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Article

Synthesis and Degradation of Polyphosphate: Isotope Effects in Enzyme- and Bacteria-Catalyzed Reactions

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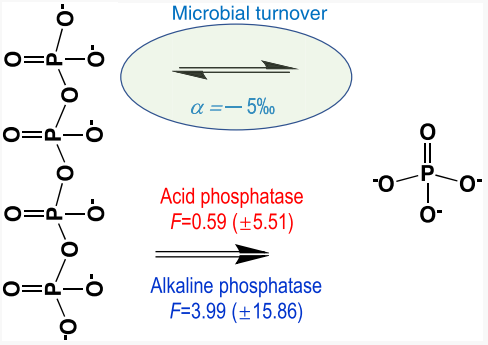
ABSTRACT: Polyphosphate (poly-P) is a chain of phosphate moieties linked through high-energy phosphoanhydride bond, and it plays an important role in regulatory functions in prokaryotic cells. Isotope effects of bacterial synthesis and degradation of poly-P may provide important insights into the roles of poly-P in environmental phosphorus (P) cycling. In this research, we investigated the enzymatic degradation of poly-P by cell-free enzyme solutions and bacterial cells (*Escherichia coli* JM103 and *Pseudomonas putida* KT2440) and analyzed phosphate oxygen isotope ratios ($\delta^{18}O_P$), enzyme activity, and dynamics of orthophosphate (P_i) and poly-P. Cell-free enzyme reaction results show that both acid and alkaline phosphatase enzymes are capable of catalyzing poly-P degradation, albeit with different efficiencies of >70 and <18%, respectively. Isotope fractionation factors during enzymatic degradation of poly-P were slightly positive [+0.59 (±5.51) to +3.99 (±15.86)‰] for acid and alkaline phosphatase, respectively, which is rather uncommon in phosphatase enzyme-catalyzed degradation of many other organic P compounds. Poly-P is synthesized during the exponential growth phase of *E. coli*, causing an isotope fractionation of −5.65 (±1.02)‰ leading to the enrichment of isotope in the residual P_i in the growth media. Degradation of poly-P occurred at the late stationary phase, with lighter isotope values of P_i in the growth media. These results imply that the specific isotopes, fractionation, and P dynamics during poly-P synthesis and degradation could serve as proxies to interpret poly-P dynamics in the environment.



KEYWORDS: *polyphosphate, degradation, oxygen isotopes, fractionation factor*

INTRODUCTION

Polyphosphate (poly-P) is a long-chain linear polymer containing three to several hundreds of orthophosphate (PO_4, referred hereafter as P_i) units linked by sharing oxygen atoms via the high-energy phosphoanhydride bond. 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).[1] While bacteria are generally known to synthesize poly-P, some of them can store a substantial amount of poly-P under specific environmental conditions. They form poly-P as a source of P and energy under favorable conditions, such as sufficient P and oxic conditions. Poly-P can be degraded to release P_i or to form ATP at any time, but particularly under P starvation or oxygen-deficient conditions.[2] The P-storage strategy is evidenced in the strong dependence of poly-P content with P_i in cell culture. It has been suggested that poly-P is an ideal and convenient compound for a large amount of P_i and energy storage in a cell because the accumulation of poly-P has little effect on the osmotic pressure within the cells.[3] Furthermore, poly-P helps to maintain the concentration of free P_i in cells at a relatively stable level, which is critical to many cell biochemical processes. Poly-P synthesis and degradation by microorganisms under different conditions maintains a considerable proportion of bioavailable P for primary production, especially in low-P aquatic environments.[4] Some other organisms such as picoplankton are known to store and liberate poly-P to support the high primary productivity during algal blooms.[5] While the specificity of conditions that trigger the synthesis and degradation of poly-P varies among species, it is clear that poly-P plays a significant role in the biogeochemical cycling of P in aquatic ecosystems.[6,7]

Given its crucial role in cell functioning and importance in P cycling in the environment, poly-P metabolism in microorganisms has been investigated in the past several decades.[8−12] Biochemical reactions involving poly-P are predominantly catalyzed by enzymes, and many enzymes have been found to respond to variations in concentrations of poly-P and P_i in the environment. At the cellular level, for

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example, in *Escherichia coli*, the activity of alkaline phosphatase (APase, EC 3.1.3.1) expression increases with P_i limitation in cells and poly-P accumulates in media deficient in both P_i and amino acids.[13] Alkaline phosphatase, which can be expressed intra- or extracellularly, is one of the most common enzymes in the natural environment and is capable of catalyzing the degradation of many phosphomonoester compounds.[14] Acid 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.[15,16] However, the specificity of these enzymes on degrading poly-P and, more specifically, the isotope effect of degradation, is yet to be investigated.

Phosphate oxygen isotopic ratios ($\delta^{18}O_P$) have been increasingly used as a proxy to trace P cycling in both extinct and extant ecosystems.[17−24] Isotopic fractionation in biological reactions occurs due to the enzyme-catalyzed oxygen isotope exchange between phosphate and water[25] and thus causes large effects on $\delta^{18}O_P$ values. Among many phosphohydrolase enzymes that catalyze phosphate bond cleavage, pyrophosphatase behaves differently: inside a cell, it catalyzes the reversible oxygen isotope exchange between P_i and water, which promotes wholesale isotope exchange and thus results in equilibrium isotope fractionation that is temperature-dependent. Equilibrium fractionation has been observed in many organisms including bacteria, algae, and tissues of many multicellular organisms due to the universal presence of the pyrophosphatase enzyme.[20,26,27] Temperature-dependent equilibrium fractionation equations have been developed to describe isotopic fractionation between water and dissolved P_i with the latest corrections for the online Thermo-Chemical Elemental Analyzer (TC/EA) measurement method on silver phosphate analyte.[28]

Many extracellular enzymes such as alkaline phosphatase (APase) catalyze unidirectional reaction (breakdown of organic P (P_o) compounds) and thus generate kinetic isotope effects that are temperature-independent.[27,29,30] Kinetic fractionation factors are enzyme- and substrate-specific.[31−33] The compound-specific isotope analyses of phosphatic compounds are just beginning to be studied. So far, isotopic fractionations during the degradation of P_o compounds have been studied on limited to selected P-diesters (RNA and DNA) and monoesters (glucose-1 phosphate, glycerol-phosphate, phytate, and 5′-nucleotide).[31−36] The distinct fractionation factors among them that are unique to phosphatase enzyme involved have provided a basis for the interpretation or revision thereof of isotope data obtained from natural environments.[21,37,38] The major objective of this research is to determine the phosphate oxygen isotope fractionation during enzymatic degradation as well as during the bacterial synthesis of poly-P. These objectives were realized from a series of incubation experiments including cell-free enzymes and two model microorganisms under environmentally relevant conditions followed by analyses on P speciation and isotopes. We anticipate both the degradation- and synthesis-related isotope effects provide additional insights into the feasibility of using $\delta^{18}O_P$ as a tracer to identify poly-P cycling in the environment.

MATERIAL AND METHODS

Pure Enzyme and Substrate Reaction and Isotopic Fractionation Factors. To determine oxygen isotopic fractionation during degradation of poly-P, sodium polyphosphate (Sigma-Aldrich) with an average chain length of 5 was incubated individually with two acid phosphatase (extracted from wheat and potato) enzymes and one alkaline phosphatase (extracted from *E. coli*) enzyme. Experiments were conducted in a series of ^{18}O-labeled waters to identify the oxygen atom incorporated during the degradation. The details of experimental and analytical methods are described below.

Enzymatic Degradation of Poly-P. The enzyme assays for two acid phosphatase enzymes (from wheat and potato) and one alkaline phosphatase enzyme (from *E. coli*) consisted of 1.5 mM poly-P and 2 UN enzyme (1 UN represents the activity required to generate 1 μmol of P_i per min). For acid phosphatase enzymes, incubations were conducted in citrate buffer (pH 4.8) with 0.9 mM Mg^{2+} used as a metal cofactor, following the manufacturer-specified method. Similar assays with alkaline phosphatase from *E. coli* were conducted in glycine buffer at pH 10.4 using Zn^{2+} and Mg^{2+} (0.97 mM $ZnCl_2$ and 0.90 mM $MgCl_2$) as metal cofactors. The optimal concentrations of metal cofactors were selected based on a series of experiments performed at various concentrations of Zn^{2+} and Mg^{2+}. All enzymatic degradation experiments were run in duplicate with reagents prepared in double-deionized (DDI) water or sterilized solutions to avoid any microbial contamination.

Degradation experiments were carried out at three different temperatures (4, 21, and 37 °C) in temperature-controlled incubators to test if the disequilibrium fractionation factors are independent of temperature. At each temperature, a series of ^{18}O-labeled waters (with $\delta^{18}O_W$ values ranging from −7.0 to +15.0‰ VSMOW) were used. Subsamples were taken from each reactor at different time points, and the reaction in the sampled solution was terminated by adjusting the pH lower than 5.0 using 1.0 M HCl.[32] After removing any enzyme residues via centrifugation, P_i formed was quantified using a colorimetric (phosphomolybdate blue) method.[39] The incubation experiments were run until no further poly-P degradation occurred which was tested by spiking of a fresh enzyme and confirming no additional P_i is released.

Processing and Purification of P_i for Isotope Analyses. Subsamples collected at different time points during various degradation experiments were purified to remove all other compounds/ions containing oxygen besides dissolved P_i. Residual poly-P was removed by treating the sample with anion resin (Dowex 1-X8 resin, 100−200 mesh; BioRad, Hercules, CA), following the established method.[40] Briefly, 50 mL of 0.2 M KCl/acetate buffer (pH 4.5) solution was eluted for every 100 μmol of P_i (of poly-P). The recovery of P_i during the separation process ranged from 86 to 99% (average, ∼93%). After the removal of poly-P, samples were further purified using the established methods.[41] In brief, a series of precipitation and dissolution reactions were undertaken to remove residual enzymes and contaminants. First, precipitation and separation of ammonium phospho-molybdate (APM) (by 0.1 μm polysulfone filter, Pall Scientific) were 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 magnesium ammonium phosphate precipitation (MAP), which removes ions soluble at high pH. The separated MAP precipitate (by filtration as above) was rinsed (with 5% ammonium hydroxide) and adjusted to neutral pH before treating with a cation exchange column (AG50W-X8; BioRad, Hercules, CA) for removing residual cations (primarily Mg^{2+} and NH_4^+). Finally, silver

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amine solution was added to the concentrated P_i solution to precipitate silver phosphate. The precipitate was separated and dried (at 110 °C for overnight) before measuring $\delta^{18}O_P$ values.

Measurement of Oxygen Isotope Ratios in Phosphate and Water. Finely ground and homogenized silver phosphate powder (250−300 μg) was packed in silver capsules and pyrolyzed in a TC/EA to release O from silver phosphate, which reacts with glassy carbon to form CO. The $\delta^{18}O$ values of CO, the analyte gas, were measured in a gas-phase isotope ratio monitoring mass spectrometer (IRMS, Thermo Delta V, Germany; precision, 0.3‰), which was coupled to TC/EA through ConFlo IV, a continuous flow system. For each sample, three replicate capsules were analyzed. Measured isotope values were calibrated against two isotope standards, YR-1a and YR-3-2, with $\delta^{18}O_P$ values of −5.49 and +33.63‰, respectively.

Water oxygen isotope ratios ($\delta^{18}O_W$) were measured using a Finnigan GasBench II coupled with IRMS following the method described in detail in Upreti et al.,[42] which was adapted from Cohn and Urey.[43] In brief, 300 μL of water samples was injected into preflushed (with 300 ppm of CO_2) Labco Exetainer vials (12 mL) to allow CO_2−H_2O equilibrium for 24 h at 26 °C. After the complete equilibrium, headspace CO_2 was introduced into IRMS and $^{18}O/^{16}O$ ratios were measured. The $\delta^{18}O_W$ values were calculated from the measured $\delta^{18}O_{CO_2}$ values and calibrated against two USGS standards [W67400 (−1.97‰) and W32615 (−9.25‰)]. The typical precision for replicate standards was less than 0.02‰. All measured oxygen isotope values of water and phosphate are reported relative to the Vienna standard mean oceanic water (VSMOW) standard in the unit of permil (‰).

Calculations of Isotopic Fractionation Factors. The equilibrium isotopic values ($\delta^{18}O_{Eq}$) resulting from the wholesale exchange of oxygen between phosphate and ambient water can be determined using established equations.[26,28,44] In this study, equilibrium isotope values were calculated using both Longinelli and Nuti (1973)[44] and Chang and Blake (2015)[28] equations as below

$$\delta^{18}O_P = 1/4.3(111.4 - t) + \delta^{18}O_W \quad (1)$$

$$\delta^{18}O_P = (\delta^{18}O_W + 10^3)\, e^{[14.43\frac{10^3}{T} - 26.54]/10^3} - 10^3 \quad (2)$$

where t and T indicate the ambient temperature in kelvin (K) and degree Celsius (°C), respectively. Similarly, $\delta^{18}O_P$ and $\delta^{18}O_W$ are the oxygen isotope values of phosphate and water, respectively. Please note that the Chang and Blake equation is more relevant to P_i precipitated as Ag_3PO_4 and measured online in TC/EA. The isotopic fractionation factor (F) of oxygen was calculated from the measured $\delta^{18}O_P$ values of P_i released from enzymatic degradation of poly-P as

$$\delta^{18}O_P = a(\delta^{18}O_W + F) + (1 - a)\,(\delta^{18}O_{poly-P}) \quad (3)$$

where $\delta^{18}O_{poly\text{-}P}$ is the oxygen isotope values of poly-P, a is the fraction of O incorporated from water, and $(1 - a)$ is the fraction of O inherited from poly-P. The value of a is nominally 0.25 (one out of four oxygens in PO_4 is derived from water). A linear relationship is expected between $\delta^{18}O_P$ and $\delta^{18}O_W$ values. The slope of the fitted line allows calculating F from the predetermined values of $\delta^{18}O_{poly\text{-}P}$, which were measured in the IRMS after direct pyrolysis in the TC/EA.

Poly-P Synthesis and Degradation by Bacteria. Poly-P metabolism in microorganisms is complex because of the variable synthesis, transformation, and degradation reactions, and each of them could be catalyzed by more than one enzymes.[45] In this study, we incubated two common bacteria, *E. coli* JM103 and *Pseudomonas putida* KT2440, both known to accumulate intracellular poly-P[45−47] to understand poly-P synthesis and degradation under aerobic growth conditions. In both cultures, the changes in cell growth, concentrations of dissolved P_i and poly-P (in the culture media and inside cells), activities of alkaline and acid phosphatase enzymes, and isotope values of dissolved phosphate and water in the media were measured during incubation. Details of the experimental setup and measurements are described below:

Bacteria Cultures. Both *E. coli* JM103 and *P. putida* KT2440 cells were grown aerobically at 30 °C in the mineral salt media with the following composition: ferric ammonium citrate, KCl, $CaCl_2$, NH_4Cl, KH_2PO_4, vitamins, and glucose as a carbon source.[48] To understand the response of the initial concentration of P_i on poly-P synthesis, two sets of growth experiments, with initial P_i concentrations of 500 and 1000 μM, were used. Cell growth was monitored via turbidity measurement at 600 nm. At specific time points, subsamples were taken for the quantitation of P_i and poly-P, alkaline phosphatase activity, and oxygen isotope values of water ($\delta^{18}O_W$). The water isotopes were used to track any changes in isotope due to water-O incorporation into released P_i from poly-P and ensure no evaporation during incubation and sample handling.

Poly-P Quantitation. Quantitation of poly-P was conducted using the 4′6-diamidino-2-phenylindole (DAPI) fluorescence method.[49,50] The binding of poly-P to DAPI shifts the emission wavelength from 475 to 525 nm, and the fluorescence intensity at 525 nm is proportional to the concentration of poly-P.[51] DAPI stock was prepared in double-deionized water at a stock concentration of 1 mM and stored frozen (in 1 mL aliquots) in the dark to minimize photodegradation and used within a week. At the time of measurement, DAPI reagent was diluted to 100 μM and experimental samples were incubated with DAPI in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES) buffer under ambient light for 7−8 min with vortexing three times to obtain a stable fluorescence signal.[50] After the completion of the reaction, an aliquot of the sample was transferred to a black 96-well plate (Corning, Inc.) and the fluorescence was measured in a Spectramax M2 spectrophotometer at 550 nm illumination wavelength after excitation at 415 nm.

Poly-P Extraction. Poly-P was extracted and purified from *E. coli* JM103 and *P. putida* KT2440 cell cultures that were sampled at different growth stages using the established extraction protocol.[4] 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 in 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 °C for 30 min with constant mixing at 300 rpm and vortexing two to three times during the incubation to achieve complete cell lysis. To separate the extracted poly-P, cell residues were pelleted by centrifugation at 5000 × g for 10 min, leaving poly-P in the supernatant. The entire reaction with proteinase K and separation was repeated three times to enhance poly-P extraction as the repeated extraction is reported to increase the recovery of poly-P as high as ∼99%.[4]

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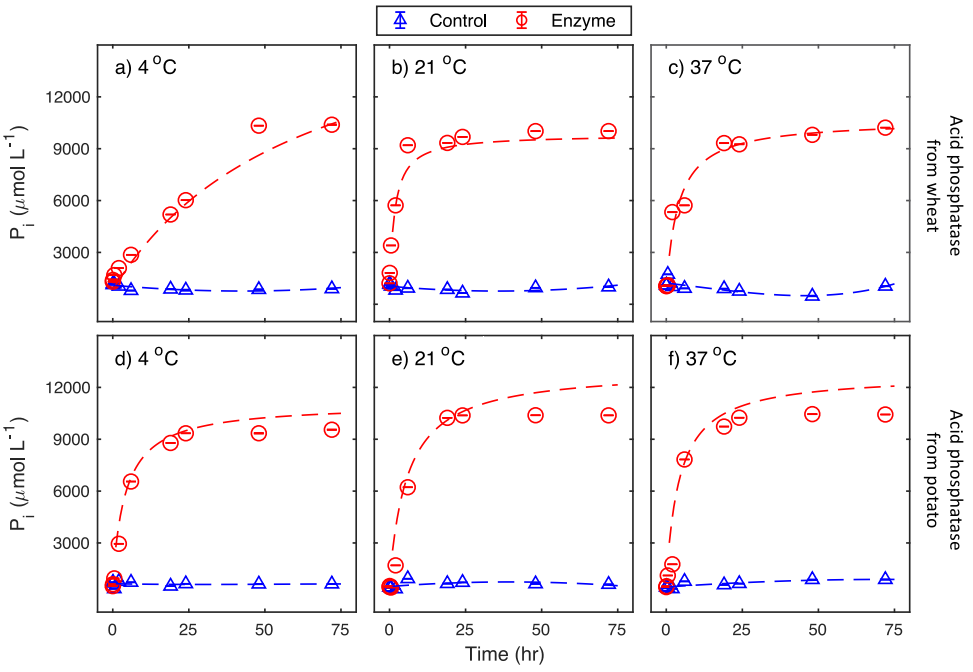

Figure 1. Inorganic P (P_i) released from poly-P degradation by acid phosphatase enzymes from wheat (a–c) and potato (d–f). Poly-P was incubated under three different temperatures. The dashed lines indicate the fitting of experimental data points by the nonlinear regression model in MATLAB.

Measurement of Alkaline and Acid Phosphatase Enzyme Activity. The activity of alkaline phosphatase (APase) was determined using a modified method of Adams et al.,[52] as described in Stout et al.[53] In brief, samples were incubated at 37 °C at pH 8.5 (Tris-HCl buffer) using *para*-nitrophenyl phosphate (*p*NPP) as a substrate. The hydrolyzed product *p*-nitrophenol (*p*NP) was then measured colorimetrically at 405 nm. APase activity was calculated as μmol of *p*NPP hydrolyzed per hour per liter of solution (μmol h^{-1} L^{-1}). Acid phosphatase activity was measured analogously in citrate (pH 6.5) buffer.

RESULTS

Kinetics of Poly-P Degradation by Acid and Alkaline Phosphatase Enzymes. Results of poly-P incubation with acid phosphatase performed at 4, 21, and 37 °C (Figure 1) show that the released P_i approached plateau concentrations in ~2 days and then gradually leveled off. The degradation kinetics of acid phosphatase from wheat and potato were slightly different but comparable among three different temperatures. While there was a slight difference in the kinetics of P_i release with the higher P_i yield at a higher temperature, the overall efficiency of degradation (ratio of released P_i to total P) was >70% in all experiments performed at three temperatures. Control experiments did not show a significant change in P_i concentration.

For alkaline phosphatase, the efficiency of release of P_i from poly-P was low (<18%) (Figure 2) compared to acid phosphatase (Figure 1). The control experiment did not show any appreciable difference during the incubation. A notable difference, however, was the high P_i at the start of the experiment. To confirm the results on limited hydrolysis and high P_i at the start, two other batches of alkaline phosphatases were ordered and experiments were repeated. Those analyses generated comparable results and confirmed the presence of contaminant P_i in the enzyme preparation.

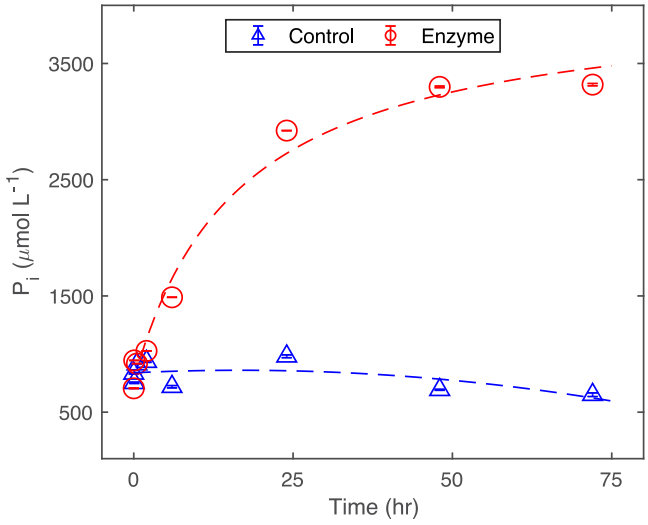

Figure 2. Phosphate released from poly-P degradation by alkaline phosphatase enzyme (from *E. coli*) at 37 °C. The dashed lines indicate the fitting of experimental data points by the nonlinear regression model in MATLAB.

Isotope Fractionation during Enzymatic Degradation of Poly-P. The relationship between the $\delta^{18}O_W$ values of water used in the incubation experiments and $\delta^{18}O_P$ values of released P_i from poly-P under enzymatic degradation is shown in Figure 3. The strong linear relationship between two parameters indicates that the oxygen atom from the water was incorporated into the released P_i. Table 1 shows the calculated oxygen isotope fractionation factors during oxygen incorporation in the P_i released from poly-P with two different enzymes under different temperatures. While the disequilibrium fractionation factors are independent of temperature, as expected, they varied in different incubations. For example, the average *F* values for acid phosphatase from wheat and potato

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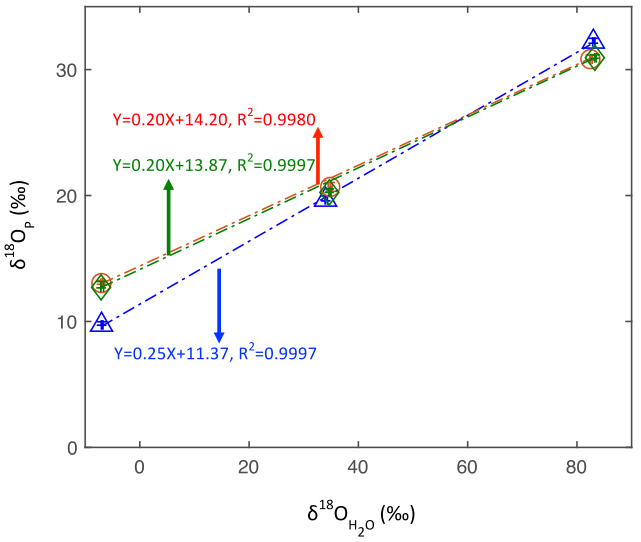

Figure 3. Phosphate-water oxygen isotope exchange catalyzed by cell-free acid phosphatase enzyme from wheat (green), acid phosphatase enzyme from potato (red), and alkaline phosphatase enzyme from *E. coli* (blue). The slopes of the linear regression of acid and alkaline phosphatase enzymes are 0.20 and 0.25, respectively.

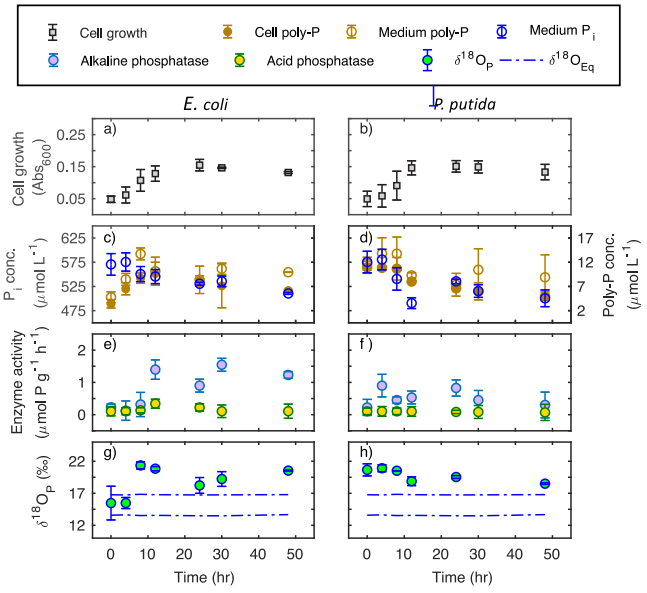

Figure 4. *E. coli* JM103 (left) and *P. putida* KT2440 (right) cell growth (a, b), concentrations of poly-P (measured as the equivalent P_i) and P_i (c, d), corresponding activities of alkaline phosphatase and acid phosphatase enzymes (e, f), and phosphate oxygen isotope values (g, h) in the residual P_i in two cultures. The upper and lower traces of lines are equilibrium isotope values calculated per Liang and Blake (2015)[28] and Longinelli and Nuti (1973),[44] respectively.

were 0.15 (±4.95) and 0.91 (±5.64), respectively, and for alkaline phosphatase was 3.99 (±15.86) (see eq 5). The potential reasons for the wide variations are included in the Discussion section.

Poly-P Synthesis in the Cell Culture of *E. coli* and *P. putida*. Figures 4 and 5 show the dynamics of P_i and poly-P in response to differences in the concentration of starting P_i along with the expression of enzyme activities (alkaline phosphatase and acid phosphatase) at different cell growth stages of *E. coli* and *P. putida*. The *E. coli* cells reached the stationary growth phase after 20 h of incubation and started to level off or slightly decrease after 24 h (Figure 4a). Correspondingly, P_i concentration decreased from 570 to 510 μM (Figure 4c). Poly-P concentration in *E. coli* cells increased in the first 10 h, but after the stationary growth phase, poly-P concentrations in both cells and medium appeared to decrease. The activity of alkaline phosphatase was low at the beginning of *E. coli* growth but started to increase after 10 h of incubation when the poly-P concentration in medium started to decrease (after the stationary phase). Acid phosphatase activity remained low during the entire two day incubation.

The growth of *P. putida* reached the stationary phase in time similar to that of *E. coli* (10 h) and remained similar until after

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*; Equation 5), Which Is Accounted for the $\delta^{18}O_P$ Values of Contaminant P_i (14.5‰)[a]

enzyme	*T* (°C)	$\delta^{18}O_W$ (‰)	$\delta^{18}O_P$ (‰)	$\delta^{18}O_{poly\text{-}P}$ (‰)	*F* (‰)	mean *F* (‰)
acid phosphatase (from wheat)	37	34.94	20.84	16.65	0.98	0.15 (±4.95)
	37	82.60	31.40		−0.26	
	21	−7.00	12.17		4.81	
	21	34.00	18.57		−8.06	
	4	−7.00	12.30		5.38	
	4	34.00	19.90		−2.21	
acid phosphatase (from potato)	37	−7.02	12.60	16.65	6.72	0.91 (±5.64)
	37	34.94	20.46		−0.69	
	37	82.46	29.94		−6.54	
	21	−7.00	13.31		9.82	
	21	34.00	20.10		−1.33	
	21	83.00	31.18		−1.63	
	4	−7.00	12.86		7.84	
	4	34.00	20.71		1.35	
	4	83.00	29.89		−7.30	
alkaline from phosphatase (*E. coli*)	37	−7.00	9.73	16.65	−10.73	3.99 (±15.86)
	37	34.00	19.66		1.94	
	37	83.00	32.21		20.78	

[a]See the text for the explanation of the variability of the fractionation factors.

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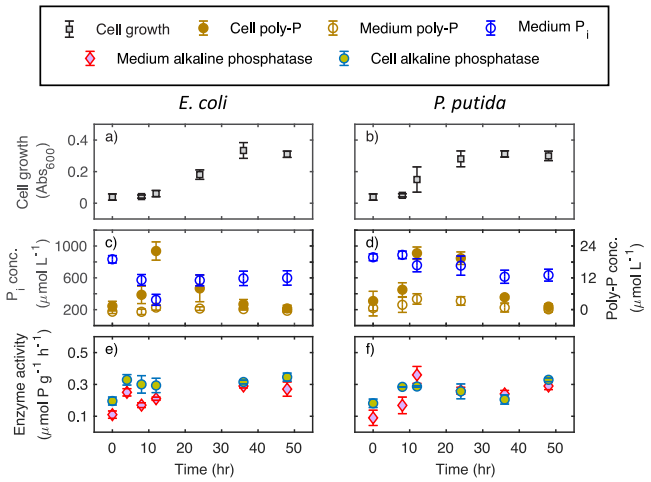

Figure 5. Increased cell growth of *E. coli* (a) and *P. putida* (b) in mineral salt medium at 30 °C when the initial P_i concentration was 1000 μM. Other parameters include poly-P and P_i concentrations (c, d) and expression of medium and cell alkaline phosphatase activity (e, f).

30 h (Figure 4b). With the growth of cells, P_i concentration in media decreased by ~100 μM in the first 12 h (during exponential growth) and decreased only slightly thereafter (Figure 4d). Concentrations of poly-P steadily decreased during the 48 h incubation from ~16 to 10 μM (measured as μM of P). Poly-P concentration in the media decreased in the first ~24 h of the incubation (exponential growth) but appeared to increase thereafter (Figure 4d). Alkaline phosphatase activity in *P. putida* cell culture was generally low compared to that of *E. coli*. Acid phosphatase activity was quite comparably low as that in the cultures of *E. coli*.

A comparison of $\delta^{18}O_P$ values of dissolved P_i in the media between two cell cultures shows some interesting relationships (Figure 4g,h). For *E. coli*, isotope values were within the equilibrium in the first 4 h of incubation but increased dramatically after 8 h of incubation. While it became slightly lighter during the 8−24 h period, there was a consistent offset of isotope values in the entire growth period. In contrast, *P. putida* growth had isotope values ~5‰ off of the equilibrium early at the start of the culture and became steadily lighter with increasing incubation time. At the end of the culture experiment, there was still a significant offset ~3‰ of the equilibrium isotope composition.

DISCUSSION

Enzymatic Degradation of Poly-P and Isotope Fractionation. The acid phosphatase enzyme was able to degrade ~70% of poly-P within 48 h, while alkaline phosphatase degraded <20% of poly-P (Figure 1). This is rather unexpected because alkaline phosphatase is one of the most common nonspecific enzymes in the natural environment and is well known for hydrolyzing a variety of phosphomonoesters.[14] Several enzyme assays were performed at different concentrations of metal cofactor and pH with varying chain lengths of poly-P to confirm the data presented are representative and to exclude the influence of other factors that may inhibit the enzyme reaction. While there were variations in degradation kinetics among the parameters studied, the optimal values of these factors were used to achieve the highest extent of degradation. Alkaline phosphatase activity was measured using the widely used *p*NPP substrate, and the results confirmed the sufficient activity of the commercial enzyme. It is unclear if unknown impurities in the poly-P, besides contaminant P_i, could be a factor. Still, the reason for the inefficient degradation of poly-P by alkaline phosphatase is not known and therefore needs further investigation.

The different degradation efficiency of the two enzymes studied was also reflected in the $\delta^{18}O_P$ values of the released P_i, in particular, the different slopes of the linear regression between P_i and water isotope values (Figure 3 and Table 1). The slopes are 0.20 (±0.03) and 0.25 (±0.005) for acid phosphatase and alkaline phosphatase, respectively. The slope of two acid phosphatases from potato and wheat was the same (Figure 3). The decrease in slope arises from contaminant P_i present in system,[33] which necessitates the modification of eq 3 (above) as

$$\delta^{18}O_P'(m+n) = m\cdot\delta^{18}O_P + n\cdot\delta^{18}O_P'' \quad (4)$$

where $\delta^{18}O_P'$, $\delta^{18}O_P$, and $\delta^{18}O_P''$ are isotope values measured of (total) P_i, hydrolysis-driven P_i from poly-P, and contaminant P_i, respectively. Similarly, m and n are P_i concentrations in the hydrolysis product and contaminant, respectively. Since $\delta^{18}O_P'$ is the measured isotope value but $\delta^{18}O_P$ is needed for eq 3, a revised equation for fractionation factor (F) that includes the contaminant contribution (n) is

$$F = \frac{1}{a}\left[\frac{1}{m}\{(m+n)\delta^{18}O_P' - n\cdot\delta^{18}O_P''\} - (1-a)\cdot\delta^{18}O_P''\right] - \delta^{18}O_W \quad (5)$$

The presence of contaminant P_i influences measured isotopes as well as F especially under when the extent of poly-P hydrolysis is low. While the data in Table 1 are calculated accounting for contaminant P_i (using eq 5), it is worthwhile to mention that the difference in F if the contaminant P_i is ignored. For example, the F value for alkaline phosphatase was −4.48 and +3.99 when the contaminant P_i is ignored (using theoretical 0.25 and 0.75 fractional contributions of oxygen in P_i from water and poly-P, respectively) and accounted for (about 26% of total P_i was contributed by the contaminant), respectively. The difference in F was insignificant in both acid phosphatase enzymes because of limited (~6%) contribution of contaminant P_i.

As described above and illustrated in Figure 6, during the cleavage of one P−O bond in a poly-P molecule, one oxygen atom from water is incorporated into the released P_i, 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 bond and leaving the last P_i intact), the slope should ideally be 0.25. On the other hand, complete degradation of poly-P should result in a slope less than 0.25 because the last P_i moiety to be released contains all oxygens inherited from its parent poly-P. The poly-P used in enzyme incubation experiments had an average chain length of ~5. It is known that the enzymatic hydrolysis of poly-P is not random and occurs only at the terminal P−O bond.[54] For the low degradation efficiency of alkaline phosphatase (<20%; Figure 2), all released P_i ions have one oxygen atom derived from water and three oxygen atoms inherited from parent poly-P, leading to the theoretical slope of 0.25. For high poly-P degradation for acid phosphatase (>70%; Figure 1), one P_i (the last one in the poly-P chain) out of five P_i (average chain length of 5) inherits oxygen entirely from parent poly-P, that is

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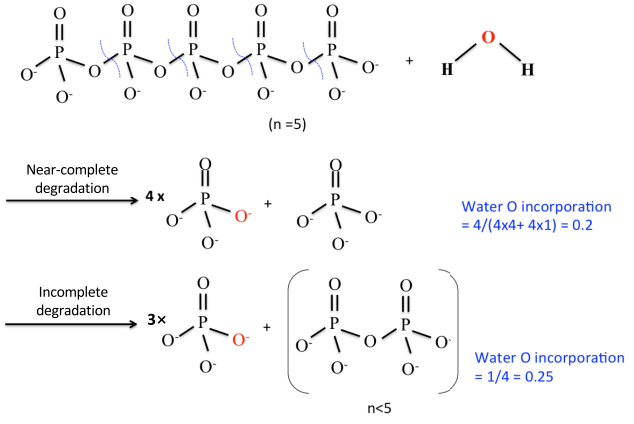

Figure 6. Variable slopes of $\delta^{18}O_P$–$\delta^{18}O_{H_2O}$ (see Figure 3) as a result of complete versus incomplete degradation of poly-P.

to say, only 4 out of 20 oxygen atoms in the released P_i come from water, leading to a slope of 0.20. There are, however, other factors that impact the regression slope. Sodium poly-P used in this study is impure and contains extra P_i (Figure 2). It is rather expected poly-P is a mixture of a variety of chain lengths. Thus, the actual fraction of water oxygen incorporation is not straightforward to estimate without complete degradation and without knowing the actual chain length distribution. This may explain the relatively large variability in the fractionation factors in acid phosphatase [0.15 (±4.95) and 0.91 (±5.64)] from wheat and from potato, respectively. The experiments were run in four different temperatures and repeated. Still, the higher uncertainties in the calculated fractionation factors for acid and alkaline phosphatase point toward the need of additional studies.

The disequilibrium isotope fractionation factors for both alkaline phosphatase and acid phosphatase enzymes from potato are slightly positive (but still around zero) under all temperatures. This is important because the disequilibrium fractionation factor during enzymatic degradation of many other organic P compounds is highly negative (range, −10 to −30‰).[30,31,55] Positive isotope fractionation factors, however, are theoretically possible and reported to occur in some natural environments.[56] For example, it has been found in RNA degradation catalyzed by alkaline phosphatase, in which the second step of the degradation is a reversible reaction that leads to equilibrium fractionation and thus yields a positive fractionation.[31] Similarly, a positive fractionation factor was also observed in phytate, a major P_o in the environment, degradation catalyzed by phytase enzyme from wheat (acid phosphatase) and *Aspergillus niger* (alkaline phosphatase).[36] Different $\delta^{18}O_P$ values of the bridging oxygen atom (C−O−P) and the three nonbridging oxygen atoms could be a potential reason for the positive isotope fractionation. However, the possibility of variable isotopes within a molecule is not expected based on the condition of synthesis and source P. The degradation mechanism of poly-P is still yet to be fully understood, especially the reason for the near-zero yet positive isotope fractionation.

Poly-P is commonly found in natural environments such as soils and sediments,[6] indicating its potential role as an important P_i source.[4,6] The use of phosphate oxygen isotope ratios to identify different organic P compounds and phosphohydrolase enzymes involved in the degradation reactions in natural systems is often challenged by the overlapping fractionation factors (range, −10 to −30‰).[57] Therefore, the unique fractionation factor of poly-P hydrolysis is encouraging and may provide opportunities to identify P_i originated from poly-P degradation.

Poly-P Cycling in *E. coli* and *P. putida* Cultures and Isotope Effects. Poly-P cycling in bacterial cultures has been studied for decades.[58,59] *E. coli* is the most commonly used bacterium 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.[1,8] The assimilation of P and formation of poly-P vary with cell activity and ambient P concentrations,[60,61] and the extent of changes in P_i in media as well as poly-P formed in this study was low. For example, the limited growth of *E. coli* was accompanied by the assimilation of ∼60 μM P_i after 48 h of incubation (Figure 4). Correspondingly, the concentration of poly-P in both the media and in cells increased during the exponential growth phase (12−20 h) and started to decrease once the cell growth reached the late stationary phase (∼30 h). The dynamics in poly-P and P_i quantified here suggest that *E. coli* assimilates P_i from surrounding environments to synthesize poly-P (Figures 4c,d and 5c,d). When the growth environment becomes more stressful (late stationary phase), poly-P starts to degrade. Interestingly, in the first set of experiments, with the decrease of poly-P concentration in the late stationary phase, P_i concentration did not increase but decreased by ∼20 μM at the end of incubation (Figure 4c). This trend may indicate that the degradation of poly-P during the late stationary phase does not release a significant amount of P_i from cells. Instead, poly-P is utilized to maintain the growth/survival, and the cells continue to assimilate P_i from the media. A similar relationship was observed in picoplankton.[5] When the initial P_i concentration is increased, there were differences in P_i and poly-P dynamics, albeit the general trend was comparable in two sets of experiments. Other possible reasons could be the difference in starting cell numbers and the metabolic status of cells. Nonetheless, these data suggest that *E. coli* assimilates P_i and synthesizes poly-P for the 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 P_i. These results of poly-P and P dynamics in *E. coli* are consistent with other studies.[9,62]

Phosphate oxygen isotope data showed consistent trends compared to poly-P and P_i variability. The increasing $\delta^{18}O_P$ values in the first 12 h may have resulted from microbial P_i assimilation. Uptake and assimilation of nutrients (e.g., NO_3^-, NH_4^+, and P_i) by microorganisms is often associated with isotope fractionation[63] as nutrients with lighter isotopes are preferentially utilized, a process that could enrich the residual nutrients with heavy isotopes. Based on the results from P_i assimilation by *E. coli*, an apparent fractionation factor of −3.27‰ was calculated in Blake et al.[27] using the simplified Rayleigh equation as follows

$$\Delta\delta^{18}O_P = \delta^{18}O_{P(t)} - \delta^{18}O_{P(0)} = 10^3(\alpha - 1)\ln f \quad (6)$$

where $\Delta\delta^{18}O_P$ is the difference of $\delta^{18}O_P$ values between time t and time 0, f is the fraction of P_i remaining in the media, and α is the kinetic fractionation factor. Results from this study, however, suggest a slightly larger fractionation (−5.65‰) for P_i assimilation by *E. coli* JM103 due to preferential uptake of lighter isotopologues over heavier isotopologues of P_i by cells. This fractionation for cyanobacterium *Synechococcus* PCC6803

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was found to be −3.33‰.[64] The fractionation, both kinetic vs equilibrium, is dependent on the type of active transporters of P_i present: P_{it} is found to facilitate the equilibrium isotopic effects by allowing the efflux of intracellular P_i, but P_{st} transporters, either high affinity or low affinity, do not allow P_i efflux extracellularly, thus imparting strong kinetic fractionation during uptake.[64] The apparently larger fractionation factor in this study during P_i assimilation is most likely due to limited P_i taken up by cells and larger uncertainty of calculation at a small P uptake. In Blake et al.,[27] the incubation time was much longer (∼600 h), and after 150 h, more than 500 μM P_i was assimilated by *E. coli*, which was >50% of the initial P_i concentration. Similarly, in Lis et al.,[64] a higher amount of P_i was taken up ($0.45 < f < 0.9$). In this study, within 12 h, only ∼65 μM P_i (∼10% of initial P_i) was assimilated (Figure 4c,d). Therefore, the larger apparent isotope fractionation in this study compared to those of Blake et al.[27] and Lis et al.[64] could be attributed to the lower amount of P_i assimilated during the incubation. It must, however, be stressed that the calculation of true fractionation requires the knowledge of isotope values both in the source (dissolved P_i) and sinks (P_i inside the cell including those in organelles). This is complicated by the fact that neither all P_i taken up by microbial cells could be extracted and measured nor the isotope values remain unchanged after biological uptake. Nevertheless, the opposite trends of the changes in isotopes and P_i and poly-P concentrations are suggestive of microbial assimilation of P_i.

When the cell growth entered the stationary phase after exponential growth, an excursion of $\delta^{18}O_P$ values toward equilibrium was observed. One possible reason could be the mixing of P_i generated from the 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, the P_i concentration did not increase with decreasing poly-P concentration but rather continued to decline (Figure 4c), suggesting a potentially P-stressed condition. The other reasons for isotope excursion are attributed to rapid microbial cycling, as microbial turnover catalyzes the exchange of water and P_i oxygen isotopes toward equilibrium.[27,29,53] In the late stationary phase (> 24 h), isotope values started to be heavier. The concentration of P used in the experiments was always high, so the P-limited condition is not expected. A detailed study using mutants of *Synechocystis* in P-replete and -deplete conditions has shown that isotope fractionation occurs under P-limited conditions.[64] However, the fractionation is reported from P-repleted condition with *E. coli* and *Klebsiella aerogenes*.[27,35] Isotope fractionation is apparent in other nutritional oxyanions, such as N, C, and S (during nitrate/sulfate assimilation and CO_2 uptake during photosynthesis).[65−67]

Trends of P_i and poly-P were different in *P. putida* than those of in *E. coli*. In the first set of experiments, *P. putida* had already accumulated a significant amount of poly-P (∼20 μM) at the onset of the experiment (Figures 4 and 5). During the 2 days of incubation, poly-P concentration decreased from 16 to 10 μM in cells, which indicates poly-P degradation. However, with the degradation of poly-P, *P. putida* still took up an additional P_i (∼100 μM) from the media. The heavier isotope values at time zero are difficult to explain, as it should be similar to that of the *E. coli* and should represent the media P_i isotope values (same media was used for both incubations). It is possible that these isotope values are impacted 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 a reduction of poly-P, likely attributed to the degradation of poly-P and rapid microbial P turnover under stressful conditions. This might possibly be originated from various factors including limitation of nutrients and impact of toxic products generated from cell metabolism, which is common in batch culture.

The microbial condition in the later stage of incubation experiments suggested by the active cycling of P_i and rapid degradation of poly-P (see above) is intriguing. Results from the first set of experiments are different from those in past publications on *P. putida*.[47] In Tobin et al.,[47] poly-P concentration increased in the first ∼25 h of incubation and then decreased but was always in opposite trend with P_i concentration. Yet, in the results obtained from the second set of experiments where the cell growth was significant (Figure 5), the opposite trend is evident. In this incubation, however, breakdown poly-P was observed even in the log growth phase. Once the cell growth reached the stationary phase, the rate of poly-P degradation increased (Figure 5 and the 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 conditions were used except for different concentrations of starting P_i. It is unclear, but the potential reasons might be different cell numbers and other variabilities (mentioned above) that may lead to the limitation or co-limitation of other nutrients,[47] which were not analyzed in this study. Further investigation is required to identify reasons for variable trends of poly-P and P_i and isotope values as a function of initial P_i.

CONCLUSIONS AND IMPLICATIONS

The results from the pure enzyme and poly-P substrate reaction suggest that some common phosphatase enzymes (i.e., alkaline phosphatase from *E. coli*, acid phosphatase from potato and acid phosphatase from wheat) are capable of catalyzing the degradation of poly-P. The extent of degradation, however, was different: alkaline phosphatase from *E. coli* catalyzes only ∼18% degradation of poly-P, whereas acid phosphatase from both potato and wheat could catalyze more than 70% degradation. Interestingly, enzymatic degradation led to a relatively small isotope fractionation: *F* for alkaline phosphatase from *E. coli* to be +3.99 (±15.86)‰, whereas for acid phosphatase from wheat and potato, +0.15 (±4.95)‰ and +0.91 (±5.64)‰, respectively. These fractionation factors are distinct from other organic P compounds and thus serve as substrate- and enzyme-specific isotope signatures. This highlights the application of phosphate oxygen isotope values as a proxy to trace environmental P_i derived from poly-P degradation.

Results from bacteria culture experiments (*E. coli* and *P. putida*) suggest that poly-P synthesis and degradation are controlled by the initial P_i concentration and dynamics is strongly associated with cell growth status: poly-P is synthesized during the exponential growth stage. Degradation of poly-P occurs when cell growth reaches the stationary phase, possibly due to the limitation of nutrients and the impact of toxic products generated from cell metabolism in batch culture. A kinetic isotopic fractionation (α) of ca. −5.65 (±1.02)‰ was observed during bacterial P_i assimilation (leading to residual P_i enriched in heavier isotopologue), which is higher than comparable studies.[27,64] The unique combination of

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fractionation factors and trends of poly-P and P_i and isotope values influenced by the exposure of cells to different starting P_i concentrations indicates the intricacies and also highlights the interdependence and feedback processes during poly-P cycling.

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Notes

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