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Analysis of oxygen isotopes of inorganic phosphate ($\delta^{18}O_P$) in freshwater: A detailed method description

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Abstract: The ability to identify the origin of phosphorus is essential to effectively mitigate eutrophication of freshwater ecosystems. The oxygen isotope composition of orthophosphate ($\delta^{18}O_P$) has been suggested to have a significant prospective as a tracer for P entering freshwater ecosystems. The $\delta^{18}O_P$ tracing method is, however, still in its preliminary stages and has proven challenging to implement for new practitioners. In order to achieve progress in developing the application of $\delta^{18}O_P$ signatures as a tracing tool, there is a need to eliminate the methodological challenges involved in accurately determining $\delta^{18}O_P$. This technical note describes the various steps needed to concentrate and isolate orthophosphate in freshwater samples into an adequately pure analyte (Ag_3PO_4), without isotopic alteration during processing. The protocol compiles the disperse experiences from previous studies, combined with our own experience.

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1. Introduction

In freshwater ecosystems, phosphorus (P) is usually the primary limiting nutrient for growth of macrophytes, algae and cyanobacteria. Increased P concentrations can therefore result in eutrophication, anoxia and degradation of water quality in lakes, rivers and streams (Blake et al., 2005; Hecky & Kilham, 1988; Wetzel, 2001). Phosphorus input to surface water aquatic ecosystems originates from various sources including septic tanks, waste water treatment plants, agricultural fertilizers, animal excreta and dissolved minerals (Heiberg et al., 2012; Marion et al., 1994; Quinton et al., 2010; Sharpley et al., 2003). Thus, identifying the various potential phosphorus sources and their relative contribution to the total phosphorus load is essential for restoration and improvement of eutrophic aquatic ecosystems (Elsbury et al., 2009; McLaughlin, Kendall, et al., 2006).

Identification of the source and apportioning the contributions of phosphorus discharging to surface water from various sources is not a trivial matter and requires an appropriate tracer, which can accommodate this complexity (Jaisi et al., 2011). An ideal tracer is part of the phosphate molecule without changing its properties.

Dissolved inorganic orthophosphate (referred to as P_i hereafter) is the primary form of P cycled through ecosystems (Moorleghem et al., 2013). Hence, the stable oxygen isotope of inorganic phosphate ($\delta^{18}O_P$, in which the subscript 'p' denotes 'phosphate') has been suggested as a significant prospective tracer for P cycling in the environment (Blake et al., 1997, 2005; Colman, 2002; Jaisi & Blake, 2014; McLaughlin et al., 2004). The $\delta^{18}O_P$ can be used as a tracer, since the P-O bond in P_i is resistant to inorganic hydrolysis at temperatures and pH levels found in natural abiotic aquatic ecosystems (Blake et al., 1997; Liang & Blake, 2007; Longinelli et al., 1976). Subsequently, the $\delta^{18}O_P$ value in abiotic aquatic ecosystems will reflect the isotopic signature of the P sources (Tamburini et al., 2010; Zohar et al., 2010). Biological mediation in aquatic ecosystems can, however, alter the source $\delta^{18}O_P$ signatures, through biological uptake and recycling. This will result in an isotopic equilibrium between the stable oxygen isotopes in the ambient water ($\delta^{18}O_w$) and the P_i sources (Blake et al., 2005). Consequently, the $\delta^{18}O_P$ value in abiotic aquatic ecosystems will only reflect the isotopic signature of the P sources when the biological activity is relatively low compared to the input of P_i.

1.1. The $\delta^{18}O_p$-method

Traditionally, the determination of $\delta^{18}O_P$ was established through fluorination (Crowson et al., 1991; Longinelli, 1966) or bromination of a phosphate precipitate, which generally was in the form of bismuth(III)-phosphate ($BiPO_4$) (Kolodny et al., 1983; Longinelli et al., 1976; Longinelli & Nuti, 1973b, 1973a; Shemesh et al., 1983, 1988). The $BiPO_4$ precipitate is a hygroscopic material that rehydrates within 15 minutes after dehydration, hence significant preparation is required before isotopic analysis. Recent methods use silver(I) phosphate (Ag_3PO_4) (Colman, 2002; Crowson et al., 1991; Lécuyer, 2004; Tamburini et al., 2010) which is less hygroscopic, is stabile, has low solubility, and results in better O yield during quantitative conversion of the PO_4-O to CO-O, and requires less preparation time (Crowson et al., 1991; Firsching, 1961). Multivalent ions and silicates interfere with Ag_3PO_4 precipitation, however low valence ions did not impact precipitation (i.e. NO_3^-, NH_4^+ and K^+) (Firsching, 1961).

Accordingly, Ag_3PO_4 precipitation has become the most popular method for $\delta^{18}O_P$ in aqueous and terrestrial environments due to improved extraction protocols enabling sufficient precipitation of Ag_3PO_4 for analysis from low inorganic phosphorus concentration matrices (Elsbury et al., 2009; Goldhammer et al., 2011; Granger et al., 2017; McLaughlin et al., 2004; Pistocchi et al., 2017; Tamburini et al., 2010; Zohar et al., 2010). $\delta^{18}O_P$ can by analyzed by thermal conversion/elemental analyser isotope ratio mass spectrometry (TC/EA-IRMS). The Ag_3PO_4 precipitation technique for TC/EA-IRMS has many advantages over the traditional fluorination technique in that (*i*) small PO_4 quantities are required for the analysis (yielding ~300-600 µg Ag_3PO_4); (*ii*) dangerous chemicals are avoided, such as BrF_5, F_2 or ClF_3; and (*iii*) measurements are automated (Vennemann et al., 2002).

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1.2. *Approaching a uniform P_i extraction method via Ag_3PO_4 precipitation*

Several detailed protocols for the extraction of P_i via precipitation of Ag_3PO_4 from different complex matrix solutions such as fresh and ocean waters and soil extractions exist (Colman, 2002; Goldhammer et al., 2011; Gruau et al., 2005; McLaughlin et al., 2004; Tamburini et al., 2010, Zohar et al., 2010). The major techniques for these protocols have been summarized by Paytan & McLaughlin (2011) and Davies et al. (2014).

For water samples, the broadly common sequence of steps for Ag_3PO_4 precipitation is this: (*i*) P_i is quantitatively removed from the sample through magnesium-induced co-precipitation (MagIC) by brucite (Karl & Tien, 1992); (*ii*) redissolution of the brucite-pallet in an acid matrix, which resuspends the P_i in solution; (*iii*) removal of other interfering sources of O, such as dissolved organic matter (DOM), by using anion exchange resins and/or sequential precipitations; (*iv*) removal of potentially interfering cations using an cation exchange resin; (*v*) precipitation of Ag_3PO_4. All steps are designed to inhibit isotopic fractionation.

One of the major challenges with all the Ag_3PO_4 precipitation methods relates to the insufficient removal of oxygen sources other than phosphate (Tamburini et al., 2010). Thus, the purification steps are of great importance. Especially DOM is of concern as the high O content of DOM can significantly interfere with the measured fractionation of $\delta^{18}O_P$ and persists throughout all sequential steps of the Ag_3PO_4 precipitation methods (McLaughlin, Paytan, et al., 2006).

There is a variety of approaches to address this problem, including *a)* adsorption of organic compounds to phosphate-free activated carbon (Gruau et al., 2005) or to a resin such as DAX-8 (Colman, 2002; Joshi et al., 2018; Tamburini et al., 2010), *b)* repetition of the MagIC step with the intention of further isolation of P_i from a matrix with potential contaminants (Colman, 2002; Goldhammer et al., 2011), *c)* acidified pH-specific precipitations of fulvic and/or humic acids (Zohar et al., 2010), *d)* sequential precipitation and re-crystallization scheme to efficiently scavenge P_i (Tamburini et al., 2010), *e)* and a final washing of the Ag_3PO_4 precipitate with hydrogen peroxide to eliminate residual organic matter by oxidation (Goldhammer et al., 2011; Tamburini et al., 2010; Zohar et al., 2010).

Despite the several existing protocols and the review papers by Paytan & McLaughlin (2011) and Davies et al. (2014) focusing on analysis of the $\delta^{18}O_P$ of inorganic phosphate, and despite numerous articles describing $\delta^{18}O_P$ application in different aquatic environments, there exists currently no collective or uniform protocol via precipitation of Ag_3PO_4 for freshwater matrices. This is further despite the fact that the method can prove challenging to implement for new practitioners. In addition, some of the common steps were originally developed and documented for other conditions than they are now applied on. For example, the MagIC steps' quantitative P_i removal was well documented, but for the matrix of oceanic seawater, which is relatively invariable compared to freshwater matrices. Nevertheless, it has nearly directly been applied on freshwater samples. Hence, to make the method as widely and practically applicable as possible, and to facilitate proper grounds for a coherent future method development aiming at freshwater systems, there is a need for a detailed method description for the Ag_3PO_4 precipitation method. The present technical note aims to address the needs with (*i*) describing each step of the Ag_3PO_4 precipitation method in detail; (*ii*) explain the historical background and reasoning behind each step; (*iii*) compile from the literature the (lacking) documentation of individual steps; (*iv*) give practical advice and suggestions to tackle potential challenges which may arise when applying the method, as it is, under different scenarios.

2. Protocol for freshwater $\delta^{18}O_P$ determination

2.1. *Reading guide for the protocol*

The protocol (Sections 2.2, 2.3 and 2.4) is about concentration and isolation of P_i in freshwater samples and result in an adequately pure solid silver phosphate crystal (Ag_3PO_4), without isotopic alteration. The description of the subsequent TC/EA-IRMS analysis of the $\delta^{18}O_P$ determination is not included. For that, we refer to Tamburini et al. (2010) or Davies et al. (2014).

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The protocol can be used when water sampling volumes are not restricted. In situations where sampling is difficult and sample volumes limited, we refer to the method presented by Goldhammer et al. (2011). If the goal is to determine $\delta^{18}O_P$ from a sediment sample, we refer to the P extraction method presented by Tamburini et al. (2010).

The here presented protocol consists of three sections: Section 2.2 *'Freshwater sampling'*, Section 2.3 *'Quantitative P_i removal by the MagIC method'*, and Section 2.4 *'Purification and silver phosphate precipitation'*. Each section is divided into main steps presented by roman numerals and each main step is further subdivided into substeps indicated by letters from the Latin alphabet.

Three different remarks will be presented throughout the protocol:

Note .....Specific concerns to be aware of when performing one of the substeps.

We experienced .....Phenomena we have experienced that have not been presented in earlier method descriptions.

Method disagreement ....Draws attention to steps where there are inconsistencies between already published $\delta^{18}O_P$ methods.

The protocol compiles the disperse experiences from previous studies, combined with our own experience. Description of the preparation of all used chemicals and reagents are provided in Appendix A.

2.2. *Freshwater sampling*

The amount of water to be sampled depends on the P_i concentration of the sampled water itself. It is recommended to sample a minimum of 20 µmols of P. This will provide enough P to allow some P losses from one step to the next and thus an easier handling of the protocol. This can lead to required water volumes of 10 to 50 L for P_i concentrations of 2 and 0.4 µM, respectively (McLaughlin et al., 2004; Tamburini et al., 2010).

It is important to take the necessary precaution in relation to the type of water being sampled. This is especially true when sampling anoxic and Fe^{2+}-rich water were P_i co-precipitation with Fe(III)-(hydr)oxides (henceforth collectively referred to as Fe-oxides), forming upon contact which atmospheric O_2, could immediately occur (Senn et al., 2015). Thus, different sampling approaches are needed when working with either oxic or anoxic samples:

Step I. Freshwater sampling

a) Prior to sampling, acid-wash, rinse with deionized distilled water (DD-H_2O), and air dry a polyethylene collection container. If planning to sample anoxic and Fe^{2+}-rich water, additionally flush the container with N_2 gas and seal the container. *b)* At the sampling site, fix a piece of nylon mesh on the opening of the collection container (oxic water sampling) or attach the nylon mesh to the tip of the sampling tube, submerged in the collection container (ferrous water sampling) to filter out coarser material. The mesh size depends on practicalities; decide on a size range which allows a decent flow of water through without clogging. We successfully used a 10 µm nylon mesh for lake, stream and groundwater, collecting about 1 L per minute using a peristaltic pump. *c)* Rinse the polyethylene container three times with sampling water before final filling. When sampling ferrous water, oxygen could enter the anoxic sample and Fe-oxides could start to precipitate. To avoid those processes rinse and fill the container by pumping water through the submerged tube into the container and let the water overflow for an extended period of time. At the final filling, prevent a headspace in the container before closing it. *d)* Collect a parallel water sample (minimum 10 mL) for measurement of P_i concentration and $\delta^{18}O$ of water, i.e. $\delta^{18}O_w$.

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Evaluation of the water sampling protocol

So far, there is no clear guideline regarding the proper filtration requirement for freshwater samples. However, the selected filtration procedure might have an effect on the final obtained purified Ag_3PO_4. If necessary particulate organic matter has typically been removed from freshwater samples by filtration through a 0.45 µm GF/F filter (Davies et al., 2014; Elsbury et al., 2009; Li et al., 2011). However, it is extremely impractical to filter many litres of water through a 0.45 micron filter. When working with freshwater samples, filtration of the HNO_3 solution after dissolution of the last MagIC pellet (Step VI) could be a solution. Nevertheless, to our knowledge, this still needs to be elucidated further.

2.3. Quantitative P_i removal by the MagIC method

The MagIC method was developed by Karl & Tien (1992) and later improved by Thomson-Bulldis & Karl (1998) to precisely determine nanomolar concentrations of SRP and total dissolved phosphorus from marine and freshwater ecosystems. The technique concentrates and isolates P_i from the majority of other dissolved ions, and ideally also from DOP and DOM, thus enabling a more manageable P_i sample for further treatment prior to the final Ag_3PO_4 precipitation.

Step II. Magnesium-induced co-precipitation of dissolved P_i (MagIC)
Magnesium-induced co-precipitation can quantitatively remove dissolved P_i by adsorption onto $Mg(OH)_2$ (brucite), initiated by addition of NaOH which raises the pH. This is utilized in the first step of the MagIC approach. Brucite can precipitate at any temperature, but temperatures should be kept low (5-10°C) in order to keep microbial activity at a minimum. Microbial activity may alter the source $\delta^{18}O_P$ signatures through biological uptake and recycling (Blake et al., 2005).
The procedure of the brucite precipitation step is as follows:

a) Discard some of the sampled water to ensure space for the reactants. *b)* Add 3 M Mg-brine to the water sample in the polyethylene container. The required volume deviate according to the sample volume. Add until the solution achieve a final concentration of ~55 mM Mg^{2+} (Karl & Tien, 1992; Thomson-Bulldis & Karl, 1998); for example, this corresponds to the addition of 1 L 3 M Mg-brine to 50 L freshwater sample. Mix well. The required Mg^{2+} concentration stems from an experimentally evaluated efficacy of P_i removal from Mg^{2+}-amended freshwater samples by Karl & Tien (1992) and corresponds to the Mg^{2+} concentration found in seawater. *c)* Then add 1 M NaOH equivalent to 0.5% of the sample solution volume (Thomson-Bulldis & Karl, 1998) and mix again. Check with pH indicator strips that the pH becomes between 9 and 10, as alkaline conditions facilitate brucite precipitation better than acidic conditions (Thomson-Bulldis & Karl,

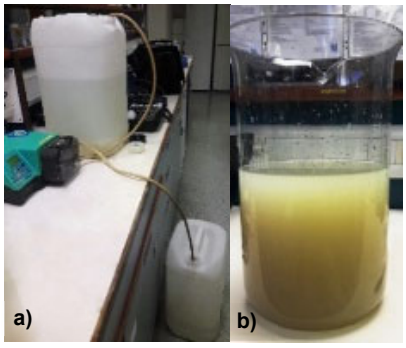

Figure 1. (a) Removing the supernatant from the brucite flocs by siphoning, using a peristaltic pump. (b) Brucite flocs left after discarding the supernatant.

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1998). If pH <9 add more 1 M NaOH and mix simultaneously. *Note* that excess NaOH does not improve P_i co-precipitation removal because the resulting higher pH decreases PO_4 adsorption. Rather, excess NaOH has the drawback that it yields a larger mass of brucite flocs which subsequently must be dissolved in a larger volume of acid (Karl & Tien, 1992). *d)* Allow the brucite flocs to settle by gravity over a couple of hours. Then remove the supernatant. If the volume of the sample solution is large, this can be done by siphoning or using e.g. a peristaltic pump (Figure 1a). The brucite flocs might make up several liters of sludge (Figure 1b). *Note* that P_i may start to desorb from the brucite flocs, probably because recrystallization of the brucite lowers the surface area if the suspension is left for longer than it takes the brucite flocs to settle (Colman, 2002). *e)* Check the absence of P_i in the supernatant, e.g. by using the spectrophotometric molybdate blue-method (Murphy & Riley, 1986). Discard the supernatant if P_i has been 100% stripped from the sample solution. If P_i is still present, add additional 1 M NaOH to the supernatant until no P_i can be detected; the supernatant at this point has a Mg concentration still matching seawater (supported by PHREEQC modelling). Combine all the precipitated brucite.

Method disagreement regarding the precipitation approach of brucite exists: Joshi et al. (2018) initially prepared a concentrated MagIC colloidal solution in a split of the sample solution (200–300 mL) and concurrently adjusted the pH of the remaining sample solution. They then subsequently mixed the two solutions. The entire volume was then gently shaken continuously to maintain a homogeneous dispersion of colloids and thus maximize the trapping of P_i. Joshi et al. (2018) state that this procedure is especially prudent when working with low P_i concentrations. This method procedure has successfully been followed by Yuan et al. (2019). Whether there are discrepancies in the results if one follows this approach instead of the magnesium-induced approach described in Step II is currently undocumented.

Step III. Sample centrifugation

The brucite flocs can be separated from the solution by centrifugation. Do the following:

a) After completing Step II, immediately centrifuge the collected brucite floc sludge at 3500 rpm for 10 minutes, and discard the supernatant. Timewise, it is recommended to use as large centrifuge tubes as possible, e.g. 250 mL tubes. (No further substeps in Step III.)

Method disagreement regarding the recommended centrifugation rotation speed: Karl & Tien (1992) recommend a low speed (1000 rpm for 1 h) as high g-forces (experienced at >12000 rpm; Karl & Tien, 1992) make the settled brucite flocs harder to dissolve subsequently and do not improve the separation from the supernatant. In contrast, Goldhammer et al. (2011) recommends a high rotation speed (10000 rpm for 15 minutes) to ensure complete settling of the fine crystalline $Mg(OH)_2$. The underlying reasoning for Goldhammer et al.'s approach is that the $\delta^{18}O_P$ of the P trapped in the fine fraction is significantly different from the $\delta^{18}O_P$ of coarser brucite flocs. In practice, these fines are not visible to the eye; the supernatant in either case should appear clear. The amount of associated P therefore must remain tiny compared to the amount in the visibly settled flocs, meaning that the difference in $\delta^{18}O_P$ needs to be comparably high. Nevertheless, we followed McLaughlin et al. (2004)'s compromise were a rotational speed of 3500 rpm for 10 minutes was used. This approach was successful followed by Young et al. (2009) and Elsbury et al. (2009), both working with freshwater samples. An alternative to centrifugation is gravitational separation used by Colman (2002).

Step IV. Brucite dissolution

The co-precipitated P_i is re-liberated by dissolving the brucite flocs in 1 M HNO_3. The technique is as follows:

a) Add 1 M HNO_3 to the centrifuge tubes used for Step III. The required added volume deviate according to the quantity of brucite flocs. Add until the brucite can be easily removed from the

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centrifuge tubes. Be sure to use the minimum amount of acid to minimize acid hydrolysis (Karl & Tien, 1992); elaborate explanation in section 2.3.1. *b)* Combine the dissolved brucite flocs from the centrifuge tubes. *c)* Adjust the final pH to ca. 1 using 1 M HNO_3 (use indicator pH test strips), as brucite is first fully dissolved under these conditions; at this point the solution will be liquid and not viscous.

Step V. Additional MagIC step

If the sample contain organic material, the color of the precipitated brucite flocs become tan or even brown (Goldhammer et al., 2011; Zohar et al., 2010) (Figure 2), whereas it should be milky whitish if purified (Karl & Tien, 1992). An additional MagIC step, Step V, is thus required leading to (*i*) further purification of P_i from a matrix with potential contaminants and (*ii*) higher concentrated P_i brucite flocs (Colman, 2002; Goldhammer et al., 2011). Step V proceeds as follows:

a) Raise the pH of the dissolved brucite to about 10-11 by adding 1 M NaOH (do not add the Mg-brine). Brucite precipitation occurs at pH 9. *b)* Then, repeat Step III and Step IV. *Note* that a final pH of 1 is still required. *c)* Repeat Step V until discoloration disappears; up to five repetitions may be necessary (Goldhammer et al., 2011).

Method disagreement exists regarding the final pH of the dissolved brucite solution. Colman (2002), Goldhammer et al. (2011) and McLaughlin et al. (2004) all recommend carefully buffering the solution back up to a pH between 4 and 6 after re-dissolution of the brucite is complete, making $H_2PO_4^-$ the main P_i species in the solution. McLaughlin et al. (2004) used 1 M potassium acetate as buffer as it is inexpensive, nontoxic, and has a low P content, whereas Colman (2002) and Goldhammer et al. (2011) used 1 M NaOH to adjust pH. However, the subsequent purification steps in these three studies (precipitation of cerium phosphate (McLaughlin et al., 2004) and a pump-based anion-exchange chromatography setup (Colman, 2002; Goldhammer et al., 2011)) differ from the purification step presented in our protocol and thy all utilizes a pH of around 6. In this protocol the subsequent purification steps utilizes the low pH (see Step VII). Adjustment of pH is therefore not applied in the MagIC protocol presented in the present study.

Step VI. Filtration

After completing Step V one should be left with a solution with a pH of ~1. The final step of the MagIC protocol separates contaminants insoluble under acid conditions and not incorporated in the brucite flocs, by vacuum filtration. Do the following:

a) Filtrate the dissolved brucite using a 0.7 µm GF/F filter. It may be necessary to centrifuge first if the floc is not fully dissolved in acid at pH 1. (No further substeps in Step VI.)

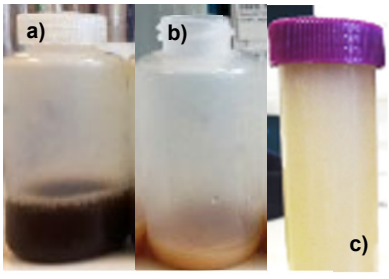

Figure 2. Brucite discoloration of sample with high dissolved organic matter (DOM) content. DOM-rich brucite flocs after (a) the first precipitation, (b) after three HNO_3 dissolution and NaOH precipitation repetitions and (c) purified brucite (Step V).

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After this step, it is recommend to proceed with the the subsequent purification step (Step VII), without waiting too long. If the samples needs to be stored and/or transported than do not dissolve the brucite flocs after the last brucite precipitation and store the sample in the fridge. The brucite flocs should first be dissolve in acid just before the first purification (Step VII).

Evaluation of the MagIC protocol

In general, we found the MagIC protocol to be an effective method to remove P_i from water samples. It is, however, obligatory to check the P_i concentration in the supernatant generated in Step II before it is discarded.

The MagIC protocol was initially developed for samples of seawater (Karl & Tien, 1992) which has a nearly constant matrix composition independent of the sampling site and a naturally high concentration of Mg^{2+} (55 mM). Also, seawater P_i concentrations are rarely high enough to challenge the quantitative P_i removal in Step II. In contrast to seawater, when working with freshwater samples, the matrix can vary significantly and Mg^{2+} needs to be added.

Karl & Tien (1992) conducted a limited preliminary investigation of the MagIC technique on freshwater samples, were the results indicated its applicability. This was later substantiated by Elsbury et al. (2009), Goldhammer et al. (2011) and McLaughlin et al. (2004). They all used the MagIC technique for isolation of P_i in freshwater samples. Nonetheless, an initial incomplete quantitative removal of P_i from the sample solution has been attributed the presence of HCO_3^-, as HCO_3^- has an affinity for brucite similar to P_i, and thus reduces the P sorption capacity of brucite (Joshi et al., 2018). An extra step prior to the MagIC treatment where HCO_3^- is removed by acid treatment forming degassing CO_2 (Joshi et al., 2018) may thus be required; this was not attempted in the present study.

Additional amendments and additions to the MagIC protocol might be necessary when working with some freshwater samples. This is an important subject, which still needs to be investigated in more detail.

The acid dissolution of brucite can be a weakness for organic rich samples (i.e. Step IV). During that step acid hydrolysis may occur, which may potentially convert organic P into new P_i in which water-O from the ambient environment may be incorporated (McLaughlin et al., 2006). The newly generated P_i will potentially be incorporated in the Ag_3PO_4 crystals, subsequently altering $\delta^{18}O_P$ signature of the sample. By repeating the MagIC step (cf. Step V) the samples are exposed for a longer acid contact period and thus, there is a higher risk of isotopic alteration driven by acid hydrolysis.

Colman (2002), Thomson-Bulldis & Karl (1998), and Jaisi & Blake (2014) all experimentally validated, that hydrolysis of a large range of DOP compounds is negligible at extreme (low and high) pH in the time frame used in routine laboratory processing of samples. Furthermore all reported hydrolysis impacts on $\delta^{18}O_P$ are below the analytical error (Paytan & McLaughlin, 2011). Yet it is important to keep in mind the significant variation of the freshwater matrix, and thus the vast array of organic P compounds with different affinities for the brucite flocs (Colman, 2002; Thomson-Bulldis & Karl, 1998).

It is therefore wise to test samples that might be susceptible to acid hydrolysis (e.g. organic-rich samples or samples with an anomalous composition of organic carbon) for isotopic contamination driven from this mechanism. ^{18}O-labeled and unlabeled reagents on replicates of the same sample can be used to trace and correlate the impact of acid hydrolysis.

The positive effect of minimizing the DOM content and other O-bearing compounds remaning in the sample by repeating the MagIC step might exceed its negative impacts. As mentioned, inefficient removal of O-bearing contaminating compounds, including DOM, nitrate (NO_3^-), sulphate (SO_4^{2-}) and calcium carbonate ($CaCO_3$) could result in inclusion of O from other compounds than PO_4 in the precipitated Ag_3PO_4 (Davies et al., 2014; Lécuyer, 2004) and could thus significantly influence the measured $\delta^{18}O_P$ signature. Especially DOM, containing up to 45% O by weight has been shown to persist until the precipitation of Ag_3PO_4 (McLaughlin et al., 2004). In literature, remaining O-bearing compounds potentially being incorporated into the Ag_3PO_4 seems to be of bigger concern (Davies et al., 2014; Goldhammer et al., 2011; Gruau et al., 2005; McLaughlin et al., 2004; Tamburini

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et al., 2010) than the probability of acid hydrolysis during brucite dissolution (Jaisi & Blake, 2014; Paytan & McLaughlin, 2011).

An alternative to the MagIC protocol is the quantitative removal of P_i by co-precipitation with Fe-oxides (Longinelli et al., 1976) which has been proven successful for freshwater samples (Gruau et al., 2005; Neidhardt et al., 2018). Fe-oxide co-precipitation is initiated by addition of 0.1 M $FeSO_4$ accompanied with aeration of the sample at a pH of 8.5±0.1 (optimal for Fe-oxide precipitation; Gruau et al., 2005). Neidhardt et al. (2018) found that it is not necessary to add $FeSO_4$ if the initial dissolved Fe^{2+} concentration in the solution samples are high (>1 mg Fe^{2+}/L). It still needs to be investigated which which of the two approaches for quantitative P_i removal is preferable.

2.4. Purification and silver phosphate precipitation

The phosphate purification protocol presented in the precent study is based on the method published by Tamburini et al. (2010). The advantage of Tamburini et al. (2010)'s protocol is that it was developed with the specific goal of minimizing the effect of organic matter on $\delta^{18}O_P$. Tamburini et al. (2010)'s purification steps of sequential precipitation and recrystallization were adapted from Kolodny et al. (1983) and modified by Liang & Blake (2006). Briefly, P_i is first precipitated as ammonium phospho-molybdate (APM), and then recrystallized as magnesium ammonium phosphate (MAP). This is combined with a subsequent cation resin treatment followed by elimination of chloride. The purification protocol is presented below.

Step VII. Ammonium phospho-molybdate (APM) precipitation

During the first step of the purification protocol, P_i is scavenged from the acidic dissolved brucite solution by precipitation of APM crystals. This enables the separation and removal of ions and contaminants that are soluble at low pH (Joshi et al., 2018). The APM precipitation procedure is as follows:

a) Initially, transfer the sample solution (i.e. the dissolved brucite) to an Erlenmeyer flask of suitable volume (sample and reactants combined volume) and place the flask in a 50 °C warm water bath shaker or on a magnetic stirrer with heating set to 50 °C. *b)* If the solution is taken directly from the refrigerator, wait until the sample is close to room temperature before continuing. *c)* Then add 25 mL 35% ammonium nitrate reagent, and then slowly add 40 mL of the 10% NH_4-molybdate solution. *d)* Adjust the final pH to ca. 1 using 1 M H_2SO_4, (use indicator pH test strips). Normally around 1 mL is enough; thereby the volume of the sample is not affected too much. *Note* that if the supernatant turns transparent bright yellow (Figure 3a) this is an indication that optimal precipitation condition with respect to APM crystals are obtained. When this color changes to milky yellow, it indicates that APM crystals are forming (Figure 3b). If no APM crystals have started to precipitate from the heated solution (> 25 °C) after around 15 min, supersaturated conditions with respect to APM crystals are likely not obtained or pH is not correctly adjusted. First check the pH and adjust if necessary. If still no APM crystals precipitate, add stepwise more 35% ammonium nitrate and 10% NH_4-molybdate solution in the same ratio as before (2.5:4) until signs of crystal precipitation. *e)* Leave the solution in the 50 °C warm water bath and shake gently overnight to ensure complete APM precipitation.

We experienced that if the supernatants were slightly alkaline after Step VIIc it became bright green (Figure 3c) and no APM started to precipitate. When adjusting the pH to 1 the supernatant turned to a transparent bright yellow color (Figure 3a) and APM crystal immediately began to form. The slightly alkaline condition could have affected the dissolution of the brucite flocs, since brucite dissolve at acidic conditions flocs. We also experienced that if the brucite flocs had not been acidified to pH 1 during the dissolution step (Step IV) and/or the additional MagIC step (Step V) was not conducted, the crystals precipitating in this purification step were white and the supernatant transparent (Figure 3d). Furthermore, we were not able to accomplish a final precipitation of Ag_3PO_4 when we tried to proceed with these white crystals. Accordingly, we suggest that the color of the

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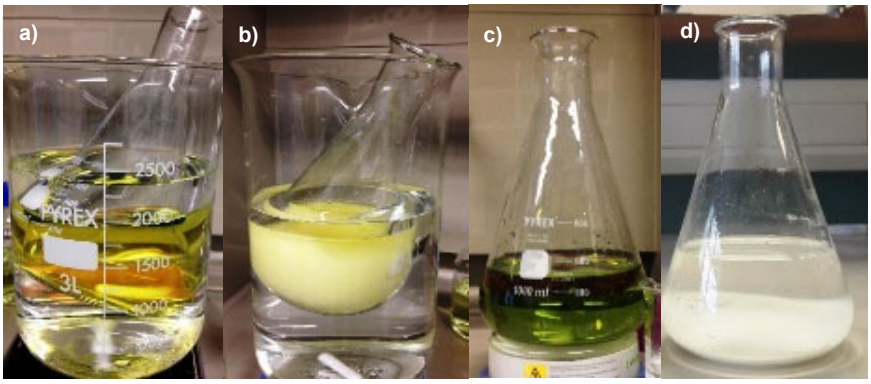

Figure 3. Color of the supernatant and the precipitate in Step VII when (a) optimal precipitation condition with respect to APM crystals are obtained, (b) APM crystals are forming, (c) alkaline condition which impede APM precipitation and (d) with an unidentified precipitate resulting from incorrect execution of the MagIC protocol (section 2.3).

supernatant and the precipitate can be used as an indicator for (*i*) optimal pH conditions for APM precipitation and (*ii*) whether it is worthwhile to continue. The adjustment of the pH and an introduction of additional MagIC steps were performed simultaneously in the present study. No examination of whether both actions are equally important has been reported nor tested in the present study.

Step VIII. APM dissolution

The P_i is released from APM by dissolution of the crystals in an alkaline solution prior to an additional purification step. Conduct the step as follows:

a) Start by separating the yellow APM crystals from the supernatant by vacuum filtration upon a 0.2 µm cellulose acetate filter and discard the supernatant. The filtration time can take several hours and more than one filter may be necessary. APM crystals from different samples may differ slightly from each other in color and size (Figure 4). *b)* Wash the crystals thoroughly with a 5% ammonium nitrate solution to rinse off contaminants. The more, the better (>200 mL). *c)* Transfer the filter containing the APM crystals to a 100 mL Erlenmeyer flask and place the flask

Figure 4. Vacuum filtered and 5% ammonium nitrate washed ammonium phospho-molybdate crystals (APM) from two different samples. The APM crystals differ in color and size.

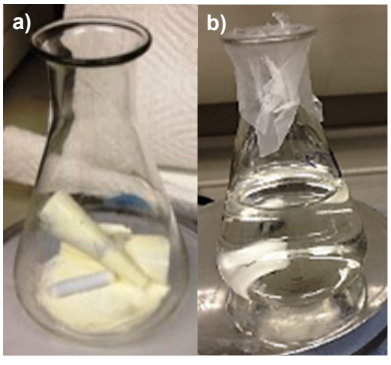

Figure 5. a) Ammonium phosphomolybdate crystals (APM) on 0.2 µm cellulose acetate filters. b) Dissolved APM crystal in a NH_4-citrate solution resulting in a transparent solution.

Figure 6. Greenish discoloration of the dissolved ammonium phosphor-molybdate crystals.

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Figure 7. Precipitated magnesium ammonium phosphate crystals .

on a magnetic stirrer. *d)* Dissolve the APM crystals in a minimum amount of NH_4-citrate solution (15-50 mL; volume depends on the quantity of formed APM crystals). Start by adding 10 mL and then add 5 mL aliquots. Work under a chemical fume hood. *e)* Gently swirl the solution while the crystals are dissolving and wait until the solution becomes transparent (Figure 5), which may take up to 15-20 minutes. Then remove and discard the filter(s). *Note* that Mg^{2+} ions could interfere with dissolution of the APM crystals leading to some crystals not dissolving. In addition, silicates may have formed in the former steps. These are not dissolvable in the NH_4-citrate solution. Accordingly, some particulate compounds might be left in the solution after it turns transparent. If so, filter again using a 0.2 µm cellulose nitrate filter and discard the filter.

We experienced that the dissolved APM solution at times had a greenish discoloration, maybe due to precipitation formation of silicate molybdate complexes (Figure 6). This could possible indicate that silicate molybdate complexes have formed. We tried to continue the protocol with these samples which still resulted in Ag_3PO_4 crystal precipitation in the last step.

Step IX. Magnesium ammonium phosphate (MAP) precipitation

In this step P_i is further purified by precipitating MAP crystals under alkaline conditions, thus enabling the removal of ions and contaminants that are soluble at high pH. The MAP precipitation procedure is as follows:

a) Initially add 25 mL Mg-reagent to the 100 mL Erlenmeyer flask, containing the dissolved APM solution, while stirring. *b)* Then slowly add about 7 mL of the 1:1 ammonia solution. *c)* Check pH. If pH < 8 carefully add more of the 1:1 ammonia solution until the solution acquires pH 8-9 which is the optimum pH for MAP precipitation. MAP crystals should start to precipitate immediately, turning the solution whitish opaque (Figure 7). *d)* Cover the Erlenmeyer flask with parafilm and make mm-size holes for venting. Leave the solution overnight on the magnetic stirrer.

We experienced that it was necessary to add a bit more Mg-reagent to some of the samples, after adjusting pH to 8-9, in order to achieve supersaturation with respect to the MAP crystals. This was true for the samples were >20 mL of NH_4-citrate solution had been used to dissolved the APM crystals.

Step X. MAP dissolution

P_i is released by dissolving the MAP crystals in a minimum amount of HNO_3.

a) Separate the white MAP crystals from the supernatant by vacuum filtration upon a 0.2 µm cellulose nitrate filter and discard the supernatant. The MAP crystals are small and may be quite

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hard to see on the filter by eye, see Figure 8. *b)* Wash the crystals thoroughly with 1:20 ammonia solution (>200 mL) to get rid of excess chloride and other contaminants. *Note* that this is of extreme importance as remaining Cl^- from the Mg solution (i.e. MgCl and HCl) will cause co-precipitation of AgCl during the final precipitation of Ag_3PO_4. *c)* Transfer the filter to a 50 mL centrifuge tube (with lid) and dissolve the MAP crystals in a minimum amount of 0.5 M HNO_3 (5-10 mL) by shaking the sample. *d)* Leave the filter in contact with the acid for at least 15-20 minutes to ensure that the MAP has dissolved. *Note* that it is difficult to assess when the crystal have fully dissolved, since the filters and the crystals are both white.

Step XI. Cation removal

The presence of cations (primarily Na^+ and multivalent cations such as Mg^{2+}) interferes with the precipitation of Ag_3PO_4. Thus a prior cation removal step is a crucial prerequisite for the subsequent successful precipitation of purified Ag_3PO_4 (Firsching, 1961). Cations can be scavenged by a proton-charged cation resin, releasing H^+ to solution, which subsequently reacts with HCO_3^- (if present), forming H_2O and CO_2 (Colman, 2002). The purification step is as follows:

a) Convert the new cation exchange resin AG50WX8 to an H^+ form by reacting the resin with 7 M HNO_3 overnight, on a horizontal shaker. A 7 M HNO_3 volume equivalent to 1.5 times the resin volume is recommended. *b)* The following day discard the HNO_3 and rinse the resin thoroughly by mixing it with 1 L DD-H_2O to bring it close to neutrality (>5). *c)* Filtrate the mixture on a 0.45 µm polycarbonate filter and discard the water. It might take up to several repetitions before a neutral pH is obtained. *d)* Add 6 mL of the obtained cation resin slurry to the sample solution. Seal the sample with a lid or parafilm and place the sample on a shaker overnight. *e)* The next day, filter the sample using a 0.2 µm polycarbonate filter and rinse the cation resin with 1-2 mL DD-H_2O. *f)* Collect the resin and recondition it in 1 M HNO_3. The resin can be re-used.

Method disagreement regarding the preparation of the cation resin. Goldhammer et al. (2011) experienced a reddish discoloration of the sample when using resin prepared the previous day. Subsequently they were unable to properly precipitate Ag_3PO_4. By preparing the cation resin within 30 min of its use they avoided this problem. They did not resolve the cause of this complication. We experienced that the samples acquired a milky white color once the resin was added, if the resin was prepared two days before its use (our resin was left in DD-H_2O overnight). The whitish coloration was avoided when using the resin the same day as it was washed in DD-H_2O. It was not possible to properly precipitate Ag_3PO_4 when using samples where the milky white color had occurred. Thus, we agree with Goldhammer et al. (2011)'s statement, that proper handling and rinsing of the resin before every application is crucial to the successful precipitation of Ag_3PO_4.

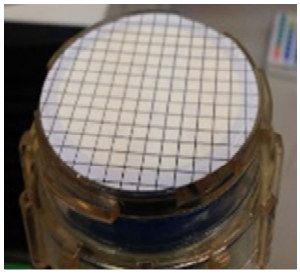

Figure 8. Vacuum filtered and 1:20 ammonia solution washed magnesium ammonium phosphate crystals.

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Figure 9. Precipitated AgCl crystals after adding $AgNO_3$ to the sample solution.

Joshi et al. (2018) adjusted the pH of the dissolved MAP solution to neutral (pH 6-8) prior to the cation removal.

Step XII. Elimination of Cl^-

Removal of Cl^- ions is extremely important, as Cl^- otherwise may react with the Ag^+ in the final precipitation of $AgPO_4$, forming AgCl which have been observed to be rimmed by silver oxide precipitates (Colman, 2002). Precipitation of AgCl hence both interfere with the Ag_3PO_4 precipitation (McLaughlin et al., 2004) and introduce non-phosphate oxygen to the sample (Colman, 2002). Chloride can be quantitatively removed by adding $AgNO_3$ crystals to the sample when the pH is acidic, causing AgCl precipitation (Figure 9) prior to the Ag_3PO_4 precipitation step. The low pH in the sample (<1) impede co-precipitation of Ag_3PO_4, and hence no P_i is lost during this step. The purification step is as follows:

a) Transfer the filtrated sample solution to a small container with a small opening (e.g. 50 mL centrifuged tube) *b)* Add a few $AgNO_3$ crystals to the sample solution. If the sample turns whitish opaque AgCl has precipitated (Figure 9). *d)* Wait at least 5 minutes and re-filter, the same filter used in Step XI can be re-used.

After this purification step, the initial freshwater sample with a volume up to 50 L has been reduced to about 10 mL highly concentered homogeneous P_i solution stripped of potential contaminants. The sample is now ready for the final Ag_3PO_4 precipitation.

Step XIII. Silver phosphate (Ag_3PO_4) precipitation

Precipitation of insoluble silver salts, such as Ag_3PO_4, can be conducted by volatilization of ammonia (Firsching, 1961). This allows a 'slow' recrystallization which facilitates the growth of large and easier-to-handle Ag_3PO_4 crystals for oxygen isotope analysis by IRMS within a few days (Firsching, 1961; Goldhammer et al., 2011). The method utilizes that Ag_3PO_4 precipitates in the solution at a pH around 7±0.5 when free Ag^+ and P_i are present. Thus the pH conditions and a high Ag^+:P_i ratio is of extreme importance to ensure complete precipitation of Ag_3PO_4. The 'slow' Ag_3PO_4 precipitation procedure is as follows:

a) Initially add the Ag-ammine solution to the sample solution, in a Ag:P_i ratio of approximately 10:1 (Colman, 2002). The sample solution turns briefly white (at pH 7), and then transparent (at pH>7) once the alkaline Ag-ammine solution has been added. *b)* The sample container is then placed in an oven at 50 °C. Yellow Ag_3PO_4 crystals start to precipitate after a few hours as the amine starts to vaporize and the Ag^+ is released (Firsching, 1961). Complete precipitation of the crystals takes up to two days. *Note* that it impotent to repeatedly add DD-H_2O to the solution to keep the volume as constant as possible. If left unattended (e.g. for one or several days) all the H_2O may evaporate, which results in uncontrolled precipitation of salts. This is still fine, as the

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salts will be dissolved when adding DD-H_2O, as they are mostly nitrate-based. If this happen it is vital to wash the Ag_3PO_4 crystals easterly well with DD-H_2O. The small diameter of the tube and the low temperature of the oven, impedes the evaporation ammonia, and thus enables a slow crystallization process (Colman, 2002). *c)* After 1 to 2 days, if no yellow Ag_3PO_4 crystals have precipitated, check the pH of the solution. If the pH of the solution differs from pH 7 (optimal pH for Ag_3PO_4 precipitation conditions; Firsching, 1961) adjust the pH by adding either HNO_3 or NH_4OH. *Note* that under no circumstances should HCl or NaOH be used to adjust the pH as Cl^- and Na^+ would interfere with the crystallization of Ag_3PO_4. *d)* When crystals have formed, vacuum filter them upon a 0.2 µm polycarbonate filter and discard the supernatant. Other filters tend to 'trap' the Ag_3PO_4 crystals on their surface. *Note* that Ag_3PO_4 crystals may form on the side of the tube, hence make sure to carefully detach these and transfer them to the filter as well. *e)* Wash the crystals extremely thoroughly with DD-H_2O to get rid of residual O-bearing compounds, as they interfere with the oxygen isotope analysis (cf. Section 2.3.1). *f)* Place the filter on a Petri dish and cover it to prevent contamination and loss of crystals. Dry the filter at 50 °C for at least 1 day. *g)* An extra elimination of residual organic matter might be necessary by introducing a final washing of the Ag_3PO_4 precipitate with hydrogen peroxide to eliminate residual organic matter by oxidation (Tamburini et al., 2010). *Note* that Crowson et al. (1991) found that contaminated silver phosphate crystals were generally dark brown to greenish brown in color and cohesive. We did not experience this discoloration but the crystal became dark under light, probably do to the photo-oxidation of silver (McLaughlin et al., 2004; Tamburini et al., 2010). *h)* If needed, the filter containing the Ag_3PO_4 crystals can be stored in a desiccator.

Method disagreement regarding the Ag_3PO_4 precipitation rate. The final precipitation of Ag_3PO_4 can be accomplished by either a 'slow' (Goldhammer et al., 2011; Tamburini et al., 2010) or 'fast' (Dettman et al., 2001; McLaughlin et al., 2004) precipitation method. In contrast to the 'slow' method presented in the present protocol, 'fast' $AgNO_3$ precipitation is achieved by first altering the solution pH to 7 by adding NH_4OH, NH_4NO_3 and nitric acid (HNO_3). Then follows the addition of $AgNO_3$ crystals dissolved in DD-H_2O, which initiate a rapid precipitation of Ag_3PO_4 within a few minutes (McLaughlin et al., 2004).

Dettman et al. (2001) compared the isotopic composition of the Ag_3PO_4 generated by the two different methods and found the resulting $\delta^{18}O_P$ values to be within expected interlaboratory variation. Tamburini et al. (2010) suggest, however, using the 'slow' precipitation method as an additional measure to minimize the disturbance by organic matter as suggested by Colman (2002).

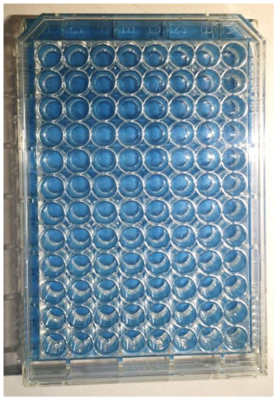

Figure 10. A microplate, used for transportation of timble capsules containing Ag_3PO_4 crystals.

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Step XIV. Ag_3PO_4 crystal preparation prior to isotope ratio mass spectrometry (IRMS)
Once the Ag_3PO_4 crystals have been precipitated, dried and stored in plastic vials they are prepared for isotope ratio mass spectrometry (IRMS) analysis. Such sample preparation involves:

a) Weighing of ~300 µg of Ag_3PO_4 crystals in triplicate from the plastic storage vial into silver timble capsules. *b)* After weighing the Ag_3PO_4 and recording the weight, add a small amount (few grains) of black carbon (no need to weigh this) to each sample. *c)* Close the capsules tight by using tweezers but absolutely do not touch them with the fingers. *d)* Place the capsules in a microplate with holes (Figure 10). Once all the samples have been weighed off, seal the plate. To do this, cover the plate with parafilm, close it with the cover lid, and then fix the lid with tape.

The Ag_3PO_4 crystal are now ready to be shipped for $\delta^{18}O_P$ analysis.

Evaluation of phosphate purification and silver phosphate precipitation

The effectiveness of the different purification steps, in producing adequately pure Ag_3PO_4, is difficult to evaluate during the execution. Oxygen contamination can not be checked for until $\delta^{18}O_P$ has been analyzed. The oxygen yield of the sample is compared to that of the pure Ag_3PO_4 used as standard (Tamburini et al., 2010). Therefore, it is important to know and pay attention to the characteristics of the precipitated crystals in each step (e.g. correct crystal color) and evaluate whether the specific purpose of the step has been obtained (e.g. whether crystals are formed or completely dissolved).

3. Final remarks

In general, it is important to keep in mind, that the amount of added reactants and chemicals can vary from water sample to water sample and in many instances it depends on yield volume or quantity from the prior step. Hence, only minimum and indicative quantities are stated in the present protocol.

As stated in the introduction, a variety of approaches have been attempted to address the problem regarding $\delta^{18}O_P$ contamination resulting from O-bearing compounds others than P_i, and from Na^+, Cl^- and multivalent cations. Bearing in mind that many methods have been tested on various water matrix compositions, their effectiveness may not be reproducible for all water samples matrices. In order to achieve progress in developing and applying $\delta^{18}O_P$ to trace P sources and cycling in freshwater ecosystems, a better understanding of the different methods' reliance on different water matrices is crucial. This does not only apply to the purification steps but applies for all sections presented in the present study.

In general, studies which have used $\delta^{18}O_P$ as a tracer emphasize the importance of additional research and knowledge regarding $\delta^{18}O_P$ data for various potential phosphate sources especially for freshwater systems (Elsbury et al., 2009; Granger et al., 2017; Tamburini et al., 2014; Young et al., 2009). The detailed protocol provided in this study will hopefully contribute to enable a broader use of $\delta^{18}O_P$ signatures as such a tracing tool.

Competing interests The authors declare to have no competing interests.

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Appendix A: Description of the preparation of all used reagents

Reagents used in Section 2.3:

- **3 M Mg-brine:** Dissolve 610 g $MgCl_2 \cdot 6H_2O$ (hexahydrate; MW 203.3 g/mol) in deionized distilled water (DD-H_2O) to a total volume of 1 L. After the salt has dissolved, filter the brine on a GF/F filter. The solution can be stored indefinitely.
- **1 M NaOH:** Dissolve 40 g NaOH pellets in deionized distilled water (DD-H_2O) to a total volume of 1 L. The solution can be stored indefinitely.
- **1 M HNO_3:** Add 66 mL of concentrated HNO_3 to 934 mL of DD-H_2O. The solution can be stored indefinitely.

Reagents used in Section 2.4:

- **35% ammonium nitrate reagent:** Dissolve 538.5 g ammonium nitrate salt (MW 80.052 g/mol) in 1000 mL DD- H_2O. Stir well to dissolve the salt completely. The solution can be stored.
- **5% ammonium nitrate reagent:** Dissolve 105.5 g ammonium nitrate salt in 2000 mL DD- H_2O. Stir well to dissolve the salt completely. The solution can be stored.
- **10% NH_4-molybdate solution:** This solution has to be prepared freshly by dissolving 53.3 g of ammonium molybdate salt (tetrahydrate form: 1235.86 g/mol) in 480 mL of DD- H_2O (enough for approximately 12 samples). The solution CANNOT be stored.
- **Ammonium-citrate solution:** Add 300 mL of DD- H_2O and 140 mL of concentrated NH_4OH to 10 g of citric acid while working under a chemical fume hood. The solution is stable at room temperature and can be stored.
- **Mg-reagent:** Dissolve 50 g of $MgCl_2$ (hexa-hydrate salt, MW 203.3 g/mol) and 100 g of NH_4Cl (MW 53.49 g/mol) in 500 mL DD-H_2O. Subsequently acidify the mixture to pH 1 with concentrated HCl. Finally, adjust the volume to 1 L with DD-H_2O. The solution is stable indefinitely and can thus be stored.
- **1:1 and 1:20 ammonia solutions:** Measure in a volumetric cylinder concentrated NH_4OH (50 mL for the 1:1 and 100 mL for the 1:20). Pour into an appropriate glass bottle and dilute with DD-H_2O (50 mL for the 1:1 and 1900 mL for the 1:20). The solution can be stored.
- **0.5 N HNO_3 solution:** Add 33 mL of concentrated HNO_3 to 967 mL of DD-H_2O. The solution can be stored.
- **Ag-ammine solution:** Dissolve 10.2 g of $AgNO_3$ salt (MW 169.87 g/mol) and 9.6 g of NH_4NO_3 in 81.5 mL of DD-H_2O. Subsequently add 18.5 mL of concentrated NH_4OH. The solution can be stored in the dark in an amber bottle.

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