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Interactive comment on "Analysis of oxygen isotopes of inorganic phosphate ($\delta^{18}O_p$) in freshwater: A detailed method description" *by* Catharina Simone Nisbeth et al.

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Interactive comment on "Analysis of oxygen isotopes of inorganic phosphate (_18Op) in freshwater: A detailed method description" by Catharina Simone Nisbeth et al.

Anonymous Referee #2 Received and published: 21 November 2019

1. General comments This manuscript synthesises previously published methodological work alongside a significant body of observation and experience from laboratory processing of samples for_18OP analysis. The aims of this contribution, i.e. to provide a methodological baseline to inform researchers who are new to the field of _18OP

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and to promote increased standardisation of methodological protocols across _18OP research, are necessary and the authors should be congratulated for focussing on these timely issues.

In general, for the purposes of a technical note, the manuscript does appear to be relatively long. This is largely due to the inclusion of previously published work, predominantly based on the methodology of Tamburini et al. (2010). I do recognise that valuable and new insights into this methodology are provided by the authors, based on their experiences of processing samples in the laboratory. However, I feel that the manuscript could be shortened in places through clearer signposting to details already published elsewhere (particularly in section 2.4), thereby re-focussing the manuscript on the body of new observations and experiences provided by the authors.

We can take this helpful advice on-board and endeavour to shorten parts of the methodology and take into account our own observations and experiences as we go through the steps. We still want to present the complete method, though, but will focus more on the relation to our own observations and experiences as we go through the steps.

2. Specific comments

- Lines 27-30 – I would avoid placing such a strong focus on sole phosphorus (P) limitation of freshwaters here. For example, the role of nitrogen (N) limitation or N/P colimitation in freshwaters is of growing interest. The potential to apply _18OP analyses to resolve questions of P, of N or of N/P co-limitation is of particular interest within them freshwater community.

Certainly, we can briefly mention the growing interest in the role of nitrogen (N) limitation and N/P colimitation in freshwaters in our introduction.

- Lines 36-39 – the _18OP community in freshwaters was initially strongly attracted to_18OP as a potential tracer of P source, with the potential to inform new source

apportionment models. However, the available evidence increasingly indicates that _18OP rarely acts as a conservative tracer of P source, certainly over larger spatial and temporal scales in catchments (as the authors note on lines 48-49). I would argue that there is far more potential power in using _18OP to understand processes controlling P cycling in freshwaters, rather than to focus too strongly on questions of source apportionment.

Certainly, as suggested we can highlight the potential power in using 18OPO4 to understand processes controlling P cycling in freshwaters, rather than focus too much on source apportionment. We will clarify this in the revised manuscript.

- Lines 40-41 – orthophosphate is indeed the primary form of P involved in transport across cytoplasmic membranes prior to intracellular metabolism. However, this does not mean that orthophosphate is the primary form of P cycled in ecosystems. For example, hydrolysis of organic compounds containing P, whether to meet metabolic demand for P or for the purposes of dephosphorylation prior to carbon (C) utilisation, may be an extremely important part of the P cycle within certain freshwater ecosystems. Processes other than uptake of orthophosphate into the intracellular environment are also associated with isotopic fractionation/isotope effects and may therefore be probed through _18OP analyses.

This is a very good point and will be included in our corrections/edits of the manuscript.

- Line 49-50 – temperature-dependent equilibrium fractionation between intracellular fluid-oxygen and phosphate-oxygen during intracellular metabolism of P is indeed an important fractionation that influences the _18OP system. However, the authors should also highlight the fact that other processes that potentially influence the P cycle within freshwaters may lead to inheritance or kinetic isotope effects. For example, the hydrolysis of organic P compounds will involve some inheritance of oxygen atoms from the phosphate moiety in the source organic compound and some incorporation of water oxygen atoms into the liberated phosphate molecule (accompanied by a kinetic

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fractionation). The balance between equilibrium and kinetic/inheritance isotope fractionation and effects will ultimately determine _18OP within a freshwater sample. Unravelling these controls on _18OP is currently one of the major challenges facing the _18OP community, but one that offers the potential to gain new insights into the range of processes influencing P within freshwaters.

Certainly, we will include this very valuable point on the inheritance or kinetic isotope effects which potentially influence the P cycle in freshwaters.

- Line 145 – the authors suggest that freshwater sample volumes of up to 50 L may be necessary to generate sufficient Ag3PO4 for analysis, assuming a P concentration of 0.4 μM. In my experience, P concentrations (as dissolved reactive P) are often Âń 0.4 μM, certainly in freshwaters in which P availability is particularly low and therefore highly likely to be limiting primary production. It is precisely these systems in which _18OP may offer new insights into the P cycle. However, this will require researchers to deal with sample volumes that often exceed 100 L, which presents additional methodological challenges.

Again, this is a very valid and important point which will be included in the corrected manuscript. Recently Tcaci (2019) published an article giving a new procedure for treating large volumes of water is described.

- Line 173 – I agree that it is impractical to filter 50-100 L of sample straight through 0.45 μm filter papers. In my experience, sequential filtration starting with filter pore sizes >0.45 μm is required to address this issue. The risk of processing samples that have not been filtered is: i) dissolution of particulate-bound inorganic P; and/or ii) acid hydrolysis of particulate organic P. Either may generate phosphate with the potential to alter _18OP, compared to the true _18OP of the original sample. In my opinion, standardising filtration of freshwater samples as part of any future _18OP analytical protocol is important.

This is an important point which we can expand and make suggestions in our cor-

rected manuscript. We have filtered samples through 100 micron plastic screens with good results but we will include the suggestion of a sequential filtering protocol if it is deemed necessary in waters with a lot of particulates. In ferrous waters, however, a lengthy filtration procedure (slow pumping velocity?) could cause more damage than good, depending on the effect of co-precipitation of PO4 with iron oxides. Clearly, the magnitude of the error introduced by allowing particulates into the high-volume sample required attention in future research. This we will point out.

- Section 2.3 – the authors focus on the use of the MagIC protocol as the initial processing step for freshwater samples. However, my experience means that I have significant doubts about the feasibility and accuracy of using MagIC in this way for large-volume freshwater samples. This is particularly true of freshwaters in which C:P ratios are high and in which there are much higher concentrations of dissolved organic matter (DOM) compared to the marine samples in which McLaughlin et al. (2004) originally developed their _18OP methodology that included MagIC. The specific challenges facing MagIC in freshwater matrices are:

i) The formation of a precipitate that does not subsequently redissolve in 1 M HNO3 (Step IV in the current manuscript). We have observed this within a number of freshwater matrices. We have not identified the precipitate, but the lack of redissolution in 1 M HNO3 suggests it is not brucite. When this occurs, our experience is that Ag3PO4 cannot be generated from a sample.

ii) Brucite is not specific for the phosphate ion and our research suggests that other competing oxyanions, including nitrate and sulphate, may be co-precipitated (this is in contrast to the statement made by the authors on lines 180-183).

iii) Dissolved organic matter, including a range of organic P compounds, can also be co-precipitated with brucite. In contrast to the research referenced on lines 309-310 of the manuscript, other observations suggest that acid hydrolysis of organic P compounds co-precipitated with brucite may indeed occur (see Davies et al. (2014) Figure

5 for example). At a minimum, we cannot yet concluded that no co-precipitation + acid hydrolysis of organic P compounds occurs in freshwater samples when using the MagIC methodology. Further work is needed to establish whether this has the potential to introduce errors into _18OP analyses in freshwaters, as a result of phosphate generated from the organic P compound that differs in isotopic composition compared to phosphate within the original sample.

In light of points i) to iii) above, the authors may want to incorporate recent research in their manuscript that has developed alternative methodologies for _18OP analysis, seeking to avoid these potential sources of error. These methodologies primarily involve initial treatment of freshwaters using anion exchange resin to isolate phosphate from contaminant sources of oxygen, whether in organic matter or in the form of other oxyanions. For example, see: Tcaci, M. et al. (2019) A New Technique to Determine the Phosphate Oxygen Isotope Composition of Freshwater Samples at Low Ambient Phosphate Concentration. Environmental Science and Technology 53: 10288-10294 and Gooddy, D.C . et al. (2015) Isotopic fingerprint for phosphorus in drinking water supplies. Environmental Science and Technology. 49: 9020-9028.

We can include the valuable new points made by the reviewer in lines 309-310. In relation to points (i) and (ii), we will refer to the Tcaci paper (2019) for large volumes and using of labelled and unlabelled acid to track the possible hydrolysis of DOP during dissolution of brucite.

- Line 200 – our observations suggest that the brucite precipitate can begin to redissolve if left after adding NaOH, likely because solution pH begins to decrease and brucite becomes unstable. Researchers should be cautious about this and be prepared to add further NaOH to maintain solution pH >9-10 in order to prevent the brucite (and co-precipitated phosphate) from redissolving into solution.

We can emphasis this point and include the additional information. We suggest to start the centrifugation and dissolution of the brucite not long after its precipitation This was

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also a point by Colman. So, I would not spend too much time here, just say that brucite should not be left for hours sitting there.

- Line 274-276 – do the authors know what these additional insoluble contaminants are and are they sure that they do not contain P?

We are not sure these insoluble contaminants do not contain P. We will change the word ‘contaminants’ to ‘particles’ to allow for a potential P content of these.

- Line 316-319 – I agree that the use of labelled and unlabelled reagents is a way to assess potential error due to hydrolysis of organic P compounds. However, are the authors suggesting that this should be incorporated as standard practice in all _18OP analyses? How feasible is this? If not feasible, then we need a protocol that we can be certain does not risk hydrolysis of organic P compounds. As I comment on above, I’m not convinced that this is the case with the use of MagIC for freshwater samples.

We do it routinely for HCl. The limitation is giving by the size of the sample. Alternatives are not really existing. One possibility could be a physical reduction of the volume, like freeze drying (possible for small volumes) or others. Another possibility would be to apply the DAX resin (it is a resin that adsorb DOP) before the magic step. But this would be costly. Other possibilities are not existing at the moment, at lest for what I know.

- Line 425-428 – Why is washing to remove Cl- so important here, given that Step XII eliminates excess Cl- prior to Ag3PO4 precipitation by adding AgNO3?

It is always important to remove Cl-. The more Cl we have, the more AgNO3 we have to add and this could then entrain the formation of AgO in the final product.

- Line 449-452 – similar to Goldhammer et al. (2011), our observations also suggest AG50WX8 resin may generate a pink colour in solution, despite resin preparation using HNO3 and DD-H2O followed by immediate use. However, in contrast to Goldhammer, we did not observe any adverse effect on Ag3PO4 precipitation, although we follow the

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precipitation stages in the McLaughlin et al. (2004) method, rather than the Tamburini et al. (2010) method.

Thank you for this additional information, we will include it in the updated manuscript.

- Line 504-505 – which O-bearing compounds do the authors expect to be removed through this washing step?

The water washing of the Ag3PO4 crystals is important because you eliminate nitrates from the previous steps. If nitrate remains, you have an extra source of oxygen, which is visible then in the Oxygen yield of the samples.

- Line 525 – prior to this step, I assume crystals need to be removed from the filter papers and added to plastic vials? What experience do the authors have with this process, for example are the Ag3PO4 crystals difficult to handle due to static electricity?

Absolutely, it is important here to be very careful. We have experienced an adverse affect from static electricity when transferring Ag3PO4 crystals to silver timbles using plastic spatulas. I would avoid plastic spatulas, use metallic as few problems have been experienced with metallic spatulas. For sure, this is a step where you lose material.

- Line 535 – one of the risks of a multi-stage purification/precipitation process is the loss of phosphate in solution or of P in a solid precipitate during processing, for example if MAP hasn't fully dissolved (lines 431-432). Do the authors have any feeling for how significant these 'losses' of P may be within their protocol? Would this ever lead to an insufficient mass of Ag3PO4 being generated for analysis, despite an apparently sufficient mass of P being present in the initial freshwater sample?

This could happen. Unfortunately, the chemistry of the samples is influencing the success of the purification. This is why it is important to pay always a lot of attention on each step and also to know what the samples are made of.

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