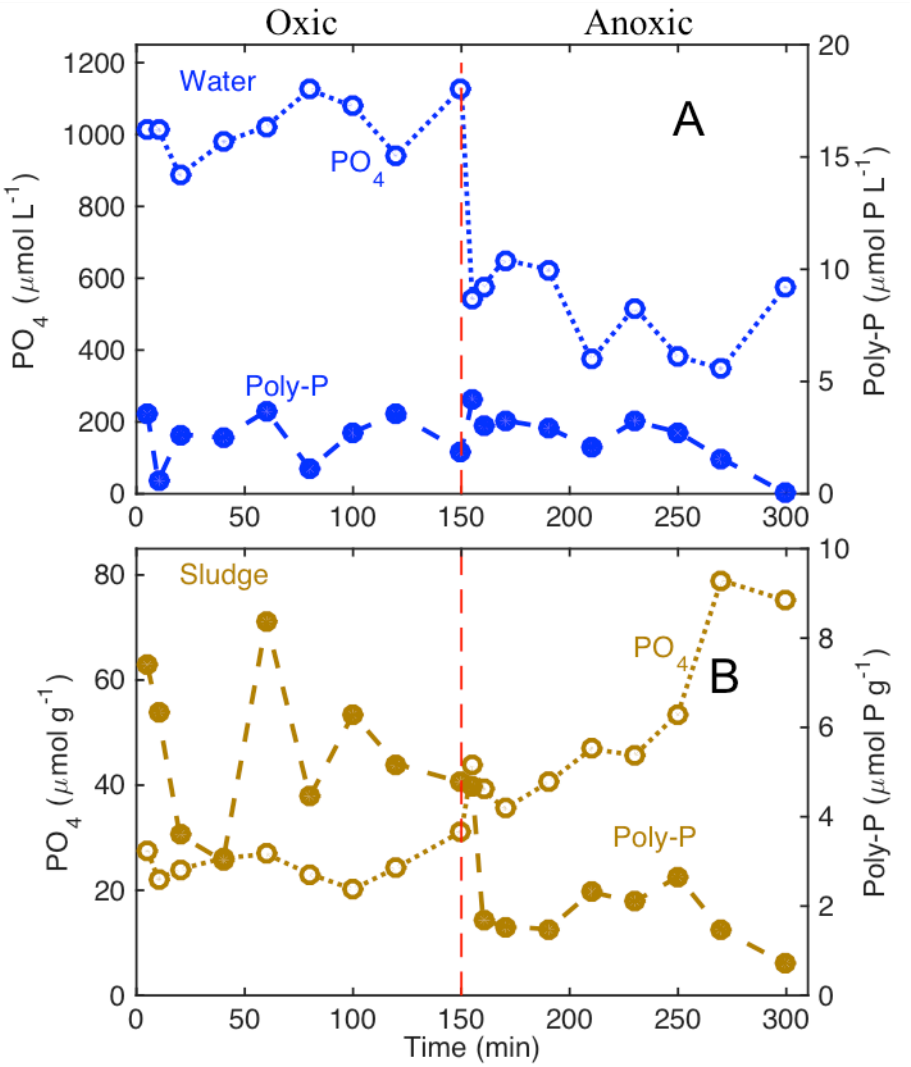

Figure 13 Concentrations of poly-P (open circle) and PO_4 (close circle) in water (A) and sludge (B) in samples collected in March 2016).

3.6 Activities of Alkaline and Acidic Phosphatase Enzymes in Wastewater

Acidic and alkaline phosphatase activities varied strongly with the changes in dissolved oxygen in the aeration basin (Figure 14). Generally activities of both enzymes were higher under anoxic condition (although exhibited large fluctuations at the later stage) than under aerobic condition. In particular, alkaline phosphatase activity responded quickly with the change in DO and the increased its activity by about 3-fold.

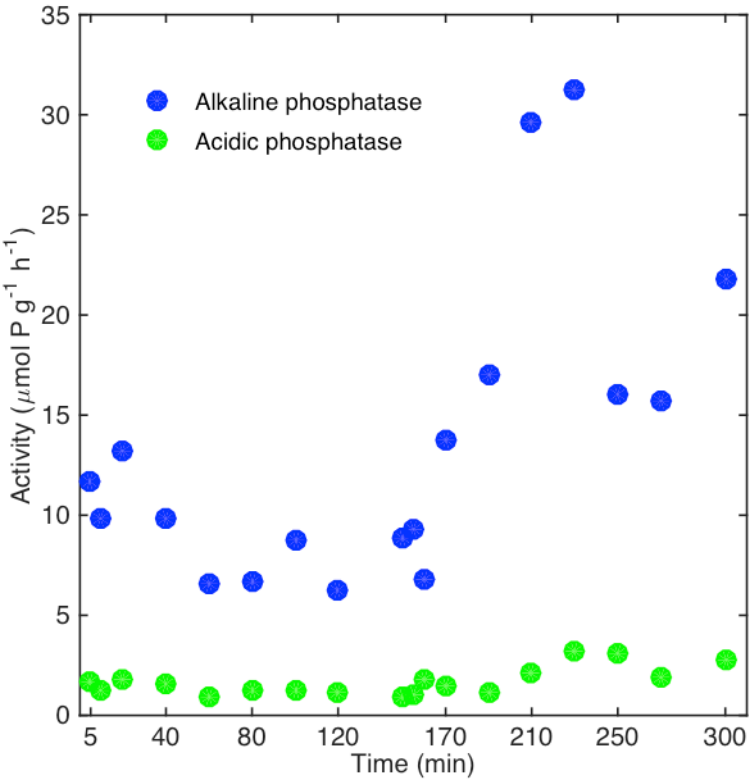

Figure 14 Alkaline (blue) and acidic phosphatase (green) activities in wastewater sludge (March 2016).

3.7 Phosphate Oxygen Isotope Ratios of Orthophosphate in Wastewater

Phosphate oxygen isotope ratios of PO_4 in KCRWTF wastewater and the expected equilibrium values are shown in Figure 15. After starting aeration (0 min), $\delta^{18}O_P$ values became gradually lighter and changed towards equilibrium values. After the onset of anoxic condition (150 min), however, isotope values became heavier dramatically, from ~ 19‰ to ~21‰ and to ~22 ‰ in March and December, respectively (Figure 15).

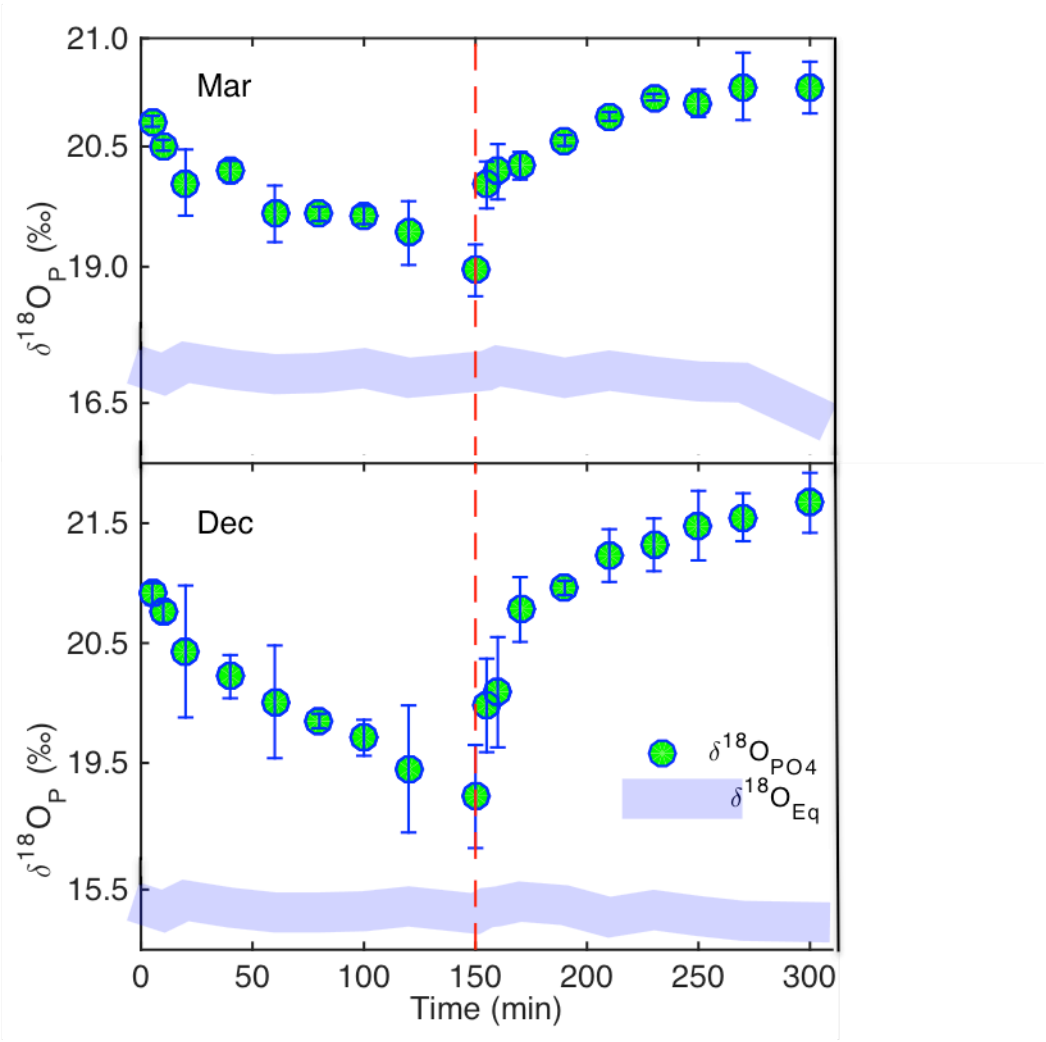

Figure 15 Phosphate oxygen isotope ratios of dissolved PO_4 in the aeration basin of KCRWTF. The vertical red dashed line represents the switching of anoxic condition (by stopping aeration) and the purple light color zone represents the calculated equilibrium values. The upper and lower boundaries of the equilibrium zone is calculated based on equations 2 and 3 after Longinelli and Nuti, (1973) and Chang and Blake (2015), respectively.

3.8 Abundance of Poly-P Accumulating Organisms in KCRWTF

Molecular microbiology analyses of sludge samples performed to identify the abundance of poly-P accumulating organisms (PAOs) expressed as the copies of PAO genes are shown in Figure 16. In general, numbers of poly-P accumulating genes increased during the aeration period (for ~3 folds; Figure 16). Numbers of PAO genes decreased during the first few h during the oxic cycle, however, increased significantly after switching into anoxic condition. The PAO response was not steady− it showed a consistent decline in gene copies before sudden spike in at 5.5 h. Interestingly, this spike occurred corresponding to DO fluctuation.

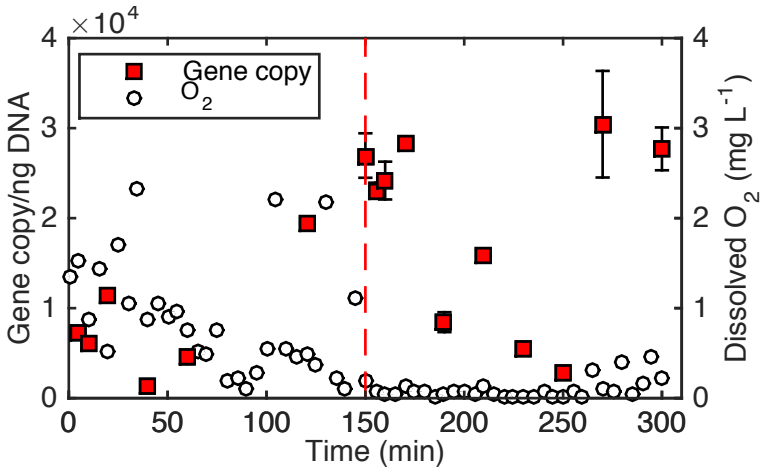

Figure 16 Copies of poly-P accumulating genes in activated sludge collected from KCRWTF.

Chapter 4

DISCUSSION

4.1 Enzymatic Degradation of Poly-P and Isotope Fractionation

Acidic phosphatase enzyme was able to degrade ~70% of poly-P within 48 h, while alkaline phosphatase degraded < 20% of poly-P. This is unexpected because alkaline phosphatase is one of the most common non-specific enzymes present in the natural environment and is well known for breaking down a variety of phosphomonoester compounds (Torriani-Gorini et al., 1994). In order to confirm these data and to exclude influence of other factors that may inhibit the enzyme reaction, several enzyme assays were performed with different concentrations of metal co-factor, pH, and poly-P with different chain lengths. While there were minor fluctuations, none of these factors, however, improved the extent of degradation. Alkaline phosphatase activity was measured by using widely used *p*-NPP substrate, which, however, limits the measurement of inactive enzyme for this particular substrate. It might be speculated that the impurities in the poly-P could be a factor. But the reason for inefficient degradation of poly-P by alkaline phosphatase in this study is not known therefore needs further investigation.

The different degradation efficiency of the two enzymes was also reflected in the $\delta^{18}O_P$ values of the released PO_4, in particular the different slopes of the linear regression between PO_4 and water isotope values (Figure 9, Table 1). The slopes are 0.20 (±0.03) and 0.25 (±0.005) for acidic phosphatase and alkaline phosphatase, respectively. As described in section 3.1 and illustrated in Figure 17, during the cleavage of one P−O bound in a poly-P molecule, one oxygen atom from water is

incorporated into the released PO_4, while the rest three oxygen atoms are inherited from parent poly-P. Therefore, an incomplete degradation of poly-P (without breaking the last P−O bound and leaving the last PO_4 intact), the slope should ideally be 0.25. On the other hand, complete degradation of poly-P will result in a slope less than 0.25 because the last PO_4 moiety to be released has all oxygens inherited from its parent poly-P. The poly-P used in enzyme incubation experiments had an average chain length of ~5. For the low degradation efficiency of alkaline phosphatase (< 20%; Figure 8), all released PO_4 have one water oxygen atom and three oxygen atoms inherited from parent poly-P, leading to a slope of 0.25 (Figure 9). For high poly-P degradation for acidic phosphatase (>70%; Figures 6 and 7), one PO_4 (the last one in the poly-P chain) out of 5 PO_4 (average chain length of 5) inherits oxygen entirely from parent poly-P, which is to say, only 4 out of (4×5 = 20) oxygen in the released PO_4 comes from water, leading to a slope of 0.20. Sodium poly-P used in this study is likely to be impure and contains extra PO_4 (Figures 6-8) and may also contain a mixture of poly-P with a variety of chain lengths. Thus the actual fraction of water oxygen incorporation is difficult to estimate without complete degradation and without knowing the detail chain lengths. This may explain the relatively large variability in the slope and calculated fractionation factors in acidic phosphatase (slopes of 0.20 ± 0.04 and 0.20 ± 0.005 for acidic phosphatase from wheat and from potato, respectively). Because of these uncertainties calculated fractionation factors for acidic phosphatase, especially for acidic phosphatase from wheat, are less reliable.

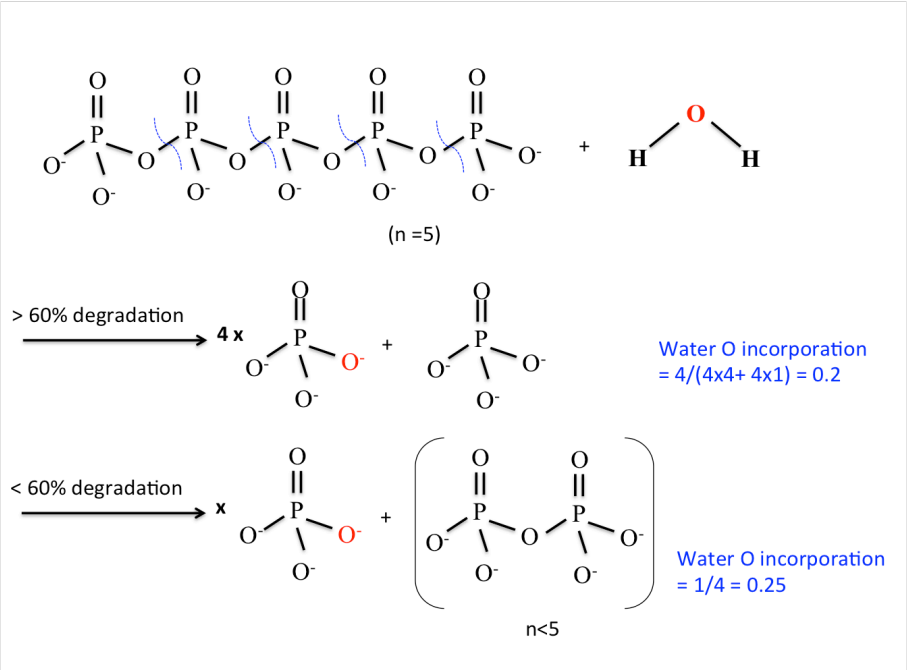

Figure 17 Illustration of variable slopes that originate from complete versus incomplete poly-P degradation.

Isotope fractionation factors for both alkaline phosphatase and acidic phosphatase enzymes from potato are positive (> 0) under all temperatures. This is very intriguing because the fractionation factor during enzymatic degradation of series of organic P compounds is negative (Liang and Blake 2006, 2009; von Sperber et al., 2013). Positive isotope fractionation factor, however, reported to occur in some natural environments (Criss, 1999). It has been also found in RNA degradation catalyzed by alkaline phosphatase, because the second step of the degradation is a reversible reaction that leads to equilibrium fractionation and thus yields a positive fractionation (Liang and Blake 2009). Positive fractionation factor was also observed

in phytate degradation catalyzed by phytase enzyme from wheat and *Aspergillus niger* (Sun et al., 2016). Different $\delta^{18}O_P$ values of the bridging oxygen atom (C−O−P) and the three non-bridging oxygen atoms (O−P) could be the reason for the positive isotope fractionation. As poly-P is an inorganic compound and does not have non-bridging oxygen atoms, this is likely not the case as well. However, the possibility of variable isotopes within a molecule is not expected based on conditions of synthesis and source P. Degradation mechanism of poly-P is still yet clear and the reason for the positive isotope fractionation needs further investigation.

Poly-P has been commonly found in natural environment such as soils and sediments (Hufer et al., 2007),indicating its potential role as an important PO_4 source (Hufer et al., 2007; Martin et al., 2012). The use of phosphate oxygen isotope ratios to identify different organic P compounds degradation reactions in natural systems is often challenged by the overlapping fractionation factors for several enzymes and substrates (range from -10‰ to -30‰; Liang and Blake 2006, 2009; von Sperber et al., 2013). The unique positive fractionation factor is encouraging and may provide opportunities to identify PO_4 originated from poly-P degradation.

4.2 Poly-P Cycling in *E. coli* and *P. Putida* Cultures and Isotope Effects

Poly-P cycling in bacteria cultures has been studied for decades (Rao et al., 2006; Rao and Kornberg, 1995). *E. coli* is the most commonly used bacteria for the study of poly-P cycling because it is easy to grow on most media and it has the ability to accumulate poly-P (Kornberg et al., 1956; Kornberg, 1957; Kornberg, 1957; Kulaev et al., 1971). Results from this study suggest that the growth of *E. coli* JM103 was accompanied by assimilation of PO_4 (its media concentration decreased by ~ 60 μM

after 48 h of incubation). Correspondingly, concentration of poly-P in both the media and in cells increased during the exponential growth phase in the first 12-20 h. However poly-P started to decrease once the cell growth reached late stationary phase (~30 h). This dynamics in poly-P and PO_4 suggest that *E. coli* assimilates PO_4 from surrounding environments to synthesize poly-P. But when the growth environment becomes more stressful (late stationary phase), poly-P starts to degrade. Interestingly, during the first set of experiments, with the decrease of poly-P concentration in the late stationary phase, PO_4 concentration did not increase, but decreased by ~20 μM at the end of incubation (Figure 11C). This indicates that, perhaps in some cases, degradation of poly-P during the stressful late stationary phase does not release significant amount of PO_4 from cells. In contrast, the cells continue to assimilate PO_4 from the media (Figure 10C). However, it remains unclear the reason for variable PO_4 dynamics in *E. coli* JM103 culture in two sets of experiments. One possible reason is the different cell number during the inoculation step. It points towards the possibility of variable growth conditions of starting culture or the metabolic status of cells between two sets. Nonetheless, these data suggest *E. coli* assimilates PO_4 and synthesize poly-P for storage of P and energy when the growth condition is favorable. When the surrounding environment becomes stressful, *E. coli* breaks down poly-P while it continues to assimilate PO_4. These results of poly-P and P dynamics in *E. coli* JM103 are consistent to other studies (Kornberg et al., 1956; Rao et al., 1985; Rao and Kornberg, 1995; Itoh and Shiba, 2004).

Phosphate oxygen isotope data showed consistent trends compared to poly-P and PO_4 variability. The gradual heavier $\delta^{18}O_P$ values in first 12 h may result from microbial PO_4 assimilation. Uptake and assimilation of nutrients (e.g., NO_4^-, NH_4^+,

and PO_4) by microorganisms is often associated with isotope fractionations (Kendall and Caldwell, 1998), as nutrients with lighter isotopes are preferentially utilized, a process that could enrich the residual nutrients with heavy isotopes. Based on the results from PO_4 assimilation by *E. coli* an apparent fractionation factor of -3.2‰ was calculated (Blake et al., 2005). Results in this study, however, suggest a larger fractionation (~-5‰) for PO_4 assimilation by *E.coli* JM103. The apparent fractionation during PO_4 assimilation varies with the portion of PO_4 assimilated from total P pool and a complete assimilation of PO_4 should results in no fractionation. In Blake et al. (2005), the incubation time was much longer (~ 600 h) and after 150 h more than 500 μM PO_4 was assimilated by *E. coli*, which was > 50% of the initial PO_4 concentration. In this study, within 12 h only ~ 30 μM PO_4 (~ 5% of initial PO_4) was assimilated (Figure 10C, D). Therefore, the larger apparent isotope fractionation in this study compared to that in Blake et al. (2005) could be attributed to the lower amount of PO_4 assimilated during the incubation. It must, however, be stressed that the true fractionation requires the knowledge of isotopes values both in source (dissolved PO_4) and sinks (PO_4 inside cells including those in organelles). This is complicated by the fact that neither all PO_4 could be extracted and measured nor the isotope values remain unchanged after biological uptake (Li et al., 2016). Nevertheless, the opposite trends of isotopes and PO_4 concentration along with that of poly-P are suggestive of microbial assimilation of PO_4.

When the cell growth entered stationary phase after exponential growth, an excursion of $\delta^{18}O_P$ values towards equilibrium was observed. One possible reason could be mixing of PO_4 generated from degradation of poly-P, which results in lighter isotope values because of the incorporation of one water oxygen ($\delta^{18}O_w$ = ~ -7‰ for

the media). However overall PO_4 concentration did not increase with decreasing poly-P concentration but rather continued to decrease (Figure 10C) suggesting potential P stress condition (see discussion above). The other reason for isotope excursion is also likely attributed to rapid microbial cycling, as microbial turn-over catalyze exchange of water and PO_4 oxygen isotopes towards equilibrium (Blake et al., 2005; Jaisi and Blake 2014; Stout et al., 2014). In the late stationary phase (> 24 h), isotope values started to be heavier. Thus is unclear the reason for variation in isotope values. However given the uncertainty of the measurement and variable cell growth in two sets of experiments, such interpretation remains speculative and requires further validation.

Trends of PO_4 and poly-P were different in *P. putida* KT2440 than that in *E. coli* results. In the first set of experiments, *P. putida* KT2440 had already accumulated significant amount of poly-P (~ 20 μM) at the onset of experiment (Figures 10 and 11). During the two days incubation, poly-P concentration decreased from 16 μM to 10 μM in cells, which indicates poly-P degradation. However, with the degradation of poly-P, *P. putida* still took up additional PO_4 (~100 μM) from the media. The heavier isotope values at time zero is difficult to explain, as it should be similar to that of the *E. coli* and should represents the media PO_4 isotope values (same media was used for both incubations). Therefore it is possible that this isotope values are caused by some unknown artifacts and likely not reliable. Similar to the experiment with *E.coli*, the decrease of $\delta^{18}O_P$ values corresponded to decrease of poly-P, likely attributed to degradation of poly-P and rapid microbial P turnover under stressful condition.

The stress condition expected to be in the later stages in the incubation experiments suggested by the active cycling of PO_4 and rapid degradation of poly-P

(see above) is intriguing, and is different from the results of the first set of experiments and that of previous publication on *P. putida* KT2440 (Tobin et al., 2007). In Tobin et al., poly-P concentration increased in the first ~25 h of incubation and then decreased but was always in opposite trend with PO_4 concentration. In this incubation, however, break down poly-P was observed even in the log growth phase. Once the cell growth reached stationary phase, the rate of poly-P degradation increased (Figure 10 and discussion above). It is speculated that the growth condition in the first set of experiments was much stressful compared to the second even though the same media and growth condition were used. It is unclear but the potential reasons might be different cell number and other variabilities (mentioned above) that may leads to the limitation of other nutrients (Tobin et al., 2007) not monitored in this study. Further investigation is required to identify reasons for variable concentrations of poly-P and PO_4 and trend of isotope values over time.

4.3 Poly-P Cycling in KCRWTF

Results on the concentration of poly-P (Figure 13) and DNA copies of poly-P accumulating organisms (PAOs) normalized to total DNA (Figure 16) suggest the strong response of poly-P synthesis to the imposed variation in DO concentrations. Poly-P concentrations in sludge decreased dramatically at the onset of anoxic condition (150 min; Figure 13), while the PAOs generally decreased (Figure 16). The fluctuations in the PAOs are most likely due to the variable heterogeneous DO (such in particulate matter, settling sludge and aggregates is expected to have low DO than the water) expected in the aeration basin (Figure 12). Despite this, a clear trend of PAOs was observed. For example, during the oxic period of ~ 40 min to 1 h, DO decreased with correspond decrease in PAOs as shown by the decrease in gene copies.

Once the system switched to anoxic condition, the number of PAOs decreased gradually, and after 2 h of anoxic condition, the PAOs reached the lowest. However, because there was a slight increase of DO after 2 h of anoxic incubation, the amount of PAOs increased correspondingly. These results strongly corroborate the role of DO on PAOs, and thus on poly-P synthesis.

The general trends of poly-P of synthesis in this study are consistent with past publications in WWTP (Xie et al., 2010; Keating et al., 2016), the variability of PO_4 in water provided some intriguing insights. It is generally believed that under anoxic conditions, PO_4 concentration should increase in both sludge and water due to the degradation of poly-P and other organic P compounds under stressful conditions (Mino et al., 1998; de Bashan and Bashan, 2004). Results in this study show that under anoxic conditions (> 150 min), the concentration of PO_4 in sludge increased as expected, but PO_4 in water decreased dramatically (Figure 13), in contrast to the conventional view that PO_4 is released into the water under anoxic conditions (van Loosdrecht et al., 1997; Mino et al., 1998). This is similar to the bacteria culture experiments (Figures 10 and 11). Decrease of PO_4 concentration in water may suggest that, under anoxic conditions, instead of releasing PO_4 to surrounding environment via poly-P degradation in cell, microorganisms may continue to assimilate PO_4. One note of caution here is that amount of poly-P which is unusually low (on average ~10% of PO_4, in sludge and <1.0% in water). Moreover, it is important to note that PO_4 in sludge is estimated using 0.5 M $NaHCO_3$ extraction (16 h), which could underestimate the total PO_4 and in sludge (cells). It means PO_4 released from poly-P degradation cannot be detected in water. This also restricts evaluating relationship of PO_4 and poly-P. The continuing assimilation of PO_4 by microorganisms under anoxic conditions

should be compensated from decrease in dissolved PO_4 in water, a trend supported by isotope data as well. With the onset of anoxic condition (150 min), isotope values in dissolved PO_4 increased noticeably, becoming still heavier than during aerobic period (Figure 15) and are consistent with microbial PO_4 assimilation in controlled experiments (Figure 10 and discussion above). These results further suggest that, under anoxic conditions, microorganisms continue to assimilate a large amount of PO_4 from the wastewater.

The excursion of PO_4 isotope values towards equilibrium under oxic condition (0–150 min) (Figure 15) suggests a faster microbial PO_4 cycling or organic matter degradation (Jaisi and Blake 2014; Liang and Blake 2006, 2009). This is expected for a complex system such as wastewater treatment plant for the following two reasons: i) under oxic condition, many microorganisms and other multicellular organisms such as protozoa, algae, and fungi actively cycle P for their metabolic and growth requirements; ii) under anoxic condition, degradation of many other organic P compounds is more efficient, thus releases more lighter PO_4 in the water. This explanation agrees well with the results from enzyme activity measurements (Figure 14). Under anoxic condition, both alkaline and acidic phosphatase activities were relatively higher, therefore, suggesting degradation of organic P compounds- a reason for generating lighter $\delta^{18}O_P$ values.

In the light of results and discussion explained above, findings from this research indicate that PO_4 cycling is more complex than generally explained in the literature. The degradation of poly-P under anoxic conditions releases PO_4 into the water and PO_4 is effectively assimilated to form poly-P. This can be realized from the WWTP system with a variety of microbial community and a large range of organic P

compounds (e.g., monoesters, diesters, DNA, RNA; Mino et al., 1999). It means a suite of microorganisms in WWTP may respond differently than mono culture of PAOs. For example, under anoxic condition, some microrganisms may continue to take up PO_4 to alleviate stressful conditions rather than releasing PO_4. This potentially new finding requires additional verification as this has large implication on the approaches being adopted in P removal in the wastewater treatment plants.

Chapter 5

CONCLUSIONS AND IMPLICATIONS

The results from pure enzyme and poly-P substrate reaction suggest that some common phosphatase enzymes (i.e., alkaline phosphatase from *E. coli,* acidic phosphatase from potato and acidic phosphatase from wheat) are capable of catalyzing the degradation of poly-P. The extent of degradation, however, was different: alkaline phosphatase from *E. coli* catalyze only ~17% degradation of poly-P, whereas acidic phosphatase from both potato and wheat could catalyze more than 70% degradation. Interestingly, enzymatic degradation led to a positive isotope fractionation factor: for alkaline phosphatase from *E. coli* it was +2.49 ‰, whereas for acidic phosphatase from potato and wheat the fractionation factors were +5.01 ‰ and +1.63 ‰, respectively. These positive fractionation factors are distinct from degradation of many other organic P compounds and thus points towards application of phosphate oxygen isotope ratios as a proxy to trace environmental PO_4 derived from poly-P degradation.

Results from bacteria culture experiments (*E.coli* JM103 and *P. putida* KT2440) suggest that poly-P synthesis and degradation is strongly associated with cell growth status: poly-P is synthesized during exponential growth. Degradation of poly-P occurred when cell growth reached stationary phase speculated due to the limitation of nutrients and impact of toxic products generated from cell metabolism in batch culture. An isotopic fractionation (~+5‰) was observed during bacterial PO_4 assimilation (leading to residual PO_4 enriched in ^{18}O). This finding is comparable with

previous study (Blake et al., 2005) and has important implications for using phosphate oxygen isotopes to understand P cycling in environments.

Poly-P cycling in KCRWTF responded strongly to variations in oxygen concentrations: under aerobic conditions, poly-P was synthesized. The lighter $\delta^{18}O_P$ values during aerobic condition suggest rapid microbial P cycling and organic P degradation. Under anoxic conditions, PO_4 concentration in wastewater decreased and $\delta^{18}O_P$ values became heavier and degradation of poly-P suggesting continued uptake of phosphate by microorganisms to survive stressful conditions. This result is different from the prevailing understanding that degradation of poly-P under anoxic conditions releases PO_4 into the water. While this condition pertains largely to PAOs, and since the amount of poly-P in KCRWTF was low, the overall response is potentially arising from other microorganisms and multicellular organisms. In summary, it suggests that understanding the mechanism of poly-P cycling in natural environment is more complex due to variable microbe community structures and corresponding synthesis and degradation of organic compounds.

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