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Advances in Oxygen Isotope Analysis of Phosphate by Electrospray Orbitrap Mass Spectrometry for Studying the Microbial Metabolism of Microorganisms

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Abstract: Understanding the impact of human activities on the metabolic state of soil and aquatic environments is of paramount importance to implement measures for maintaining ecosystem services. Variations of natural abundance $^{18}O/^{16}O$ ratios in phosphate have been proposed as proxies for the holistic assessment of metabolic activity given the crucial importance of phosphoryl transfer reactions in fundamental biological processes. However, instrumental and procedural limitations inherent to oxygen isotope analysis in phosphate and organophosphorus compounds have so far limited the stable isotope-based evaluation of metabolic processes. Here, we discuss how recent developments in Orbitrap high resolution mass spectrometry enable measurements of $^{18}O/^{16}O$ ratios in phosphate and outline the critical mass spectrometry parameters for accurate and precise analysis. Subsequently, we evaluate the types of ^{18}O kinetic isotope effects of phosphoryl transfer reactions and illustrate how novel analytical approaches will give rise to an improved understanding of $^{18}O/^{16}O$ ratio variations from biochemical processes affecting the microbial phosphorus metabolism.

Keywords: Microbial metabolism · Orbitrap mass spectrometry · Oxygen isotope ratios · Phosphate · Phosphoryl transfer reactions

Nora M. Bernet received her Bachelor's and Master's degree in Chemistry from ETH Zurich. She carried out her Master thesis in the group of PD Dr. Thomas Hofstetter at the Department of Environmental Chemistry at Eawag working on elucidating the role of substrate oxygenation efficiency in the adaptation of Rieske non-heme iron dioxygenases to new contaminants. In October 2022, she joined Hofstetter's group as a doctoral student to investigate the effect of environmental changes on the microbial metabolism using oxygen isotopes in phosphate.

Thomas B. Hofstetter is a senior scientist at the Department of Environmental Chemistry at Eawag and Privatdozent (senior lecturer) in Environmental Chemistry at the Department of Environmental System Science of ETH Zurich. He obtained his PhD in Environmental Chemistry under the supervision of René Schwarzenbach from ETH Zurich and was a postdoctoral researcher at the Woods Hole Institution of Oceanography as well as a visiting scientist at the University of Minnesota. His research group studies the mechanisms of enzymatic and abiotic reactions of organic contaminants and explores the application of stable-isotope based methods to track such reactions in natural and engineered environments.

1. Introduction

Variations of isotope ratios play important roles for studying the biochemistry of metabolic processes in natural and perturbed environments. Subtle changes of natural abundance $^{13}C/^{12}C$- and $^{2}H/^{1}H$-ratios of methane, for example, allow one to interpret biogenic formation of methane, a major greenhouse gas, in terms of the biochemically available energy and thus metabolic state of the responsible microorganisms (*i.e.* hydrogenotrophic methanogens).[1,2] Large variations of $^{2}H/^{1}H$-ratios in microbial lipids *vs.* $^{2}H/^{1}H$-ratios of water, to add another example, stem from $NADP^+$/NADPH balancing reactions and have been found to correlate with the energy metabolism of aerobic heterotrophic microorganisms.[3] These and many other studies illustrate the added value of consideration of natural variations of isotope and isotopologue ratios. They provide complementary evidence of environmental processes when insights cannot be obtained from the quantification of metabolic species concentrations and fluxes.[4] However, despite these apparent advantages, stable isotope analysis is exploited relatively rarely for disentangling specific biochemical reactions in the context of metabolic processes in the environment.

One major practical reason for not considering the evidence from stable isotope ratios more widely is the fact that isotope ratio mass spectrometry (IRMS) could, so far, be carried out almost exclusively with specialized analytical instrumentation. Unfortu-

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nately, isotope ratio mass spectrometers are not very sensitive and require extensive sample treatment and processing to obtain one of the analyte gases, from which compound-average ratios of rare (*i.e.* heavy) and abundant (*i.e.* light) isotopes of an element can be measured at adequate precision.[5-7] However, recent developments show that the Orbitrap high-resolution mass spectrometers can achieve similar performance while providing position-specific isotope ratios within the studied molecules.[8,9] Such instrumentation would, in principle, be much more abundant and thus potentially easier to access, for example, in facilities for metabolomic and proteomic analyses. Moreover, Orbitrap high resolution mass spectrometry (abbreviated here as Orbitrap HRMS), might be applied more versatilely both from an analytical and conceptual perspective. First, the scope of compounds that can be analyzed will be very broad.[9-13] Second, given the ability to examine molecules and their fragments not only for the ratios of the most abundant isotopes but also compounds with multiple substitutions with rare isotopes (so-called 'clumped' isotopologues), additional means for inferring a compound's origin and fate become available.[1,14-16] Important examples for the feasibility of HRMS-based isotope analysis include works on oxyanions,[17] primarily nitrate,[18] acetate,[19] and selected amino acids.[16,20] Yet, several procedural questions such as the compatibility of different sample matrices for HRMS operation for typical high-resolution *vs.* isotope ratio applications remain to be examined. Ensuring the potential 'interoperability' of HRMS devices for different kinds of analyses might trigger the development of additional sample preparation protocols aiming at maintaining versatile mass spectrometer use.

In our laboratories, we pursue such novel avenues for HRMS-based stable isotope analysis. Specifically, we explore how analyses of $^{18}O/^{16}O$ ratios of phosphate and organophosphate compounds can be applied as tracers for metabolic processes of microorganisms in soil and aquatic ecosystems. Phosphate is both reactant and product in phosphoryl transfer reactions which are pertinent to fundamental biochemical processes involved, for example, in signaling, energy transduction, and transcription of genetic information.[21-23] The overarching hypothesis of our work is that variations of $^{18}O/^{16}O$ ratios of phosphate convey changes in phosphorus homeostasis of living cells and finally provide a holistic approach to probe for changes of metabolism due to environmental change. To test this hypothesis, two specific objectives must be met, which we outline here: First, methods and procedures are needed for stable O isotope analysis of phosphate from biological samples of high throughput; Second, the magnitude of isotope effects of phosphoryl transfer reactions needs to be characterized to interpret changes in $^{18}O/^{16}O$ ratios of phosphate that arise from metabolic activity of microorganisms under different environmental conditions.

2. Analysis of Oxygen Isotope Ratios in Phosphate

The instrumental approach established for determination of small $^{18}O/^{16}O$ ratio variations in phosphate relies on the combination of elemental analysis and isotope ratio mass spectrometry (EA/IRMS, Fig. 1a).[24] The strategy used to obtain the high precision required to determine rare and abundant isotopes of an element at natural isotopic abundances relies on the conversion of analytes to small molecule gases, from which the ions of different isotopologues can be quantified simultaneously.[5–7] For $^{18}O/^{16}O$ ratio analysis of phosphate, this approach necessitates preparation of phosphate samples in the form of solid Ag_3PO_4.[25] Pyrolysis thereof transforms the O of phosphate to CO, from which $^{18}O/^{16}O$ and $^{17}O/^{16}O$ ratios are quantified. Apparently, this requirement comes with a series of procedural challenges for the selective extraction of the metabolically relevant, cytosolic phosphate and purification of samples from very different matrices (*e.g.* cell culture experiments, soil and water samples) prior to chemical conversion of phosphate. The extracted phosphate typically undergoes two precipitation/dissolution cycles through $(NH_4)_3PMo_{12}O_{40}$ and NH_4MgPO_4 salts, as well as cation exchange steps before the final Ag_3PO_4 product is obtained with the necessary purity.[25,26]

Advances in Orbitrap HRMS applications for stable isotope analyses[8,17,18] could offer alternative avenues for $^{18}O/^{16}O$ ratio measurements in phosphate and other oxyanions. First, Orbitrap HRMS enables direct quantification of phosphate isotopologues without prior conversion of phosphate to CO. This principle could lead to simplified sample preparation protocols and increase sample throughput. In addition, Orbitrap HRMS makes it possible to measure phosphate isotopologues with alternative and multiple rare isotope substitutions (*e.g.* $P^{16}O_3{}^{17}O$, $P^{16}O_2{}^{18}O_2$, and $P^{16}O_2{}^{17}O^{18}O$, Fig. 2). Information from clumped O isotopes in phosphate is largely unexplored and could potentially reveal processes that are not accessible from $^{18}O/^{16}O$ ratios. Finally, Orbitrap HRMS also provides the option to fragment phosphate and organophosphate molecules and determine their O isotope ratios. Once implemented, this option might offer additional insights into metabolic processes involving phosphorus, for example, by resolving isotopic compositions of metabolic sources and sinks of phosphate.

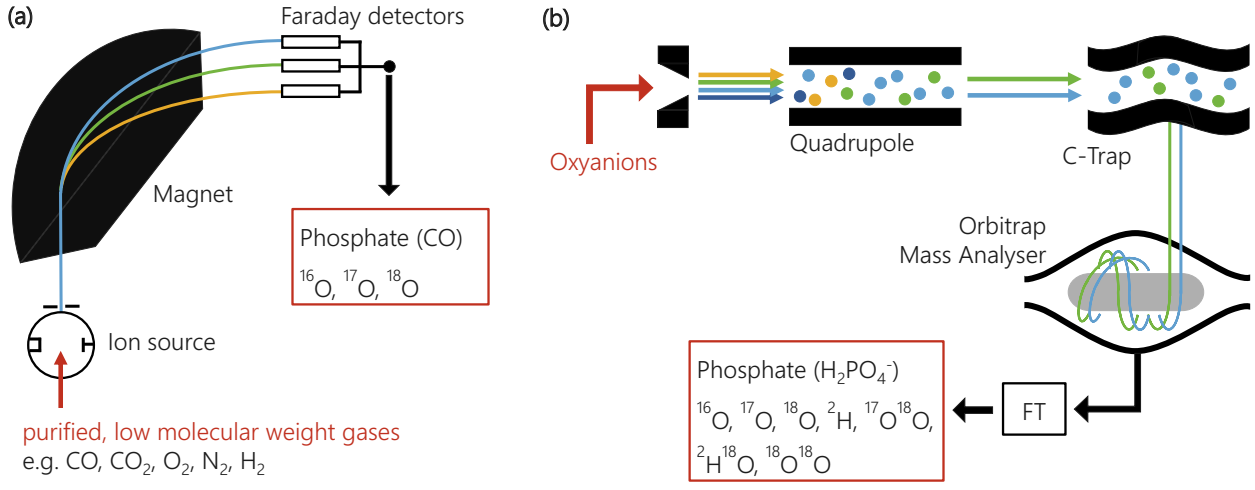

Fig. 1. Scheme of (a) isotope-ratio mass spectrometer (IRMS) and (b) ESI-Orbitrap high-resolution mass spectrometer (HRMS). Scheme modified from Hilkert *et al.* ref. [18].

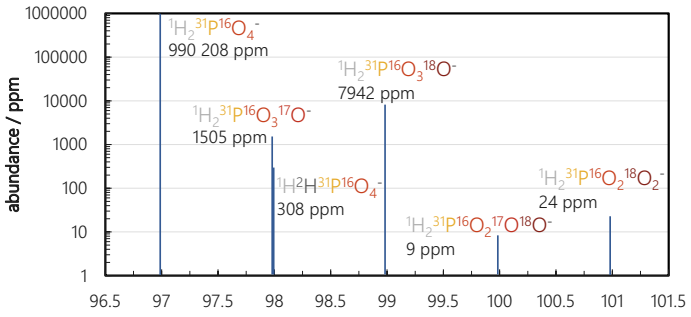

Fig. 2. Theoretical mass spectrum of the different $H_2PO_4^-$ isotopologues detected by Orbitrap mass spectrometers. The monoisotopic $H_2P^{16}O_4^-$ peak at a *m/z* of 96.97 and the singly-substituted $H_2P^{16}O_3{}^{18}O^-$ isotopologue at a *m/z* of 98.97 are detected at highest relative abundance and used for $^{18}O/^{16}O$ ratio quantification. Other singly- and doubly-substituted $H_2PO_4^-$ isotopologues existing at lower relative abundances.

Thorough evaluations of how to quantify isotope ratios by Orbitrap HRMS with comparison to data from traditional IRMS have been performed for only a few compounds. Excellent works for nitrate[17,18] and acetate[19] stand out because these careful studies also provide roadmaps for the method development for other analytes. Here, we outline such a roadmap for Orbitrap HRMS-based $^{18}O/^{16}O$ ratio measurements of ^{16}O and ^{18}O isotopologues of $H_2PO_4^-$ (Fig. 1b) whose principal feasibility has been reported earlier.[17] We note, however, that previous studies focused exclusively on oxyanions in methanolic solutions and that questions regarding sample cleanup and interferences of matrix constituents with the performance of the mass spectrometer remain to be elucidated.

The analytical precision is controlled by counting statistics as the precision of any measurement is the product of the time of observation and the intensity of the least abundant ion beam analyzed.[8] A higher precision can be achieved by a combination of longer measurement time and maximized ion counting.[19] However, longer measurements require larger sample sizes of a given concentration. Early examples with phosphate showed that by maximizing the ion count for the targeted phosphate isotopologues ($^{1}H_2P^{16}O_4^-$, $^{1}H_2P^{16}O_3{}^{18}O^-$) the acquisition error of $^{18}O/^{16}O$ ratio measurement can be reduced towards the theoretical limit defined by counting statistics (Fig. 3).[17] From a practical perspective, the acquisition error of the $^{18}O/^{16}O$ ratio can be minimized by optimizing (i) the electron spray ionization (ESI) conditions through manipulation of sample infusion flow rate, ionization spray voltages, capillary temperatures, and gas flow rates with the goal to increase and stabilize the total ion current, and (ii) mass spectrometer parameters such as the advanced quadrupole system range, automatic gain control (AGC) target and mass resolution with the goal to maximize the number of ions counted.

To illustrate some of the critical mass spectrometry parameters here, we virtually follow the path of phosphate ions through the instrument as outlined in Fig. 1b. Phosphate ions generated in the ESI source are then filtered through the advanced quadrupole system (AQS) according to a predefined mass range, for example 96.5 to 101.5 amu (see Fig. 2) to include important isotopologues of $H_2PO_4^-$ while excluding other, interfering ions.

The filtered ions subsequently accumulate in the C-trap up to a threshold set as automatic gain control (AGC) target before the ions are injected in the Orbitrap for mass analyzer. Optimizing AGC targets requires consideration of two factors. AGC target should be high to increase the number of collected phosphate ions. However, space-charge effects, namely coalescence at higher ion densities in the Orbitrap, might alter the abundance of isotopologue ions in the Orbitrap and cause erroneous O isotope ratios. Finally, because Orbitrap mass analysis time is directly proportional to the mass resolution, the lowest mass resolution at which isotopologue masses can be resolved should be selected to allow for obtaining the highest number of scans per time.

A complementary strategy for evaluating $^{18}O/^{16}O$ ratios of phosphate in a metabolic context is the quantification of isotopologues of PO_3^- fragment ions[27] instead of those of $H_2PO_4^-$. A fragment-based approach might reduce interferences of other ions (*i.e.* sulfate) in the mass range of $H_2PO_4^-$. Moreover, this approach likely allows for determining O isotope ratios of phosphate groups of metabolically important organophosphates (*e.g.* glycerol phos-

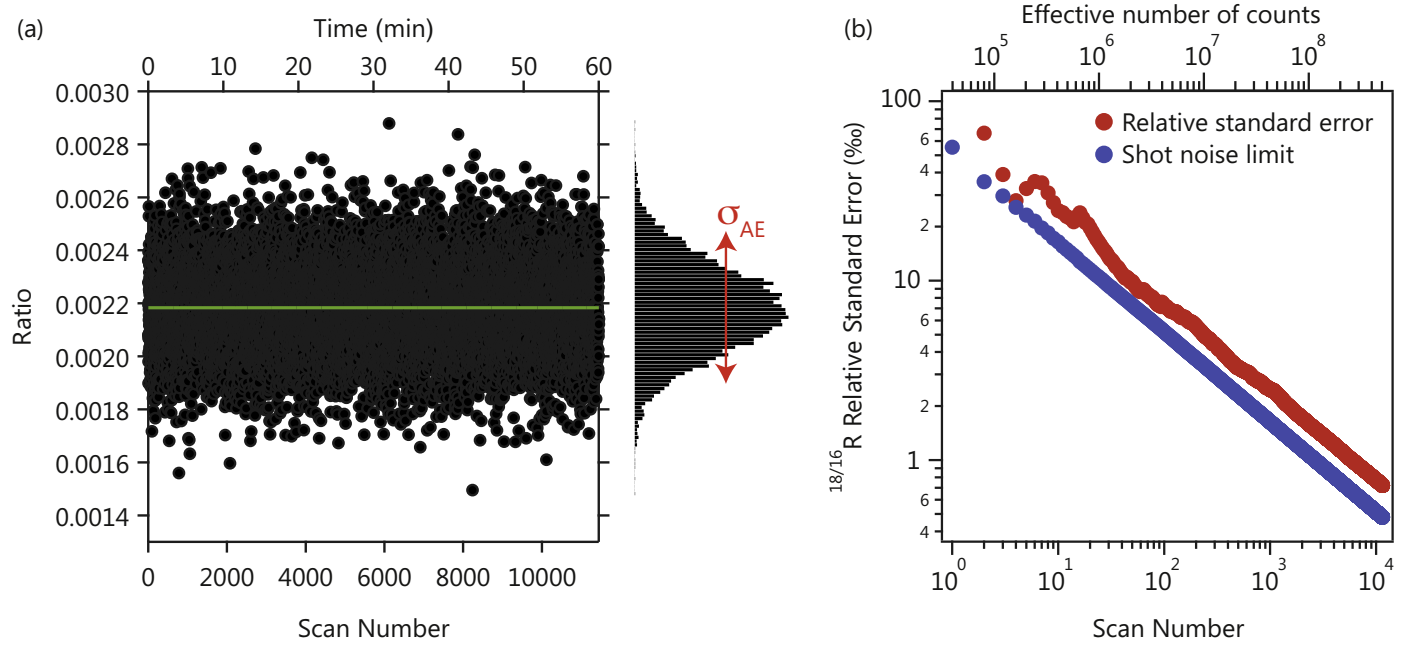

Fig. 3. (a) $^{18}O/^{16}O$ ratios determined by Orbitrap HRMS over a 60-minute acquisition time. Individual $^{18}O/^{16}O$ ratio measurements in each scan (black circles) produce a normal distribution of $^{18}O/^{16}O$ ratios around an average value (green line) with σ_{AE} as the standard acquisition error. (b) Shot noise limit and relative standard error plotted versus the effective number of ion counts during a 60-minutes measurement cycle.

phate or adenosine monophosphate).[28] Such information will be particularly insightful for studying changes of O isotope ratios in phosphoryl (PO_3^{2-}) transfer reactions; the most important reaction in the phosphorus metabolism of living organisms.

3. Oxygen Isotope Effects of Phosphoryl Transfer Reactions

A potential new avenue for assessing metabolic activity holistically is the evaluation of phosphorus metabolism through the monitoring of variations of $^{18}O/^{16}O$ ratios in phosphate at natural isotopic abundance. $^{18}O/^{16}O$ ratios in PO_4^{3-} have previously been suggested as a biomarker in the search for life[30] and more specifically for assessing microbial activity in environmental systems.[31] Given that many important biomolecules contain phosphate and that reactions involving phosphoryl transfers play an essential role in many fundamental biochemical processes,[21–23] understanding how phosphate is cycled in cellular reactions provides a promising way to look into the functioning of microbial metabolism. Phosphoryl transfer reactions involve the nucleophilic displacement of a PO_3^{2-}-group between phosphate esters and nucleophiles (*i.e.* H_2O in Fig. 4).[23,32,33] These reactions require enzyme catalysts because phosphate esters are inert under typical environmental conditions.[23,29,34] The low reactivity of phosphate derivatives is due to the negatively charged phosphate and the resulting charge repulsion with the attacking nucleophile. These circumstances lead to a very high activation barrier for phosphate(ester) hydrolysis.[21,33]

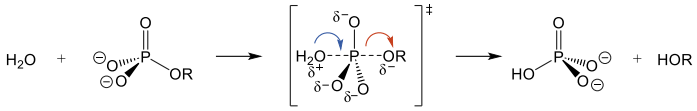

Fig. 4. Generalised mechanism of a phosphoryl transfer reaction through a pentavalent transition state with water as incoming nucleophile and R-OH as leaving group.[29]

Enzyme-catalysed phosphoryl transfer reactions, such as the phosphorylations and dephosphorylations of organic molecules, can modulate the $^{18}O/^{16}O$ ratio of intracellular PO_4^{3-}. $\delta^{18}O(PO_4^{3-})$, the representation of $^{18}O/^{16}O$ ratios of phosphate in the delta notation, can thus be seen as isotopic 'footprints' of the combined phosphoryl transfer processes of the entire phosphorus metabolism. Variations of $\delta^{18}O(PO_4^{3-})$ as a consequence of external forces, such as anthropogenic impacts, would then represent changes of metabolic activity that are induced by phosphorus homeostasis. In fact, experimentally observable changes of $\delta^{18}O(PO_4^{3-})$, the so-called oxygen isotope fractionation of PO_4^{3-}, depend not only on the kind of enzyme that catalyzes the specific metabolic reaction but also on the type of the phosphate ester substrate. While the processes determining the observable $\delta^{18}O(PO_4^{3-})$ are understood only phenomenologically, the chemical origins of this oxygen isotope fractionation are well known.[29,32,33] To that end, one considers bond-specific ^{18}O-kinetic isotope effects, ^{18}O-KIE, of phosphoryl transfer reactions of phosphate mono-, di- and triesters (Equation 1),

$$^{18}\text{O-KIE} = \frac{^{16}O k}{^{18}O k} \quad (1)$$

where $^{16}O k$ and $^{18}O k$ are the reaction rate constants for the transfer of phosphoryl group with light (^{16}O) and heavy (^{18}O) isotopic substitution. Phosphoryl group transfers involve changes of P-O bonding which are the source of ^{18}O-KIEs. The magnitude of these ^{18}O-KIEs can be rationalized approximately by the zero-point vibrational energy differences assuming that bonds between P and ^{18}O are stronger than those containing ^{16}O.[29] ^{18}O-KIEs are large and up to values of 1.025 if ^{18}O-substitution is located at the P-O bond to be broken (or formed) as compared to the small secondary KIEs relevant to cases where ^{18}O substitution occurs at the so-called non-bridging O atoms of the phosphoryl group.[29,32,33]

These general considerations of ^{18}O-KIE in phosphoryl transfer reactions are primarily based on the examination of leaving group isotope effects and thus deliberately neglect additional contributions to the $\delta^{18}O(PO_4^{3-})$. In phosphoryl transfer reactions leading to PO_4^{3-} release, for example in processes catalyzed by phosphatases, phosphoryl groups are transferred to the oxygen-containing nucleophiles H_2O and OH^-. The nominal exchange of one out of four oxygen atoms comes with an additional contribution to the observable $\delta^{18}O(PO_4^{3-})$. The $\delta^{18}O$ of the incoming nucleophile can make up ¼ of the $\delta^{18}O(PO_4^{3-})$ in a phosphoryl transfer reaction. Reactions of the incoming nucleophiles, however, also exhibit ^{18}O-KIEs. The $\delta^{18}O$ of the O-atoms transferred to PO_4^{3-} therefore does not necessarily correspond to the $\delta^{18}O$ of the nucleophile. Consequently, $\delta^{18}O(PO_4^{3-})$ can deviate from the weighted average of $\delta^{18}O$ of the phoshporyl group and the nucleophile according to the nominal 3:1 oxygen atom ratio. Data from studies of acyl group transfers to esters[35] suggest that nucleophile ^{18}O-KIEs are approx. 1.01 and thus in the lower range of PO_4^{3-} leaving group isotope effects. There will therefore be a preference for transferring ^{16}O from the nucleophile to the PO_4^{3-} released in a phosphoryl transfer reaction.

A combined evaluation of both leaving group and nucleophile ^{18}O-KIEs in phosphoryl transfer reactions from changes in $\delta^{18}O(PO_4^{3-})$, however, has so far not been attempted. While contribution of nucleophile ^{18}O-KIEs were of limited relevance for deciphering the biochemical reaction mechanisms,[23,33] understanding their contribution is essential to interpret variations of $\delta^{18}O(PO_4^{3-})$ of metabolic processes in the environment given the variability of $\delta^{18}O$ of H_2O.[36] Empirical relationships indeed describe the combined leaving group and nucleophile ^{18}O-KIEs by comparing $\delta^{18}O(PO_4^{3-})$ and $\delta^{18}O(H_2O)$ in terms of an operational O isotope enrichment factor.[37–40] These isotope enrichment factors are indeed quite variable pointing to the different mechanisms of enzyme-catalyzed phosphoryl transfers. Disentangling these observations in terms of leaving group and nucleophile ^{18}O-KIEs and interpreting them as part of reaction sequences in metabolic networks, therefore, remains elusive. More conclusive evidence could be obtained from the elucidation of how $^{18}O/^{16}O$ ratios of phosphate and organophosphates change with reaction progress. Such procedures for deriving KIEs are indeed very common in many fields of stable isotope science[41] but traditional procedures for $^{18}O/^{16}O$ ratio measurements of phosphate by IRMS prevented such analyses of phosphoryl transfer reactions. It is our goal to take advantage of the emerging opportunities of Orbitrap HRMS to derive such ^{18}O-KIEs for reactions of major relevance in the microbial phosphorus metabolism.

4. Conclusion & Outlook

Assessing the metabolic activity of the environmental microbiome under influence of anthropogenic stressors is a complex task. Because of the fundamental relevance of phosphoryl transfer reactions in metabolic processes, variations of $^{18}O/^{16}O$ ratios of phosphate offer a promising proxy for detecting shifts in metabolic activity of microorganisms. To that end, novel Orbitrap HRMS-based procedures for quantifying $^{18}O/^{16}O$ ratios in phosphate need to be implemented that allow for processing a larger number of samples compared to existing approaches. Moreover, these approaches will also allow for quantifying the ^{18}O kinetic isotope effects of various phosphoryl transfer enzymes. Such data will be invaluable to interpret how changes in metabolic activity and

phosphorus metabolism are reflected in $^{18}O/^{16}O$ ratios of intracellular phosphate.

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