Review

Phosphate oxygen isotopes within aquatic ecosystems: Global data synthesis and future research priorities

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HIGHLIGHTS

- Oxygen isotope ratio in dissolved inorganic phosphate a novel stable isotope tracer.
- Theoretical basis for application of this tracer in aquatic ecosystems reviewed.
- Protocols for determining phosphate oxygen isotope ratio summarised.
- Synthesis of global data from marine and freshwater ecosystems reported.
- Priorities for future research in this rapidly evolving field identified.

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ABSTRACT

The oxygen isotope ratio of dissolved inorganic phosphate ($\delta^{18}O_p$) represents a novel and potentially powerful stable isotope tracer for biogeochemical research. Analysis of $\delta^{18}O_p$ may offer new insights into the relative importance of different sources of phosphorus within natural ecosystems. Due to the isotope fractionations that occur alongside the metabolism of phosphorus, $\delta^{18}O_p$ could also be used to better understand the intracellular and extracellular reaction mechanisms that control phosphorus cycling. In this review focussed on aquatic ecosystems, we examine the theoretical basis to using stable oxygen isotopes within phosphorus research. We consider the methodological challenges involved in accurately determining $\delta^{18}O_p$, given aquatic matrices in which potential sources of contaminant oxygen are ubiquitous. Finally, we synthesise the existing global data regarding $\delta^{18}O_p$ in aquatic ecosystems, concluding by identifying four key areas for future development of $\delta^{18}O_p$ research. Through this synthesis, we seek to stimulate broader interest in the use of $\delta^{18}O_p$ to address the significant research and management challenges that continue to surround the stewardship of phosphorus.

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1. Introduction

Phosphorus (P) is an essential element for all life, being integral to the structure and function of key biomolecules including DNA, RNA, adenosine triphosphate (ATP) and phospholipids. Under ambient conditions in natural ecosystems, P is tightly cycled within the biosphere and can limit or co-limit primary producer and microbial communities (Ruttenberg, 2003). However, inputs of P to terrestrial and aquatic ecosystems from a range of sources, alongside fluxes of P between these ecosystems, have increased significantly as a result of global population growth and attempts to expand and intensify food production for human society (Filippelli, 2008; Liu et al., 2008). Recent increases in bioavailable P fluxes and concentrations have been linked to undesirable ecosystem changes, including increases in primary production, shifts in community composition, increased frequency of algal blooms and hypoxia, and reduced biodiversity within aquatic ecosystems (Sondergaard and Jeppesen, 2007; Smith and Schindler, 2009).

Inefficient P use and the lack of effective recycling of P within urban and agricultural systems define a 'broken' P biogeochemical cycle, leading to concern over whether human use of P resources is sustainable (Cordell et al., 2009; Childers et al., 2011; Elser and Bennett, 2011). Whilst estimates of mineral P reserves have increased dramatically in recent years (Jasinski, 2012), these estimates remain highly uncertain and future exhaustion of reserves is likely without major advances in P mining and recycling technologies (Vaccari and Strigul, 2011; Seyhan et al., 2012). Securing access to P remains a globally significant issue, given the reliance of agricultural production on inorganic P fertilisers and the current lack of alternative sources for P beyond mineral reserves (Beardsley, 2011).

Despite the importance of P to human society, and the implications for natural ecosystems of perturbations to the P cycle, understanding of the sources and reaction mechanisms controlling biogeochemical cycling of P within ecosystems remains limited (Blake et al., 2005; Slomp, 2011). A critical reason for this is the lack of inherent tracers for analysing the sources and the metabolism of P in natural ecosystems (Karl, 2000). For example, quantifying the importance of different sources of P in aquatic ecosystems has previously relied on mass flux budgets, spatial and temporal analysis of P concentration, export coefficient models and indirect tracers such as boron as a marker for waste water treatment plant (WWTP) effluent, or microbial source tracking to identify human versus agricultural sources of faecal contamination (Dillon and Kirchner, 1975; Smith et al., 1989; Neal et al., 2000; Jarvie et al., 2002; Scott et al., 2002; Simpson et al., 2002; Holt et al., 2003; Bowes et al., 2008). However, none of these approaches provides an inherent label for P. As a result, none offers a direct means of tracing specific sources or biogeochemical processes relevant to the P cycle.

The stable oxygen isotope ratio within phosphate (hereafter, $\delta^{18}O_p$) has recently emerged as a novel and potentially powerful inherent tracer for the sources and metabolism of P in natural ecosystems (McLaughlin et al., 2004; Elsbury et al., 2009; Young et al., 2009; Goldhammer et al., 2011a; Li et al., 2011). This review focusses on the use of $\delta^{18}O_p$ in aquatic ecosystems and particularly within freshwater ecosystems, complimenting a recent review dealing with $\delta^{18}O_p$ in soil–plant systems (Tamburini et al., 2014). Our objectives are to: i) examine the theoretical basis to the use of $\delta^{18}O_p$ in P research; ii) consider the methodological challenges involved in the analysis of $\delta^{18}O_p$ in

aquatic ecosystems; and iii) synthesise global data from initial application of $\delta^{18}O_p$ within aquatic ecosystems. We conclude by identifying future priorities for $\delta^{18}O_p$ research within freshwater ecosystems, aiming to stimulate further development and application of this technique.

2. Stable isotopes and P biogeochemistry: theoretical background

Phosphorus has three major isotopes, ^{31}P (stable), ^{32}P ($t_{1/2} = 14.36$ days) and ^{33}P ($t_{1/2} = 25.34$ days) (Smith et al., 2011). Biogeochemical cycling of P in aquatic ecosystems has previously been explored using the radioactive isotopes ^{32}P and ^{33}P (Benitez-Nelson and Buesseler, 1999; Benitez-Nelson, 2000). However, the use of radioisotopes is constrained by short isotope half-lives, perturbation of experimental systems associated with labelling, or the use of incubations which omits irregular events that occur in natural ecosystems, such as seasonal algal blooms (Levine et al., 1986; Thingstad et al., 1993; Benitez-Nelson, 2000).

Stable isotope ratios have been widely used to understand long-term trends and processes in both local and global biogeochemical cycles (Beveridge and Shackleton, 1994; Newton and Bottrell, 2007 and references therein). Stable isotope analysis requires an element to have a minimum of two naturally-occurring stable isotopes, with changes in the ratio of individual isotopes of an element in a sample, against a known ratio in a reference, providing insight into biogeochemical processes controlling the element cycle. Carbon (C), nitrogen (N) and oxygen (O) are commonly used in stable isotope research on the basis of ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$ respectively. However, stable isotope analyses cannot be conducted on the P atom in P-containing compounds, because ^{31}P is the only stable P isotope.

In natural aquatic ecosystems, P is often bound strongly to O in the form of the dissolved inorganic phosphate ion, defined hereafter as P_i. Therefore, attention has recently focussed on whether the stable isotope composition of O in P_i can be used to gain insight into the biogeochemical cycling of P. Isotope fractionation involves the preferential incorporation of one isotope from a starting material over another into a reaction product. Kinetic fractionation describes a process that preferentially selects one isotope (generally the lighter isotope) due to a faster reaction rate in a unidirectional reaction. In contrast, equilibrium fractionation is a thermodynamic effect in which a system has time to exchange isotopes continuously in order to achieve the lowest energy system, wherein the heavier isotope forms the strongest bond possible (Hoefs, 2008). The P–O bonds in P_i are resistant to inorganic hydrolysis under typical temperature and pressure conditions in the Earth's surface water and groundwater ecosystems (O'Neil et al., 2003). Therefore, without biological mediation, negligible isotope exchange occurs between P_i and water within these ecosystems (Tudge, 1960; Blake et al., 2001). Whilst the initial stages of some abiotic reactions, including the sorption of P_i to solid-phase iron-oxide or the formation of apatite, may be associated with kinetic fractionation, this does not persist and through time the stable isotope composition of the solid-phase P_i approaches that of the aqueous-phase P_i (Liang and Blake, 2007; Jaisi et al., 2010, 2011). In contrast, enzyme-catalysed processes cleave P–O bonds leading to kinetic or equilibrium fractionation between the isotopes of O in P_i and O in a surrounding fluid, either within a cell or within the extracellular environment (Blake et al., 2005). The fractionation between the stable isotopes of O (^{16}O and ^{18}O) within a

sample is expressed as $\delta^{18}O$, relative to Vienna Standard Mean Ocean Water (VSMOW), defined as $0\%_{oVSMOW}$ on the $\delta^{18}O$ scale (Eq. (1)).

$$\delta^{18}O_{sample} = 1000\left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} - 1\right]\%_{oVSMOW} \qquad (1)$$

Phosphate oxygen isotopes have previously been used within both the archaeological and environmental communities to determine palaeoclimate conditions at the time of formation of biogenic phosphatic material, such as apatite in fossil bones and teeth, as well as to reconstruct historical salinity and water temperatures from biogenic phosphate derived from fish remains (Longinelli and Nuti, 1973; Bryant et al., 1994; Fricke and O'Neil, 1996; Lécuyer et al., 1996; Stephan, 2000). Recent methodological advances in the analysis of $\delta^{18}O_p$ now provide the opportunity to use $\delta^{18}O_p$ as both a tracer of source and of the extent of metabolism of P in aquatic ecosystems (McLaughlin et al., 2004; Elsbury et al., 2009; Goldhammer et al., 2011b).

3. Isotope effects controlling $\delta^{18}O_p$ during biogeochemical cycling of P

A range of isotope effects associated with metabolic processes have the potential to influence $\delta^{18}O_p$. These effects are summarised in Fig. 1 and described in Sections 3.1 to 3.3.

3.1. Intracellular metabolism of P_i

A variety of P-containing compounds are present within aquatic ecosystems, including P_i, inorganic polyphosphates and organic compounds such as proteins, sugars and phospholipids (Karl and Tien, 1997). However, P_i is the species preferentially utilised by organisms as it is capable of diffusion across cytoplasmic cell membranes (Björkman and Karl, 1994; Liang and Blake, 2006b). Consequently, P_i is required for a number of intracellular reactions, including cellular signalling through phosphorylation and dephosphorylation, as well as being incorporated within biomass during cell growth (Blake et al., 1997, 2005). The major enzyme that catalyses intracellular P cycling is inorganic pyrophosphatase, due to its ability to mediate reversible reactions such as the cleavage of adenosine-5′-triphosphate to adenosine-5′-diphosphate (Cohn, 1958; Blake et al., 2005; Fig. 2). Intracellular reactions catalysed by inorganic pyrophosphatase cause complete and very rapid (in the order of minutes) O isotope exchange between O

atoms in P_i and O atoms in surrounding water molecules that donate O atoms to newly produced P_i molecules (Kolodny et al., 1983; Blake et al., 1997). Thermodynamic fractionation characterises the exchange of O between P_i and the surrounding fluid, resulting in temperature-dependent equilibrium between O in P_i and O within intracellular water ($\delta^{18}O_w$, expected to be identical to extracellular $\delta^{18}O_w$ (Fricke et al., 1998)).

Release of P from biomass into the extracellular environment is driven by two mechanisms. Firstly, from live biomass as a by-product of uptake and intracellular metabolic reactions, in order to maintain non-toxic intracellular P concentrations (Cooperman et al., 1992). Secondly, following the death and lysis of cells (see Section 3.3). Within environments in which P_i is extensively taken up and recycled through live biomass, an initially distinctive $\delta^{18}O_p$ composition in the extracellular environment, potentially reflecting sources of P, could be overprinted following intracellular, equilibrium fractionation and release of P_i to the extracellular environment. The $\delta^{18}O$ value of a P_i molecule in equilibrium with surrounding water can be predicted, based on the measured $\delta^{18}O_w$ and temperature of the surrounding water, using the empirically-derived Eq. (2) (Longinelli and Nuti, 1973). This is the basis for using historical $\delta^{18}O_p$ in solid-phase samples, such as tooth enamel, as a palaeothermometer, given an ecosystem in equilibrium via intracellular metabolism (Kolodny et al., 1983; Fricke et al., 1998).

$$T(°C) = 111.4 - 4.3\left(\delta^{18}O_p - \delta^{18}O_w\right) \qquad (2)$$

where T is the average growth temperature during shell formation for sampled organisms and $\delta^{18}O_p$ and $\delta^{18}O_w$ refer to the isotopic composition of P_i and environmental water (at 25.2 °C) respectively. Whilst predominantly based on biogenic apatite from marine organisms, one freshwater species, *Unio pictorum*, was included in the data from which Eq. (2) was derived, suggesting no significant difference between seawater and freshwater equilibria equations. Although Eq. (2) has been used widely, more recent development of techniques for purification of P_i from solid materials and for determination of $\delta^{18}O_p$ led Pucéat et al. (2010) to propose a revised phosphate–water fractionation equation based on empirical work (Eq. (3)):

$$T(°C) = 118.7(\pm 4.9) - 4.3(\pm 0.20)\left(\delta^{18}O_p - \delta^{18}O_w\right) \qquad (3)$$

Based on empirical Eqs. (2) or (3), if both the ambient water temperature and $\delta^{18}O_w$ are determined then the expected equilibrium value for $\delta^{18}O_p$ can be calculated. Comparing the theoretical value for $\delta^{18}O_p$ at
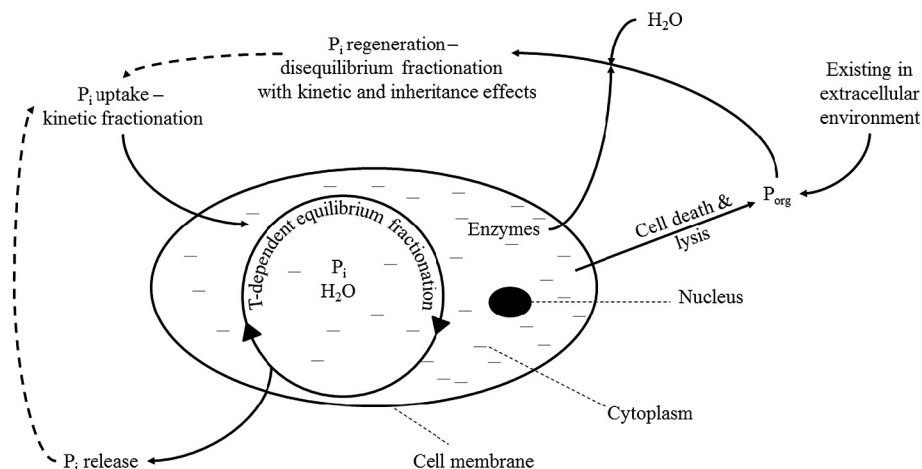

Fig. 1. Schematic diagram describing the major isotope effects that can occur within the intracellular or extracellular environment due to metabolic processes. P_{org} = organic P compounds; P_i = dissolved inorganic phosphate ion; H_2O = water molecule, T = temperature.
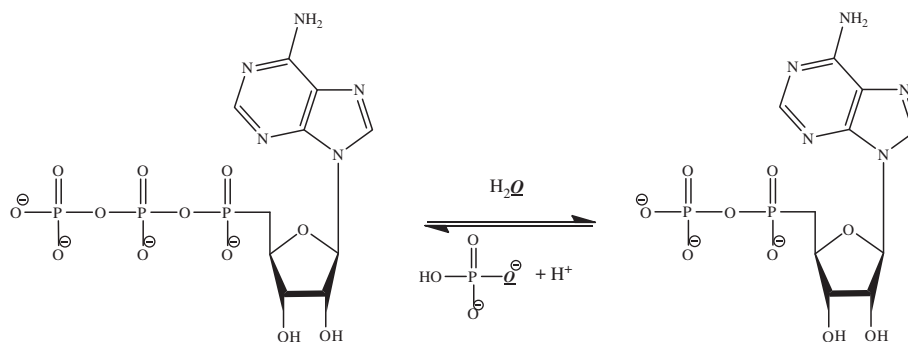
Fig. 2. Reversible reaction of the dephosphorylation of adenosine-5′-triphosphate to adenosine-5′-diphosphate, in which P_i is generated as a by-product (Gajewski et al., 1986).

equilibrium with $\delta^{18}O_p$ observed in an environmental sample can provide insight into the extent to which P_i has been recycled through intracellular metabolic reactions, assuming that $\delta^{18}O_p$ in the sources of P to an ecosystem was originally at disequilibrium.

3.2. Uptake of extracellular P_i

Many biologically-mediated processes associated with isotope effects are kinetically controlled, for example bacterial sulfate reduction and methanogenesis (Nakai and Jensen, 1964; Whiticar, 1999). During bacterial sulfate reduction, bacteria preferentially reduce ^{32}S- and ^{16}O-containing sulfate molecules, because these molecules have lower bond energies due to a lower combined mass. Thus, bacteria can metabolise isotopically lighter sulfate at a faster rate, resulting in the formation of isotopically light sulfide relative to the starting sulfate pool, assuming that only partial reduction of the available sulfate occurs (e.g. Habicht and Canfield, 1997). A similar kinetic fractionation may also be imparted on the P_i pool during biological uptake of P_i from the extracellular environment. Initial research reported by Blake et al. (2005) has demonstrated that *Escherichia coli* grown in controlled laboratory conditions preferentially take up $^{31}P^{16}O_4^{3-}$ compared to the isotopically-heavier $^{31}P^{18}O_4^{3-}$, resulting in enrichment of the extracellular environment with $^{31}P^{18}O_4^{3-}$. If this kinetic isotope effect operates more widely in natural ecosystems, the effect would only be observed
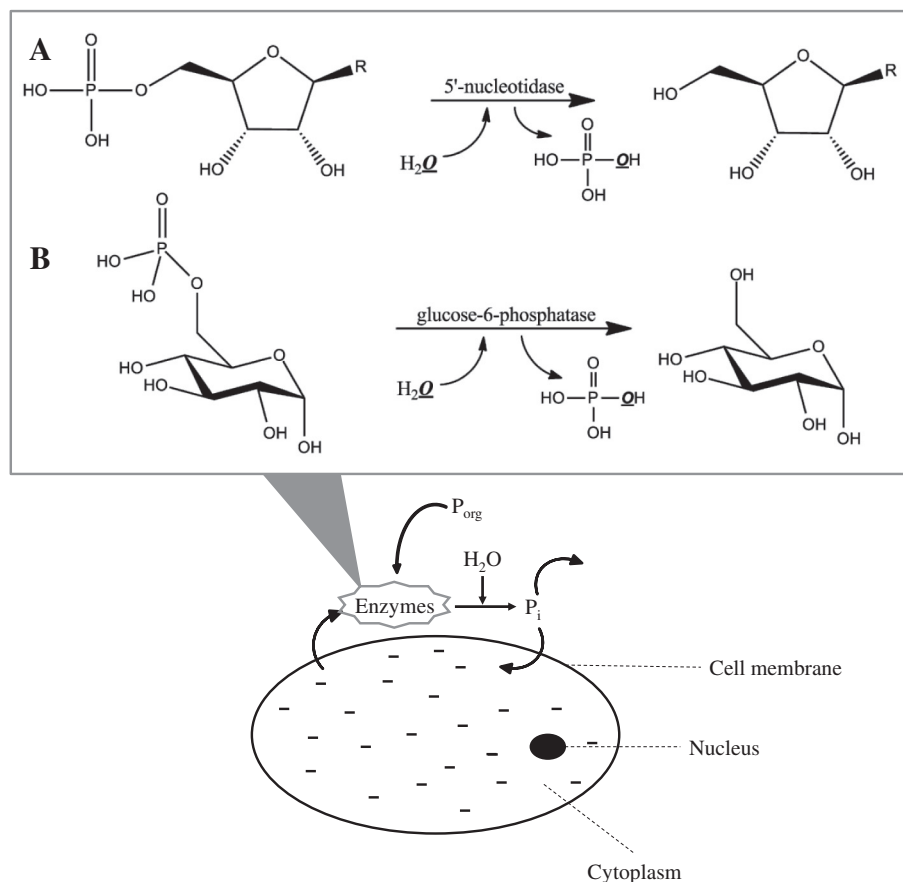
Fig. 3. Schematic diagram representing extracellular metabolism of P_i, in which enzymes are released to the extracellular environment beyond the cytoplasmic membrane to metabolise P_{org} and release P_i which is subsequently transported across the cell membrane. Reactions include: A: Hydrolysis of a 5′nucleotide molecule through the action of 5′nucleotidase; and B: Hydrolysis of glucose-6-phosphate through the action of glucose-6-phosphatase. Both reactions yield a P_i molecule as a by-product.

given unidirectional reactions in which the reactants are not fully consumed and where competing fractionations do not over-print that associated with biological uptake of P_i.

3.3. Extracellular hydrolysis of organic P compounds

Organic P (P_{org}) compounds can be used to support metabolism given low bioavailable P_i concentrations within the extracellular environment. However, P_{org} compounds are too large for direct diffusion across cytoplasmic cell membranes (Liang and Blake, 2006b). Therefore, P_{org} must be hydrolysed through enzyme-mediated reactions involving phosphohydrolases that are either attached to the outside of a cell membrane or are secreted by organisms into the extracellular environment (Liang and Blake, 2006b). These extracellular metabolic reactions release P_i as a by-product (Fig. 3) that can be transported into a cell via diffusion or via ATP-driven pumps or transport proteins (Ammerman, 1991).

During enzyme-catalysed hydrolysis of P_{org}, $P-O$ bonds are cleaved and O atoms within the original P_{org} compound are replaced with O atoms from surrounding water molecules in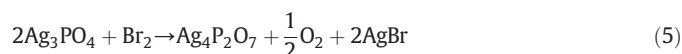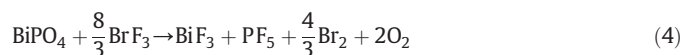 the newly created P_i. The specific enzyme and associated hydrolysis pathway will influence the extent of O isotope exchange. For hydrolysis catalysed by a phosphomonoesterase, one O atom within the newly produced P_i will be incorporated from the surrounding water-O and the remaining three O atoms are inherited from the P_{org} compound. With hydrolysis catalysed by a phosphodiesterase, two O atoms will be incorporated from the surrounding water-O (in a two-stage process involving incorporation of one O atom due to the action of the phosphodiesterase and the second O atom due to phosphomonoesterase-catalysed hydrolysis of the newly produced monoester), with the remaining two O atoms inherited from the P_{org} compound. Because extracellular hydrolysis of P_{org} does not result in complete exchange of all four O atoms in P_i with O atoms in water, inheritance isotope effects occur in which $\delta^{18}O_p$ of the newly produced P_i lies between that of the starting P_{org} compound and $\delta^{18}O_w$ (Blake et al., 1997; Colman et al., 2005). Incorporation of water-O within P_i during extracellular hydrolysis of P_{org} is also accompanied by a kinetic fractionation, due to more rapid incorporation of the isotopically lighter ^{16}O atom compared to the ^{18}O atom from water molecules in the resulting P_i (Blake et al., 2005). To date however, the magnitude of the fractionation factor associated with this process has only been quantified for a small number of enzyme–substrate combinations (e.g. Liang and Blake, 2006b).

In many aquatic environments, extracellular hydrolysis is significant in the regeneration of P_i (Ammerman and Azam, 1985; Chrost, 1991; Colman et al., 2005; Goldhammer et al., 2011a). The fate of P_i regenerated from P_{org} depends on the initial driver for P_i regeneration from organic matter. Under conditions of P_i limitation, regenerated P_i is required to meet the metabolic demand for P among heterotrophic microorganisms. Alternatively, dephosphorylation of particulate or dissolved P_{org} may be required prior to uptake of C compounds to meet intracellular energy or C requirements among heterotrophic organisms (Colman et al., 2005; Goldhammer et al., 2011a). Because C rather than P demand drives dephosphorylation under these conditions, not all P_i regenerated from P_{org} is necessarily taken up and recycled extensively through biomass. For example, it has been estimated that in coastal waters only 10–15% of P_i produced through the action of secreted 5′-nucleotidase was taken up by microorganisms (Ammerman and Azam, 1985). The remaining 85–90% may enter the P_i pool of the water column and affect the bulk $\delta^{18}O_p$ value of the extracellular environment. Effects on extracellular $\delta^{18}O_p$ due to regeneration of P_i from P_{org} that is driven by C-limitation have also been observed in deep-ocean water and in marine sediment porewater (Colman et al., 2005; Goldhammer et al., 2011a).

4. Analytical protocols for determining $\delta^{18}O_p$

Historically, $\delta^{18}O_p$ has been determined through fluorination or bromination of a phosphate precipitate, traditionally bismuth(III) phosphate ($BiPO_4$) although currently silver(I) phosphate (Ag_3PO_4) (Lécuyer,

2004). Fluorination (Eq. (4)) and bromination (Eq. (5)) halogenate the metal ion, reducing the bromine present and releasing gaseous O_2 which can be quantitatively converted into CO_2 for analysis (Vennemann et al., 2002; Lécuyer, 2004). However, fluorination (which can also be achieved using BrF_5) and bromination reactions can suffer from the use of hazardous materials, poor O_2 yields, time intensive preparation (precipitation of $BiPO_4$ requires 6 days of extraction time relative to the 3 days of the Ag_3PO_4 precipitation method) and the requirement for large sample sizes in both methods (typically 4–5 mg Ag_3PO_4) (Lécuyer et al., 1993; Vennemann et al., 2002; Lécuyer, 2004; Gruau et al., 2005).

$$BiPO_4 + \frac{8}{3}BrF_3 \rightarrow BiF_3 + PF_5 + \frac{4}{3}Br_2 + 2O_2 \tag{4}$$

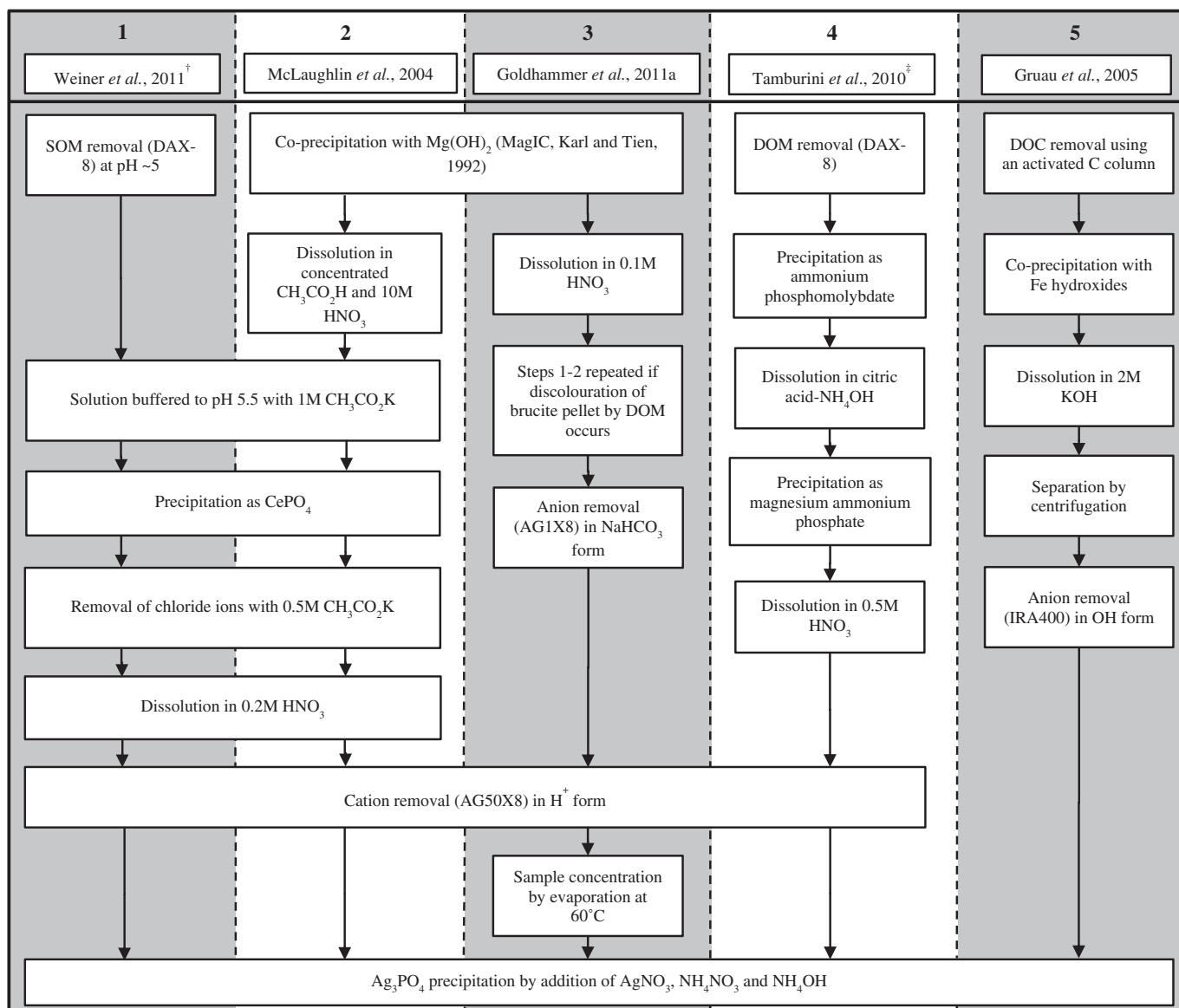$$2Ag_3PO_4 + Br_2 \rightarrow Ag_4P_2O_7 + \frac{1}{2}O_2 + 2AgBr \tag{5}$$

Isotope ratio mass spectrometry (IRMS) allows for the precise measurement of small mass differences between different isotopes within a compound and, when coupled to a thermal conversion/elemental analyser (TC/EA), the system can carry out relatively rapid on-line measurements of isotope ratios (LaPorte et al., 2009). These systems have been automated, whilst retaining both precision and accuracy, for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ solid sample analysis. A further advantage of TC/EA-IRMS is the relatively small sample mass required for analysis (typically 400–500 μg Ag_3PO_4). To establish similar TC/EA-IRMS protocols for $\delta^{18}O$ measurements in P_i, aqueous samples containing P_i must be converted from dissolved to solid form. In this conversion, particular care must be taken to ensure that: i) the original isotope composition of P_i is retained by using non-fractionating reactions or ensuring reactions go to completion; ii) P_i is not introduced through hydrolysis within P_{org}-rich natural samples; and iii) other O-bearing compounds including dissolved organic carbon (DOC), nitrate (NO_3^-), sulfate (SO_4^{2-}) and calcium carbonate ($CaCO_3$) are excluded from the final solid product.

Bismuth(III) phosphate ($BiPO_4$) is a hygroscopic material, with full rehydration following dehydration possible after 15 min in air. The $BiPO_4$ precipitate requires significant preparation before isotopic analysis to remove adsorbed water which can otherwise increase O yields by up to 12.5% in $\delta^{18}O_p$ analysis (Karhu and Epstein, 1986), with $\delta^{18}O_p$ enrichment possible when drying at temperatures greater than 200 °C (Mooney-Slater, 1962; Shemesh and Kolodny, 1988). Such constraints limit the utility of $BiPO_4$, leading to consideration of alternative solid-phase compounds for analysis of $\delta^{18}O_p$. Silver(I) phosphate (Ag_3PO_4) has been identified as a suitable alternative, due to its weakly hygroscopic nature, stability, low solubility and relatively short preparation time (Lécuyer, 2004; McLaughlin et al., 2004; Tamburini et al., 2010). Firsching (1961) was among the first to precipitate Ag_3PO_4 in order to gravimetrically determine the phosphate concentration of a homogenous solution. Whilst silicates and multivalent ions were observed to interfere with Ag_3PO_4 precipitation, ions with lower valences did not significantly interfere (e.g. NO_3^-, ammonium (NH_4^+) and potassium (K^+)), or exerted only a slight effect at high concentrations (sodium (Na^+) and SO_4^{2-}). However, Ag_3PO_4 has only more recently become a viable basis for analysis of $\delta^{18}O_p$ in aqueous environmental samples, following advances in extraction protocols that enable the precipitation of a sufficient mass of Ag_3PO_4 for analysis from complex matrices that often contain low concentrations of P_i (Colman, 2002; McLaughlin et al., 2004).

4.1. P_i extraction protocols for aqueous matrices

The major published protocols for extraction of P_i via precipitation of Ag_3PO_4 are summarised in Fig. 4. None of the protocols in Fig. 4 currently represents a definitive approach for all aquatic matrices. Development

1	2	3	4	5
Weiner *et al.*, 2011[†]	McLaughlin *et al.*, 2004	Goldhammer *et al.*, 2011a	Tamburini *et al.*, 2010[‡]	Gruau *et al.*, 2005

SOM removal (DAX-8) at pH ~5

Co-precipitation with Mg(OH)$_2$ (MagIC, Karl and Tien, 1992)

DOM removal (DAX-8)

DOC removal using an activated C column

Dissolution in concentrated CH$_3$CO$_2$H and 10M HNO$_3$

Dissolution in 0.1M HNO$_3$

Precipitation as ammonium phosphomolybdate

Co-precipitation with Fe hydroxides

Solution buffered to pH 5.5 with 1M CH$_3$CO$_2$K

Steps 1-2 repeated if discolouration of brucite pellet by DOM occurs

Dissolution in citric acid-NH$_4$OH

Dissolution in 2M KOH

Precipitation as CePO$_4$

Anion removal (AG1X8) in NaHCO$_3$ form

Precipitation as magnesium ammonium phosphate

Separation by centrifugation

Removal of chloride ions with 0.5M CH$_3$CO$_2$K

Dissolution in 0.5M HNO$_3$

Anion removal (IRA400) in OH form

Dissolution in 0.2M HNO$_3$

Cation removal (AG50X8) in H$^+$ form

Sample concentration by evaporation at 60°C

Ag$_3$PO$_4$ precipitation by addition of AgNO$_3$, NH$_4$NO$_3$ and NH$_4$OH

Fig. 4. Categorisation of published protocols for the precipitation of Ag$_3$PO$_4$ from aqueous samples. [†]Weiner et al. (2011) method is based on soil extracts using anion-exchange resin. [‡]Tamburini et al. (2010) method is based on HCl extracts of soil samples.

and evaluation of extraction protocols are required across the range of aquatic samples in which $\delta^{18}O_p$ research is undertaken, to ensure that accurate determination of $\delta^{18}O_p$ is achieved within these complex matrices.

There are commonalities across these protocols, particularly across procedures 1–4: i) concentration of P$_i$ through co-precipitation of P$_i$ with brucite following the magnesium-induced co-precipitation (MagIC) method of Karl and Tien (1992); ii) redissolution of the brucite precipitate in an acid matrix; iii) removal of other potential sources of O using anion exchange resins and/or sequential precipitations; iv) removal of cations that have the potential to interfere with Ag$_3$PO$_4$ precipitation, using a cation exchange resin; iv) precipitation of Ag$_3$PO$_4$. The method of Gruau et al. (2005) in Fig. 4 was developed for freshwaters and includes an initial dissolved organic matter (DOM) removal step using activated carbon. This protocol is based on the BiPO$_4$ method of Longinelli et al. (1976), with the benefit of a weakly hygroscopic end precipitate (Ag$_3$PO$_4$) relative to BiPO$_4$.

Final precipitation of Ag$_3$PO$_4$ is accomplished using either 'slow' or 'fast' approaches. Both precipitation methods involve the addition of silver nitrate (AgNO$_3$), ammonium hydroxide (NH$_4$OH) and ammonium nitrate (NH$_4$NO$_3$) to form a near-neutral pH solution. Whilst 'slow'

precipitation is achieved by holding a solution at ~50 °C over several days to allow large Ag$_3$PO$_4$ crystals to grow, 'fast' precipitation can achieve full precipitation in a matter of minutes (Dettman et al., 2001; Tamburini et al., 2010). The 'fast' precipitation also has the added advantage of 100% Ag$_3$PO$_4$ yields independent of sample sizes, whereas lower recovery has been observed using the 'slow' precipitation, particularly for smaller P$_i$ masses (Dettman et al., 2001). However, the isotopic difference in Ag$_3$PO$_4$ generated by the two methods has been found to be within expected interlaboratory variation (Dettman et al., 2001).

Some research has applied the McLaughlin et al. (2004) protocol that was originally developed for marine systems to freshwater matrices without modification, whilst other research has adapted one of the major Ag$_3$PO$_4$ precipitation protocols to include clean-up stages for samples containing high concentrations of particulate organic matter and DOM. Particulate organic matter in environmental samples is typically removed through filtration at a maximum filter pore size of 0.45 μm (Elsbury et al., 2009; Li et al., 2011). In organic-rich sample matrices, including many freshwaters, inefficient removal of DOM could significantly influence measured $\delta^{18}O_p$, because DOM can consist of up to 45% O by weight and has been shown to persist until the precipitation

of Ag_3PO_4 (Ma et al., 2001; Lécuyer, 2004; McLaughlin et al., 2004; Zohar et al., 2010). For accurate determination of $\delta^{18}O_p$, all O-containing contaminants, especially DOM, must be removed from the Ag_3PO_4 precipitate prior to TC/EA-IRMS. This remains a significant challenge for research seeking to apply $\delta^{18}O_p$ to complex freshwater matrices. Removal of DOM from samples has been attempted through adsorption of organic compounds to activated carbon (Gruau et al., 2005), washing the final Ag_3PO_4 precipitate with hydrogen peroxide to oxidise organic compounds (Tamburini et al., 2010; Zohar et al., 2010), using resins to specifically remove DOM (Tamburini et al., 2010), repetition of precipitation steps such as MagIC in order to isolate P_i from a matrix of potential contaminants (Goldhammer et al., 2011b), and pH-specific precipitations to remove fulvic and/or humic acids (Zohar et al., 2010).

Beyond a source of contaminant O, DOM includes P_{org} compounds that may undergo acid hydrolysis during the protocols described in Fig. 4, for example during the dissolution of brucite. Hydrolysis of P_{org} that is co-precipitated with brucite can yield P_i, with potential incorporation of water-O from the extracellular environment during acid hydrolysis (McLaughlin et al., 2006d). For example, in synthetic samples, 98.3% of the mass of P in ATP has been shown to co-precipitate with brucite, and 10.3% of the mass of P co-precipitated as ATP can undergo acid hydrolysis to yield P_i using the 1:1 concentrated acetic acid (CH_3CO_2H) and 10M nitric acid (HNO_3) system within Protocol 2 (Davies et al., 2014, Fig. 5). The P_i generated from hydrolysis of P_{org} will subsequently be incorporated within a Ag_3PO_4 precipitate, potentially altering the bulk $\delta^{18}O_p$ composition. Therefore, if the original $\delta^{18}O_p$ of P_i is to be retained, effective removal of DOM must be achieved prior to introduction of conditions that could hydrolyse P_{org} compounds.

Although research to date has focussed on analysis of $\delta^{18}O_p$ in P_i, the oxygen isotope composition of the phosphate moieties within P_{org} compounds ($\delta^{18}O_{Porg}$) is also of significant interest, both as a constraint on the inheritance effects that influence $\delta^{18}O_p$ in P_i regenerated from P_{org}, but also, potentially, as an inherent tracer of P_{org} cycling within aquatic ecosystems. Analysis of $\delta^{18}O_{Porg}$ requires extraction of P_i from P_{org} followed by precipitation of a solid-phase P compound. Following a review of several extraction methods, Liang and Blake (2006a) concluded that extraction using UV radiation does not result in significant isotope effects on $\delta^{18}O_{Porg}$. However, we are not aware of any published research that has sought to characterise $\delta^{18}O$ in P_{org} compounds from aquatic samples using the apparently robust UV radiation extraction method.

4.2. Determination of $\delta^{18}O_p$ through EA-IRMS

On-line EA-IRMS has been utilised for analysis of Ag_3PO_4. Dried samples (typically 400–500 μg) are introduced in silver capsules as the traditional and cheaper tin alternatives can lead to significant deterioration of chromatography results (Lécuyer et al., 2007). Conversion of Ag_3PO_4 to CO for analysis is achieved through pyrolysis at 1270 to 1450 °C in the presence of a carbon source to aid full conversion, typically nickelised graphite and/or glassy carbon (Vennemann et al., 2002; Lécuyer et al., 2007; Halas et al., 2011). Water vapour is removed through a water trap and CO is separated from other gaseous impurities through gas chromatography using purge-and-trap technology or a packed GC column (Meier-Augenstein, 1999; Fourel et al., 2011). Helium is used as a carrier gas to transfer the sample gas into a mass spectrometer via a continuous flow mode. Mass signals of 28 ($^{12}C^{16}O$) and 30 ($^{12}C^{18}O$; $^{14}C^{16}O$; $^{13}C^{17}O$) are integrated and compared to those in an independently introduced pulse of pure CO reference gas to calculate the $^{18}O/^{16}O$ ratio. Subsequently, these ratios are calibrated to the VSMOW scale in per mill notation (‰$_{VSMOW}$) using international reference materials, typically benzoic acids and barium sulfates (LaPorte et al., 2009; Halas et al., 2011). The precision for isotopic analysis is generally quoted as better than ± 0.3‰$_{VSMOW}$ (1 standard deviation) (Lécuyer et al., 2007; LaPorte et al., 2009; Halas et al., 2011).

A major analytical challenge remains the lack of internationally certified Ag_3PO_4 standards, despite the long-term use of Ag_3PO_4 in the determination of $\delta^{18}O_p$ in archaeological and palaeoclimate studies (Stephan, 2000; Vennemann et al., 2002; Halas et al., 2011). Using materials other than Ag_3PO_4 as calibration standards, such as benzoic acids, can introduce matrix effects in which differences in chemical composition between materials can influence the analysis, for example through differing temperatures at which efficient pyrolysis is achieved (Lécuyer et al., 2007). Some researchers have produced internal synthetic Ag_3PO_4 standards from either internationally recognised $\delta^{18}O$ standards such as NBS120c (phosphate rock distributed by NIST), or KH_2PO_4 solutions equilibrated with ^{18}O-enriched water (Lécuyer et al., 2007; Fourel et al., 2011; Halas et al., 2011). These synthetic Ag_3PO_4 materials have been shown to be stable over long periods (at least 8 years), a key requirement for any international reference material (Lécuyer et al., 2007). A number of these uncertified compounds have been distributed to other laboratories
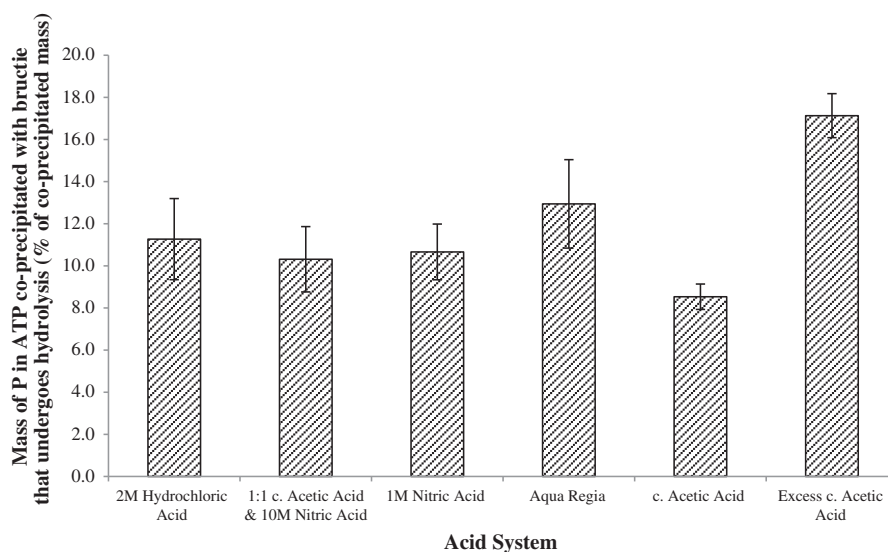

Fig. 5. Extent of ATP hydrolysis after co-precipitation with, and dissolution of, brucite using minimum volumes (for complete dissolution) of five different acid systems, and one system (Excess c.Acetic Acid) when an 8-fold volume excess of acid was used. Mean values given, error bars show ± 1 standard deviation (n = 3 for each acid system; Davies et al., 2014).

and initial inter-laboratory comparison undertaken (Lécuyer et al., 2007; Halas et al., 2011). However, finite availability and the lack of a quality control network in production represent significant challenges for broader use of these materials.

Linearity effects, referring to a change in the reported isotope ratio due to a change in sample mass introduced to the EA-IRMS and thus the peak size of the CO ion in the mass spectrometer (Brand, 2004), can also affect determination of $\delta^{18}O_p$. If this effect is consistent and can be quantified then the measured isotope ratios can be corrected (Brand, 2004). One alternative solution to address linearity effects is to match CO ion peak heights across a run for both samples and standards by using a narrow sample mass range. However this is made more complicated by variable Ag_3PO_4 pyrolysis efficiency and CO yield between samples, which can cause variations in peak heights. Difficulties have been found in ensuring complete conversion of Ag_3PO_4 to CO in the TC/EA, with Ag_3PO_4 requiring pyrolysis in the hot spot of a furnace (LaPorte et al., 2009). Whilst many compounds can achieve complete conversion easily (especially at temperatures exceeding 1400 °C), thereby ensuring accurate analysis of the bulk $\delta^{18}O$, incomplete conversion of other matrices, such as carbonates, has been implicated in isotope fractionation (Koziet, 1997; Kornexl et al., 1999). If conversion to CO is not complete then the reaction order of the O atoms in a compound becomes significant, with those being most strongly bound to the P atom being under-represented in the CO product. If this fractionation can be shown to affect all samples equally then the results can be properly calibrated. However, if the kinetic fractionation is not equivalent across all samples due to variable conversion to CO,

then correction becomes problematic. This highlights the need to ensure the pyrolysis of Ag_3PO_4 is efficient and standardised across a sample run, which requires precise pyrolysis of samples within the hot spot of a furnace.

5. Synthesis of global $\delta^{18}O_p$ data from aquatic ecosystems

Recent research has highlighted the potential to use $\delta^{18}O_p$ as both a tracer of P sources and as a dynamic tracer of metabolic processes affecting P cycling in aquatic ecosystems. The characterisation of significant sources of P_i within an ecosystem can be achieved using $\delta^{18}O_p$ assuming:

  i) $\delta^{18}O_p$ for major sources of P_i is constrained;
 ii) Individual sources of P_i possess distinct $\delta^{18}O_p$ signatures;
iii) $\delta^{18}O_p$ for P_i sources is not equal to the theoretical equilibrium;
 iv) $\delta^{18}O_p$ signatures for P_i sources are maintained and not rapidly over-printed by fractionation associated with metabolic processes.

Given these pre-requisites, if $\delta^{18}O_p$ within an ecosystem tends towards the value of a particular source, inferences regarding the dominant P_i sources within ecosystems can be made. However, under conditions in which iv) above is not met and $\delta^{18}O_p$ tends towards the theoretical equilibrium (Eqs. (2) or (3)), $\delta^{18}O_p$ can then be used to probe the extent of intracellular metabolism of P_i. Insights into other metabolic processes, including biological P_i uptake and extracellular hydrolysis of P_{org}, may be gained through the kinetic and inheritance isotope effects associated with these processes. Table 1 synthesises published $\delta^{18}O_p$ data currently available across a range of aquatic

Table 1
Synthesis of global $\delta^{18}O_p$ data derived from a range of aquatic ecosystems.

Type	Setting	Min $\delta^{18}O_p$/‰	Max $\delta^{18}O_p$/‰	Av $\delta^{18}O_p$/‰	1σ	Number of samples	Geographical locations	Reference
Marine	Coastal surface waters	15.9	22.3	20.0	1.34	141	Monterey Bay, California; Long Island Sound, USA	McLaughlin et al. (2004); Colman et al. (2005); McLaughlin et al. (2006b)
	Open ocean surface (<200 m depth)	13.8	23.7	18.5	3.27	17	Monterey Bay, California; Sargasso Sea	McLaughlin et al. (2004); McLaughlin et al. (2013)
	Deep ocean (>200 m depth)	17.4	25.4	22.6	2.08	31	North Atlantic and Pacific Ocean tropical gyres; Sargasso Sea	McLaughlin et al. (2004); Colman et al. (2005); McLaughlin et al. (2013)
Estuarine		7.8	20.3	15.9	2.78	54	North San Francisco Bay California; Elkhorn Slough, California;	McLaughlin et al. (2004); McLaughlin et al. (2006a); McLaughlin et al. (2006c)
Freshwater	River (main)	8.6	15.2	12.4	1.46	32	San Joaquin River and Lake Tahoe California; Lake Erie, USA	Elsbury et al. (2009); McLaughlin et al. (2006a); Young et al. (2009)
	River (tributaries)	9.2	17.2	12.5	2.03	32	Elkhorn Slough, San Joaquin River and Lake Tahoe California; Lake Erie, USA	McLaughlin et al. (2006a); Young et al. (2009)
	Groundwater	15.1	22.4	18.6	2.13	9	Elkhorn Slough and San Joaquin River, California	Blake et al. (2001); McLaughlin et al. (2006a); Young et al. (2009)
	Lake (surface water – 1 m)	10	17.1	14.1	1.57	34	Lake Erie, USA	Elsbury et al. (2009)
	Lake (5.5–22 m)	8.4	15.8	13.6	1.58	25	Lake Erie, USA	Elsbury et al. (2009)
	Lake (58 m)	–	–	12.7	–	1	Lake Erie, USA	Elsbury et al. (2009)
	WWTP final effluent	8.4	18.4	12.9	4.02	14	Brittany, France;	Gruau et al. (2005); Young et al. (2009); McLaughlin et al. (2006c)
	Constructed wetlands (canals, inlet, interior and outlet)	20.0	25.5	21.5	1.32	25	Florida Everglades, USA	Li et al. (2011)
	Marsh	21.6	25.1	23.5	1.61	4	Florida Everglades, USA	Li et al. (2011)
Sediments	Marine porewater	12.8	26.6	22.2	2.59	119	Benguela Upwelling, South Atlantic	Goldhammer et al. (2011a)

ecosystems. Below, we consider the ways in which recent research has begun to explore the use of $\delta^{18}O_p$ in aquatic ecosystems, highlighting the insights into P biogeochemistry that can be derived using this stable isotope technique.

5.1. Marine ecosystems

Marine upwelling zones, in which nutrient-rich bottom water is driven towards the surface, are areas of enhanced primary production and potentially rapid P turnover (Pennington and Chavez, 2000). Research in upwelling zones has reported $\delta^{18}O_p$ values that approach equilibrium with water-O, indicating substantial intracellular metabolism of P_i in these coastal ecosystems (Colman et al., 2005; McLaughlin et al., 2006b). However, a lack of complete equilibrium between $\delta^{18}O_p$ and water-O in upwelling zones suggests biological turnover of P_i relative to inputs from terrestrial and deep-water sources can be lower in coastal upwelling zones compared to the open ocean (McLaughlin et al., 2006b).

In the surface water of the open ocean, P_i concentrations in the photic zone can be extremely low (e.g. 0.2–1 nM in the Sargasso Sea), often limiting or co-limiting primary production (Wu et al., 2000). Under these conditions, $\delta^{18}O_p$ values that tend towards temperature-dependent equilibrium with water-O have been reported, indicating extensive cycling of P_i through biomass (Colman et al., 2005). Insights into temporal variations in P sources and the extent of biological turnover of P within marine ecosystems can also be gained through $\delta^{18}O_p$. For example, in Monterey Bay, California, McLaughlin et al. (2006b) observed $\delta^{18}O_p$ values in the upper water column (<200 m) that were significantly influenced by source contributions from terrestrial freshwater and from deeper water (500–800 m). However, during the spring–summer periods of upwelling, $\delta^{18}O_p$ in the upper water column was isotopically heavier relative to non-upwelling autumn/winter periods, and more closely reflected the $\delta^{18}O_p$ value expected at theoretical equilibrium. The most likely explanation for this trend is enhanced intracellular cycling of P_i by phytoplankton relative to P_i inputs during periods of upwelling. Upwelling periods provide nutrients to the surface zone that, combined with a greater intensity of sunlight in the spring–summer months, can enhance productivity and consequently biological turnover of P_i (McLaughlin et al., 2006b). Extensive extracellular–intracellular–extracellular cycling of P_i during the upwelling periods would lead to an over-printing of source $\delta^{18}O_p$ signatures and a shift of $\delta^{18}O_p$ in the extracellular environment towards equilibrium values. However, the data reported by McLaughlin et al. (2006b) suggest that the extent of extracellular–intracellular–extracellular was still not sufficient, even in the spring–summer period, to establish complete equilibrium between $\delta^{18}O_p$ and water-O.

The preferential remineralisation of P over C from dissolved organic compounds has been observed in marine ecosystems, particularly in the euphotic zone (Clark et al., 1998; Aminot and Kerouel, 2004). This selective remineralisation of P_{org} is caused by the presence of extracellular enzymes and their action on P_{org} substrates, meaning that the turnover rate of P_{org} molecules in this environment is dependent on the specific enzymes and P_{org} compounds present. For example, it has been shown that the flux of dissolved ATP through an oligotrophic ocean ecosystem can be up to fivefold greater than for the bulk P_{org} compound pool (Bjorkman and Karl, 2005). McLaughlin et al. (2013), in research conducted in the Sargasso Sea, used $\delta^{18}O_p$ to investigate the role of P_{org} hydrolysis in P-cycling in marine ecosystems. Based on the fractionation imparted on $\delta^{18}O_p$ when P_i is regenerated from a specific P_{org} molecule following a specific hydrolysis pathway, McLaughlin et al. used Eq. (6) to estimate the fraction of P_i in samples that had been regenerated from P_{org}:

$$\%P_i \text{ from } P_{org}\text{remineralisation} = \frac{\left(\delta^{18}O_{sample} - \delta^{18}O_{eq}\right)}{\left(\delta^{18}O_{rem} - \delta^{18}O_{eq}\right)} \times 100\% \qquad (6)$$

where $\delta^{18}O_{rem}$ is the calculated value of $\delta^{18}O_p$ based on a particular combination of P substrate and hydrolysis pathway; $\delta^{18}O_{eq}$ is the expected

equilibrium value of $\delta^{18}O_p$ and $\delta^{18}O_{sample}$ is the measured $\delta^{18}O_p$ in a sample. McLaughlin et al. concluded that 5–80% of P_i present in the upper 200 m of an oligotrophic marine ecosystem was the product of P_{org} remineralisation, dependent on which enzyme/substrate system was used in Eq. (6). These data, combined with expressed enzyme activity and low P_i concentration, suggest that P_i regeneration from P_{org} was coupled with uptake by organisms to support metabolism. The proportion of P_i thought to derive from P_{org} remineralisation was significantly reduced (12–35%) at a monitoring station closest to the shoreline, suggesting increased P_i availability in this region and thus reduced requirement for P_{org} mineralisation. Despite the limited number of samples and large contrasts between differing enzyme/substrate systems, this research highlights the potential for using $\delta^{18}O_p$ to gain insights into P cycling within the marine ecosystem, without the need to directly determine the concentrations or fluxes affecting P_i and P_{org} pools.

Colman et al. (2005) also demonstrated that $\delta^{18}O_p$ was close to or at theoretical equilibrium in deep water samples from both the Atlantic (900–4000 m) and Pacific (300–3000 m) oceans. These data are consistent with intracellular equilibration between $\delta^{18}O_p$ and water-O in surface waters, followed by advection of P_i to depth. However, small offsets ($-1.5\%_{VSMOW}$) from the expected theoretical equilibrium in P_i from deep-ocean samples collected below the thermocline were used by Colman et al. to infer that significant P_{org} mineralisation occurred in this zone, imparting a disequilibrium fractionation on $\delta^{18}O_p$. The disequilibrium effects were not fully overprinted by equilibrium fractionations, supporting estimates that 80–95% of the remineralised P_i was not utilised by the surrounding microorganisms in these deep-ocean waters to meet their P requirements, because metabolism in these zones is hypothesised to be limited by C and energy rather than by P. However, the size of the $\delta^{18}O_p$ offset from equilibrium was smaller than that expected on the basis of P_i regeneration from P_{org} through the action of phosphohydrolases, suggesting partial biological turnover of P_i in these deep ocean environments (Colman et al., 2005).

5.2. Estuarine ecosystems

Source $\delta^{18}O_p$ signatures have been shown to be retained within some estuaries (e.g. McLaughlin et al., 2006a). Source signals representing inputs derived from surrounding land (e.g. chemical fertilisers associated with runoff from agricultural land) and from other aquatic ecosystems (e.g. groundwater and surface water tributaries) are thought to dominate the bulk $\delta^{18}O_p$ composition in many estuarine ecosystems, due to the short residence time of P compounds in tidal estuaries (McLaughlin et al., 2006a). The limited contact time with biomass in these ecosystems constrains the opportunity for metabolism of P, increasing the potential for source $\delta^{18}O_p$ signatures to be retained. McLaughlin et al. (2006a) demonstrated that towards the mouth of Elkhorn Slough in California, $\delta^{18}O_p$ was dominated by that of oceanic-derived P_i (~20‰$_{VSMOW}$) due to tidal flushing of the ecosystem in this area. Similar $\delta^{18}O_p$ values were also found upstream in the estuary. However, these were attributed to chemical fertilisers, entering the estuarine ecosystem through runoff from agricultural fields, that have an isotopic composition similar to that of the marine ore from which the fertilisers were formed. One mid-estuary site had a significantly lower $\delta^{18}O_p$ composition. This observation, combined with the use of other natural tracers, suggested that groundwater discharge significantly affected bulk $\delta^{18}O_p$ at this sampling location. $\delta^{18}O_p$ can also provide insights into the presence of temporal and spatial patterns in P_i sources to estuaries. For example, it was observed that $\delta^{18}O_p$ composition throughout the main estuary channel was not affected by either tidal or seasonal variations (McLaughlin et al., 2006a). However, a nearby harbour, close to the mouth of the estuary, did show evidence of tidal patterns through $\delta^{18}O_p$, with high tides associated with $\delta^{18}O_p$ values higher and closer to that of oceanic-derived P_i and low tides associated with lower $\delta^{18}O_p$ values towards those observed upstream in the estuary.

5.3. Sediments within aquatic ecosystems

$\delta^{18}O_p$ has also been used as a tracer of particulate inorganic P sources and sinks within aquatic ecosystems (Markel et al., 1994; Jaisi and Blake, 2010). The first major lacustrine site for this application was Lake Kinneret, situated in the Dead Sea Rift Valley (Markel et al., 1994). Markel et al. (1994) showed that $\delta^{18}O_p$ in sediment bound-P_i varied significantly with sediment grain size, with clay bound-P_i demonstrating significant enrichment of $\delta^{18}O_p$ compared to silt or sand bound-P_i. These $\delta^{18}O_p$ signatures enabled an internal P-cycling model of Lake Kinneret to be developed, which included major processes such as the sedimentation of both detrital and authigenic apatite and CCP (Ca$_3$(-HCO$_3$)$_3$(PO$_4$)), as well as the dissolution of these materials. In conjunction with other internal P-cycling studies of Lake Kinneret, $\delta^{18}O_p$ was used as a tool to elucidate processes such as the release of P through dissolution of authigenic apatite and to support the quantitative analyses of sediment–lake interactions found in other studies. Further work has also shown that $\delta^{18}O_p$ may act as a tracer of P source and as a paleotemperature proxy (particularly in detrital and authigenic P phases) in other ecosystems, such as the sediments of continental margins (Jaisi and Blake, 2010).

More recent methodological advances that enable accurate measurements of $\delta^{18}O_p$ in samples containing a small mass of P_i have underpinned studies of $\delta^{18}O_p$ within the porewaters of deep marine sediments (Goldhammer et al., 2011a,b). These studies demonstrate that $\delta^{18}O_p$ in extracted porewater, P_i concentration profiles and two-endmember isotope mixing models can be used to determine whether marine sediments are dominated by P release (regeneration of P_i during microbial respiration of organic matter and the desorption of P_i from mineral phases), P burial from sources in the overlying water column, or P_i movement through advection or diffusion within the sediment column (Goldhammer et al., 2011a,b). In particular, it was shown that marine sediment porewaters demonstrate significant disequilibrium in $\delta^{18}O_p$, reflecting the dominance of disequilibrium effects associated with P_i regeneration from organic matter (Goldhammer et al., 2011a). However, it was noted that simple isotope mass balance models can overlook processes which could be significant within these environments and thus improvements need to be made for future analyses, including the construction of more complex and realistic isotope models that allow for the incorporation of physical mixing processes, such as diffusion and advection.

6. Freshwater ecosystems

Beyond the relatively intensive study of Lake Erie reported by Elsbury et al. (2009), the catalogue of $\delta^{18}O_p$ data for freshwater ecosystems remains more constrained than for marine and terrestrial ecosystems. However, a limited number of initial studies have explored the use of $\delta^{18}O_p$ to assess sources and metabolism of P_i in freshwater ecosystems.

6.1. Application of $\delta^{18}O_p$ to identify sources of P in freshwater ecosystems

A range of potential sources of P to freshwater ecosystems may be distinguished on the basis of $\delta^{18}O_p$, as long as the source isotope signature is not rapidly over-printed by isotopic fractionations associated with metabolism (e.g. Young et al., 2009; Li et al., 2011). However, this emerging dataset also shows potential for significant overlap in $\delta^{18}O_p$ between a number of individual sources. For example, whilst $\delta^{18}O_p$ may be used to distinguish P derived from fertilisers compared to WWTP effluents, no significant differences were found between $\delta^{18}O_p$ across other potential sources, such as vegetation and detergents (Young et al., 2009). Table 2 synthesises currently available data regarding $\delta^{18}O_p$ in potential sources of P to freshwaters, with average $\delta^{18}O_p$ in these sources ranging from 13.2 to 21.5‰. However, note that for many of these sources, the size of the available dataset is currently small and geographically constrained.

Gruau et al. (2005) compared $\delta^{18}O_p$ across three commonly used inorganic P fertilisers and effluent samples from three WWTPs in France. The origin of the phosphatic rock used in the production of the fertilisers was found to be significant in the resulting fertiliser $\delta^{18}O_p$ (19.6–23.1‰$_{VSMOW}$), which was consistent with the typical ranges for the two largest phosphatic rock suppliers to France–Morocco (18.5‰$_{VSMOW}$ to 20.5‰$_{VSMOW}$) and Florida (17.2‰$_{VSMOW}$ to 23.2‰$_{VSMOW}$) respectively. Laboratory dissolution experiments demonstrated no significant fractionation of $\delta^{18}O_p$, suggesting that fertiliser $\delta^{18}O_p$ could be preserved in runoff from agricultural land (Gruau et al., 2005). Phosphate in WWTP effluents from the study by Gruau et al. was assumed to originate from two main sources: human waste, including faeces, urine and waste food disposal (30–50%) and phosphate-based detergents (50–70%). For these WWTP effluents, $\delta^{18}O_p$ values fell in the narrow range of 16.6 to 18.1‰$_{VSMOW}$, whilst $\delta^{18}O_p$ for a phosphate builder used in detergents fell within the same range (17.9‰$_{VSMOW}$), indicating possible maintenance of source $\delta^{18}O_p$ composition in the final WWTP effluent. However, the $\delta^{18}O_p$ composition of WWTP effluents could also reflect microbial metabolism of P_{org}, as reported values also fall in the $\delta^{18}O_p$ range expected following fractionation due to the degradation of P_{org} associated with microbial metabolism (Blake et al., 1997; Gruau et al., 2005). The difference between $\delta^{18}O_p$ ranges for fertiliser- and WWTP-derived phosphate (<2‰$_{VSMOW}$) reported by Gruau et al. was statistically significant. However, given the relatively small absolute difference, and the variability in $\delta^{18}O_p$ of the two sources (ranges for both sources were ≥1.5‰$_{VSMOW}$), these authors concluded that $\delta^{18}O_p$ may not be suitable for determining the sources of anthropogenic P_i.

However, the $\delta^{18}O_p$ of other potentially significant sources of P_i to freshwaters has not been thoroughly characterised, including soil leachate, waste from aquaculture and, in particular, groundwater and septic tank discharge. Where sources such as inorganic fertiliser or WWTP effluent have begun to be characterised, available data tend to focus on specific geographical regions such as California (USA) (Young et al., 2009), the Great Lakes (USA) (Elsbury et al., 2009; Young et al., 2009)

Table 2
Observed $\delta^{18}O_p$ values for potential P sources to freshwater ecosystems (Ayliffe et al., 1992; Gruau et al., 2005; McLaughlin et al., 2006a; Young et al., 2009; Li et al., 2011; Gross et al., 2013).

Source	Min $\delta^{18}O_p$/‰	Max $\delta^{18}O_p$/‰	Av $\delta^{18}O_p$/‰	1σ	Number of samples	Geographical location
Chemical fertilisers	15.5	25.3	22.1	2.32	33	–
Fertiliser ore and processing	18.2	21.6	20	1.43	5	Israel
[†]Aerosols and dust	14.2	24.9	20.1	2.17	17	Israel
WWTP final effluent and during processing	8.4	18.4	13.5	3.5	18	USA; France
Detergent	13.3	18.6	16.8	1.83	7	–
Toothpaste	–	–	17.7		1	–
[†]Animal faeces	15.7	23.1	20	1.82	11	South Pacific; California, USA
[†]Soil leachate	17.9	19.1	18.5	0.474	5	Israel
Compost	23.3	27	25.2	2.62	2	California, USA
[†]Vegetation leachate (Live, Dead and Decayed)	14.2	23.1	16.8	2	27	California, USA

[†] Water soluble P_i fractions.

and Brittany (France) (Gruau et al., 2005). A larger body of research is required to extend the isotopic characterisation of sources of P_i to other geographical locations. Further, the majority of existing studies provide only snapshots in time, and the potential for temporal changes in $\delta^{18}O_p$ of sources has not been fully constrained, for example within WWTP effluent in which the dominant sources and treatment efficiency may change significantly over annual, seasonal or daily timescales.

Analysis of $\delta^{18}O_p$ in natural freshwater samples has demonstrated that disequilibrium isotopic compositions can persist in these environments (Young et al., 2009; Li et al., 2011). Young et al. (2009) compared $\delta^{18}O_p$ in the tributaries to three major US freshwater bodies, Lake Erie, the San Joaquin River and Lake Tahoe, to a theoretical equilibrium value. Only two of the 40 samples analysed in the study fell within the 95% confidence limit for the theoretical isotopic equilibrium. This was also true for two groundwater samples in the San Joaquin catchment, in which data indicated that both groundwater samples were enriched in ^{18}O relative to the expected equilibrium isotopic composition.

Similar conclusions were reached by Li et al. (2011) who showed that anthropogenic phosphate fertilisers could be traced within a freshwater wetland in the Everglades National Park, USA. Inputs of P from artificial fertilisers in the Everglades Agricultural Area (EAA) have contributed to mean concentrations of P in runoff being 20-fold greater than in non-agricultural areas of the national park. The average $\delta^{18}O$ of fertilisers used within the EAA was found to be $24.4 \pm 0.7‰_{VSMOW}$ between April 2005 and March 2006. Analysis revealed that $\delta^{18}O_p$ was at equilibrium with the ambient water in one constructed wetland (April 2005), although later sampling in July and March 2006 determined that $\delta^{18}O_p$ was strongly governed by fertiliser inputs, being enriched with $\delta^{18}O$ compared to the expected equilibrium, particularly close to the inlet from the EAA. Seasonal variation was also observed, with $\delta^{18}O_p$ being enriched in the colder, winter months relative to samples collected in July. These observations may reflect differences in metabolic activity between summer and winter months, or alternatively reflect the timing of fertiliser applications. At least within the specific catchments reported in research by Li et al., disequilibrium isotope composition was shown to be maintained in P_i under certain circumstances. However, the maintenance or otherwise of disequilibrium $\delta^{18}O_p$ remains to be evaluated across a wider range of spatial and temporal scales for freshwater ecosystems.

If disequilibrium isotope compositions do persist in freshwater ecosystems and the major P_i sources differ significantly in $\delta^{18}O_p$, then it would be possible to utilise $\delta^{18}O_p$ in source apportionment calculations. Li et al. (2011) estimated the proportion of P_i derived from artificial fertiliser inputs (F) using a two-system mass balance model (Eq. (7)), the theoretical equilibrium isotope composition ($\delta^{18}O_{recycled}$), the isotopic composition of fertiliser ($\delta^{18}O_{fertliser}$) and the isotopic composition of P_i measured in a sample ($\delta^{18}O_p$).

$$F = \frac{\left(\delta^{18}O_p - \delta^{18}O_{recycled}\right)}{\left(\delta^{18}O_{fertliser} - \delta^{18}O_{recycled}\right)} \times 100\% \qquad (7)$$

This mass balance equation is based on the assumption that only fertiliser inputs and biologically recycled phosphate make up the P_i of the wetland. Using this equation, it was estimated that artificial fertiliser inputs into the wetland contributed between 15 and 100% of the total dissolved P_i content, with the lowest and highest proportions found in samples collected in April and in March respectively. Clearly, more complicated mass balance approaches would be required for more complex ecosystems with multiple sources of P. However, if sources, and any fractionation effects, could be sufficiently well constrained, then it would be theoretically possible to build mixing models to estimate the relative contribution from different sources to P within receiving waters.

6.2. Evidence of metabolism of P in freshwater ecosystems from $\delta^{18}O_p$ analysis

Evidence of temperature-dependent equilibrium fractionation between $\delta^{18}O_p$ and $\delta^{18}O_w$ has also been used to infer full or partial metabolism of P_i in some freshwater ecosystems (Blake et al., 2001; Elsbury et al., 2009). For example, Blake et al. (2001) analysed $\delta^{18}O_p$ within groundwater samples from a shallow P_i-rich aquifer in Cape Cod, Massachusetts, USA. Significant inputs of P_i to groundwater resulted from sewage contamination, causing P_i concentrations to rise to 30–108 μmol/L. Full equilibration between $\delta^{18}O_p$ and $\delta^{18}O_w$ in the surrounding groundwater was not achieved, probably due to low DOC concentrations meaning that elevated P_i concentrations were above metabolic requirements. However, a strong positive correlation was found between $\delta^{18}O_p$ and $\delta^{18}O_w$, suggesting partial metabolism of P and that $\delta^{18}O_p$ could be used to provide insights into metabolism of P_i in groundwater.

The first major study of the application of $\delta^{18}O_p$ in the water column of a lacustrine setting was conducted in Lake Erie, USA and emphasises the potential to use $\delta^{18}O_p$ within freshwater P cycling studies to identify trends that cannot be detected through analysis of concentration alone (Elsbury et al., 2009). Despite strict regulations on point sources of P since the 1970s and no observed increase in influx to the lake, P_i concentration in the Central Basin of Lake Erie has increased since 1990 (Elsbury et al., 2009). As changes in inflow P_i fluxes could not explain the observed increase in P_i concentration, analysis of $\delta^{18}O_p$ was initially used in an attempt to identify additional sources of P_i.

Weighted average riverine $\delta^{18}O_p$ feeding the lake was considerably depleted in ^{18}O compared to that of the theoretical equilibrium $\delta^{18}O_p$, whereas the $\delta^{18}O_p$ values observed in Lake Erie were generally enriched in ^{18}O compared to those of the seven sampled tributaries, particularly in the Central Basin. Any samples with $\delta^{18}O_p$ between that of the theoretical equilibrium and the weighted riverine signal ($+11‰_{VSMOW}$) could potentially be attributed to incomplete equilibration of the O within riverine phosphate with water-O in the lake though metabolism of P_i. Consequently, Elsbury et al. (2009) concluded that samples with $\delta^{18}O_p$ values outside of the potential mixing region must be derived from an additional (unconstrained) source of P_i. Potential sources included smaller tributaries that were not sampled in the study; however this was deemed improbable given the large P_i fluxes required to achieve the observed concentration increase in the Central Basin. Alternatively, disequilibrium isotopic effects could be operating in the lake, leading to an increase of $\delta^{18}O_p$ in lake P_i though processes associated with a kinetic fractionation (Blake et al., 2005). However, the most likely explanation for the observed increase in P_i concentration was concluded to be end-member mixing of riverine P_i with an isotopically enriched source(s) of P_i ($> +17‰_{VSMOW}$), which was suggested by Elsbury et al. to be associated with remineralisation of ^{18}O-enriched P_{org} within the bed sediments, followed by release of P_i from the lake sediments to the water column.

7. Concluding remarks and priorities for future research

The use of $\delta^{18}O_p$ in research examining P cycling in natural ecosystems is at an embryonic stage, particularly with respect to freshwater ecosystems. This stable isotope tracer has the potential to offer new insights into both the relative importance of different sources of P to ecosystems, and the extent to which P from individual sources is linked to metabolic activity within ecosystems. These insights would have important implications for understanding the reaction mechanisms controlling P biogeochemistry in nature, and for the design and targeting of future policies and practices to deliver more sustainable stewardship of P. Below, we conclude by identifying four key areas for development of $\delta^{18}O_p$ research that would enable the potential of this isotope tracer to be more fully realised:

- *Develop robust analytical protocols for $\delta^{18}O_p$ in freshwater matrices*. The primary challenge here is to avoid errors in the determination of $\delta^{18}O_p$

due to incorporation of contaminant O in a precipitate that is subsequently pyrolysed. In freshwater matrices this involves excluding O derived from DOC compounds and from oxyanions other than P_i from the final precipitate. However, it also requires removal of P_{org} compounds from an aquatic matrix at the appropriate stage of an analytical protocol to ensure that hydrolysis of P_{org} and production of contaminant P_i is avoided. Finally, despite recent inter-laboratory comparison of the isotope composition of various sources of Ag_3PO_4, the need for an internationally certified Ag_3PO_4 standard has yet to be addressed.

- *Expand the global library of $\delta^{18}O_p$ within sources of P to freshwaters.* Proper characterisation of $\delta^{18}O_p$ in P sources provides the basis for using this stable isotope tracer as a source apportionment tool. Initial data suggests that distinguishing certain sources on the basis of $\delta^{18}O_p$ may be possible, whilst the isotopic composition of other sources of P_i may overlap. However, the global database of $\delta^{18}O_p$ in sources of P remains limited. The priorities are: i) to characterise sources that are not currently included in the global database, for example P_i within potable water supplies and within septic tank discharges; ii) to extend the geographical extent to which individual sources are characterised, for example covering regions in which inorganic P fertiliser is derived from differing rock P reserves, or regions in which differing $\delta^{18}O_w$ may drive differences in $\delta^{18}O_p$ within the final effluent of WWTPs; and iii) to constrain the temporal variation in $\delta^{18}O_p$ within key sources of P, for example associated with seasonal or diurnal cycles in the final effluent from WWTPs.

- *Focus on the potential of $\delta^{18}O_p$ to provide insight into biogeochemical processes controlling P cycling.* $\delta^{18}O_p$ will not act as a conservative tracer for the source of P where metabolic reactions significantly influence the P cycle within an ecosystem. Whilst clearly a constraint on source apportionment studies, the isotope fractionations that occur alongside metabolic processes provide the opportunity to use $\delta^{18}O_p$ to explore the reaction mechanisms controlling P cycling. Specifically, the balance between equilibrium and disequilibrium fractionations can provide insight into the relative importance of intracellular metabolism of P_i versus the extracellular regeneration of P_i from P_{org}. This requires future research to determine and interpret variations in $\delta^{18}O_p$, both through time (for example, from inter-annual or seasonal cycles to event-driven variations within lake or river ecosystems) and through space (for example, tacking downstream changes in $\delta^{18}O_p$ associated with point or diffuse inputs of P to aquatic ecosystems). Further research could also focus on analysis of $\delta^{18}O$ within the phosphate moieties of P_{org} to better understand the cycling of P_{org} within aquatic ecosystems.

- *Couple $\delta^{18}O_p$ with parallel techniques to develop an integrated isotope framework across C, N and P cycles.* Whilst analysis of $\delta^{18}O_p$ is beginning to emerge as a research tool in aquatic ecosystems, parallel stable isotope techniques have a longer history of application for N ($^{15}N/^{14}N$ in ammonium and $^{15}N/^{14}N$, $^{18}O/^{16}O$ in nitrate) and for C ($^{13}C/^{12}C$). Coupling analysis of $\delta^{18}O_p$ with that of stable isotopes in N and C compounds would potentially offer new insights into process interactions between C, N and P biogeochemistry. A coupled stable isotope framework could draw on natural abundance approaches or, ultimately, on isotopic labelling studies based on production of labelled $\delta^{18}O_p$ to complement isotopically labelled sources of N and C. Finally, by coupling stable isotope and radioisotope techniques, processes that govern the cycling of P within aquatic ecosystems across both the short and long term could be more readily constrained.

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