Joseph Adu-Gyamfi · Verena Pfahler
Editors

Oxygen Isotopes of Inorganic Phosphate in Environmental Samples

Purification and Analysis

Joint FAO/IAEA Centre
Nuclear Techniques in Food and Agriculture

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Preface

The Soil and Water Management & Crop Nutrition (SWMCN) Subprogramme of the Joint Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA) Centre of Nuclear Techniques in Food and Agriculture supports IAEA Member States and FAO Member Countries in the use of stable isotope techniques for assessing the hydrological cycle in agro-ecological systems, evaluate evaporative losses and mixing of different water sources with the aim to improve water management and quality. Multi-tracer stable isotopes of nitrogen ($\delta^{15}N$, $\delta^{15}N\text{-}NO_3$, $\delta^{18}O\text{-}NO_3$), water ($\delta^{2}H\text{-}H_2O$, $\delta^{18}O\text{-}H_2O$), sulphur ($\delta^{34}S\text{-}SO_4$, $\delta^{18}O\text{-}SO_4$) and carbon ($\delta^{13}C$), have been used as forensics in water quality investigations. However, identifying sources and transport of phosphorus (P), as an agro-contaminant in agricultural catchments and environment has a drawback because it has only one stable isotope (^{31}P) but many radioisotopes with short half-lives, making it difficult to use for long-term P cycling studies.

Phosphorus in most organic and inorganic forms is strongly bound to oxygen (O), which has three stable isotopes to enable tracking P cycling and transformation using the stable isotopes of O in phosphate (PO_4), ($\delta^{18}O_P$). In recent years, various studies have indicated that the analysis of the stable isotopic composition of oxygen (O) bound to P ($\delta^{18}O_P$) to better understand P cycling in the environment, has become a promising tracer (surrogate) to investigate soil P transformation, plant P uptake, and to trace the sources of P from the soil to water bodies and the environment.

The SWMCN Subprogramme through a Coordinated Research Project (CRP) has actively sought to modify the methodological problems of the $\delta^{18}O_P$ protocols encountered in the field such as high loads of organic matter in the samples which makes purification challenging, and the need to improve sampling strategies in the field. This was done in collaboration with the Swiss Federal Institute of Technology in Zurich (ETH), the Trinity College, Dublin, Ireland, and scientists from developing countries. This guideline reflects the latest development and the state of art related to the use of stable isotopes of oxygen in phosphate to address the challenges encountered by scientists in the laboratory and in the field.

This publication is structured into six chapters outlines the background and examples of $\delta^{18}Op$ studies in sediments, soils, fresh water, mineral fertilizers and plants,

it presents a modified stepwise extraction and purification protocols, sample preparation for analysis, planning and designing of a study using δ^{18}Op. The aspect of external quality assurance was provided with an example of an inter-laboratory study for silver phosphate standards and data interpretation, and finally the conclusions, future trends and opportunities for scaling out of the method from laboratory to field studies. It is expected that this guideline would be extensively applied in research geared to understand phosphorus dynamics in different agro-environments. The SWNCM Subprogramme wishes to thank all the contributors involved in the preparation of this publication.

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Contents

Chapter 1
The Use of the $\delta^{18}O_P$ to Study P Cycling in the Environment

V. Pfahler, J. Adu-Gyamfi, D. O'Connell, and F. Tamburini

Abstract Phosphorus (P) fertilizers are known to increase crop productivity; however, when applied in excess, it can cause serious environment pollution. Monitoring P pollution in natural environments using stable isotopes has been difficult because P has only one stable isotope (^{31}P) making the use of P stable isotope tracing not an option. Radioactive P isotopes (^{32}P and ^{33}P) have been used but its drawbacks are the short half-life, health risks and safety procedures required to apply them in agricultural catchments. Phosphorus in organic and inorganic P forms is strongly bonded to oxygen (O), which has three stable isotopes, providing a system to track P cycling in agricultural catchments and environment using the stable isotopes of O in phosphate ($\delta^{18}O$-PO_4). In recent years, various studies have indicated that the analysis of the stable isotopic composition of oxygen (O) bound to P ($\delta^{18}O_p$) to better understand P cycling in the environment, has become a promising tracer (surrogate) to investigate soil P transformation, plant P uptake and to trace the sources of P from the soil to water bodies and the environment. The chapter outlines the background and examples of $\delta^{18}O_p$ studies in sediments, soils, fresh water, mineral fertilizers and plants.

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1.1 Background

Phosphorus (P) is an essential nutrient for all living organisms as it is part of many biochemical compounds like DNA and ATP. Available P levels in the soil are, however, often limiting crop production and farmers need to apply P fertilizer. The primary source for P fertilizer is rock phosphate, which is widely distributed throughout the world both geographically and geologically with USA, China, Morocco and Western Sahara and Russia accounting for 72% of the world total (Zapata and Roy 2004). In addition, farmers in many developing countries cannot always afford P fertilizer, but the soils in those countries are often amongst those with the lowest P availability. Besides the positive impacts of P fertilizers increasing crop productivity, P can cause negative impacts on the environment when applied in excess. Algal blooms in aquatic systems due to large input of P are still an issue in many aquatic systems including the Great Lakes in North America (Paytan et al. 2017). We therefore need to better understand P cycling in the environment. Radioisotopes and stable isotopes are a useful tool to investigate nutrient cycling in the environment (Di et al. 1997; Hogberg 1997; Barbour 2007). In regard to P, researchers relied for a long time mainly on the two artificial radioisotopes of P, ^{32}P and ^{33}P, since P only has one stable P isotope (^{31}P) and several radioisotopes (from ^{26}P to ^{30}P and from ^{32}P to ^{38}P). The radioisotope approach is often used to study the uptake of P from different phosphate rocks and fertilizers by plants (Zapata and Roy 2004; Frossard et al. 2011; IAEA 2013, 2016a, b; Nanzer et al. 2014; Nguyen et al. 2017; Scrase et al. 2020; Wolff et al. 2020), the effects of organic manure and inorganic fertilizer application on soil P cycling (Ma et al. 2020) or the residence time of P in different P pools in soils or plants (Mimura et al. 1996; Helfenstein et al. 2018). It is a useful tool to study P cycling, but its drawbacks are the short half-life of the two radioisotopes (14.3 days for ^{32}P and 25.4 days for ^{33}P) and possible health risks, which has led to the restrictions of applying them in the field, and the need for special safety equipment and national regulations to monitor radiation. Obtaining the licence to work with radioisotopes of P that emit high beta (β) radiation will require operational radiation protection and safety procedures, as well as adequate training of personnel to handle these nuclides safely (IAEA 2016a, b). There is therefore a need for a stable isotope approach to investigate P cycling in the environment.

In recent years, various studies have indicated that the analysis of the stable isotopic composition of oxygen (O) bound to P ($\delta^{18}O_P$) to better understand P cycling in the environment, has become a promising tracer (proxy) to investigate soil P transformation and to trace the source of P from the soil to water bodies and the environment. The two main underlying facts for using this method to study P cycling are (1) P is mainly bound to O in the environment and (2) the bond between P and O is stable under earth surface conditions and in the absence of biological activity (Winter et al. 1940). The two ways of altering the $\delta^{18}O_P$ signature are by (a) sorting between heavier and lighter isotopologues or (b) cleaving the P–O bond.

1.1.1 Sorting of Different Isotopologues

The sorting of heavier and lighter isotopologues could happen due to biological P uptake or inorganic processes, like the preferential sorption onto iron oxides (Jaisi et al. 2010). There are only a few studies which investigated the effect of P uptake by biota. Blake et al. (1997) report a fractionation factor of around −3‰ for *Escherichia coli*. More recently Ferrera et al. (2015) investigated the effect of P uptake by different coral species on the $\delta^{18}O_P$. Depending on the coral species, fractionation factors varied between −18.80 and +0.91‰, with the majority of the investigated species having negative fractionation factors. If an uptake effect, however, is visible in environmental systems, is questionable as a significant amount of P (>50%) must be removed from a P pool without being replenished in order to observe an effect on $\delta^{18}O_P$ values. This seems rather questionable in case of P in the soil solution since it is usually constantly replenished. Another kind of sorting can occur due to inorganic processes like sorption/desorption of P onto iron or aluminium oxides and precipitation/dissolution of P minerals (Jaisi et al. 2010). These processes play an important role in the environmental P cycle (Arai and Sparks 2007), but fractionation factors associated with inorganic processes seem to be rather small and are still debated (Jaisi et al. 2010; Melby et al. 2013).

1.1.2 Cleaving the P–O Bond

Biological activity like the hydrolysis of organic P by enzymes, can cleave the P–O bond and thus lead to an exchange of O between phosphate and water (Blake 2005). This leads to a change of the initial $\delta^{18}O_P$ value (Table 1.1).

Inorganic pyrophosphatase (PPase), an ubiquitous enzyme catalyses the hydrolysis of pyrophosphate (= diphosphate) into phosphate (Cohn 1958; Blake et al. 1997) and leads to a temperature-dependent equilibrium between O in water and in phosphate. It can be calculated by the equation of Chang and Blake (2015), as rearranged by Pistocchi et al. (2017):

$$EQ\delta^{18}O_P = -0.17 \cdot T + 26.5 + \delta^{18}O_w \quad (1.1)$$

where $EQ\delta^{18}O_P$ is the $\delta^{18}O_P$ value of phosphate at equilibrium with O in the ambient water in ‰,

T is the ambient temperature in °C and $\delta^{18}O_w$ is the isotopic composition of O in water in ‰.

What makes the inorganic PPase so important for the $\delta^{18}O_P$ is that O exchange can not only occur during the hydrolysis of pyrophosphate but also by locking a phosphate molecule into the active site of the enzyme (Cohn 1958; Blake et al. 1997). When the inorganic PPase is involved, the initial $\delta^{18}O_P$ signature is erased completely (see Table 1.1).

Table 1.1 Examples for the effect of different enzymes on $\delta^{18}O_P$ values

Enzyme(s)	$\delta^{18}O_P$ of substrate (‰)	$\delta^{18}O$ of water (‰)	Temperature (°C)	Fractionation factor (‰)	$\delta^{18}O_P$ of product (‰)	References
Inorganic PPase	20	−10	20	EQ[§]	13.1	Chang and Blake (2015)
	0	−10	20		13.1	
	20	−10	5		15.7	
	20	0	20		23.1	
Acid phosphatase	20	−10	nr	−10 ± 3	10 ± 0.75	Von Sperber et al. (2014)
	0	−10			−5 ± 0.75	
	20	0			12.5 ± 0.75	
Alkaline phosphatase	20	−10	nr	−30 ± 8	5 ± 2	Liang and Blake (2006a)
	0	−10			−10.0 ± 2	
	20	0			7.5 ± 2	
DNase 1+AlPase	20	−10	nr	−25 ± 6	−7.5 ± 3	Liang and Blake (2009)
	0	−10			−17.5 ± 3	
	20	0			−2.5 ± 3	

Inorganic PPase = inorganic pyrophosphatase; EQ = Equilibrium fractionation; nr: not relevant (reaction is temperature-independent); DNase 1= deoxyribonuclease 1; AlPase = alkaline phosphatase

Hydrolytic enzymes like acid and alkaline phosphatases only lead to the exchange of 1–2 oxygen atoms and are temperature-independent (Liang and Blake 2006a; von Sperber et al. 2014, 2015). The associated fractionation factors vary greatly, from + 20 to −30‰, and consequently the $\delta^{18}O_P$ of the released phosphate varies depending on the involved enzymes. Some fractionation factors like the one of phytase and acid and alkaline phosphatase are substrate dependent (von Sperber et al. 2015; Bai et al. 2020).

1.2 From Past to Present

Early ^{18}O studies worked with ^{18}O-enriched samples rather than natural abundance (Winter et al. 1940; Cohn and Hu 1978; Larsen et al. 1989) and were designed to understand the mechanism of the studied enzymes. Natural abundance studies only came later and were not that common, most likely due to the challenges of purifying extracts for $\delta^{18}O_P$ analysis. At the beginning, bismuth phosphate ($BiPO_4$) was used as analyte (Tudge 1960), however, $BiPO_4$ is highly hydroscopic, making it more

difficult to handle and store (Vennemann et al. 2002). Nowadays, Ag_3PO_4 is used as analyte (Firsching 1961; Crowson et al. 1991), which is easier to handle and faster to precipitate. Advancements have also been made in the analysis of Ag_3PO_4 (Vennemann et al. 2002). The analysis with a high temperature conversion elemental analyser (TC/EA) coupled to an isotope ratio mass spectrometer (IRMS) is now the most common method.

The origins of the ^{18}O method lie in chemical, biochemical and paleotemperature studies (Tamburini et al. 2014). In the early 2000s, Blake et al. (2001) postulated that the $\delta^{18}O_P$ method could be used to study biological P cycling. From then onwards, the $\delta^{18}O_P$ method became more widespread and scientists used it in a diverse range of environmental samples, including lake and marine sediments, salt and fresh water, soils, mineral and organic fertilizers, rocks, dust and plants (Tamburini et al. 2014). Most naturally occurring $\delta^{18}O_P$ values, reported so far, range between 10 and 30‰, with the exception of igneous rocks, which range between −0.8 and +12‰ (Smith et al. (2021)). For a more detailed overview of $\delta^{18}O_P$ values in the environment, the reader is referred to Bauke (2020) for $\delta^{18}O_P$ values in soils; Smith et al. (2021) for $\delta^{18}O_P$ values from rocks; for aquatic ecosystems, see for example Davies et al. (2014) and Gooddy et al. (2015). General overviews of $\delta^{18}O_P$ values can, for example, be found in Tamburini et al. (2014) and Jaisi & Blake (2014). Figure 1.1 shows examples

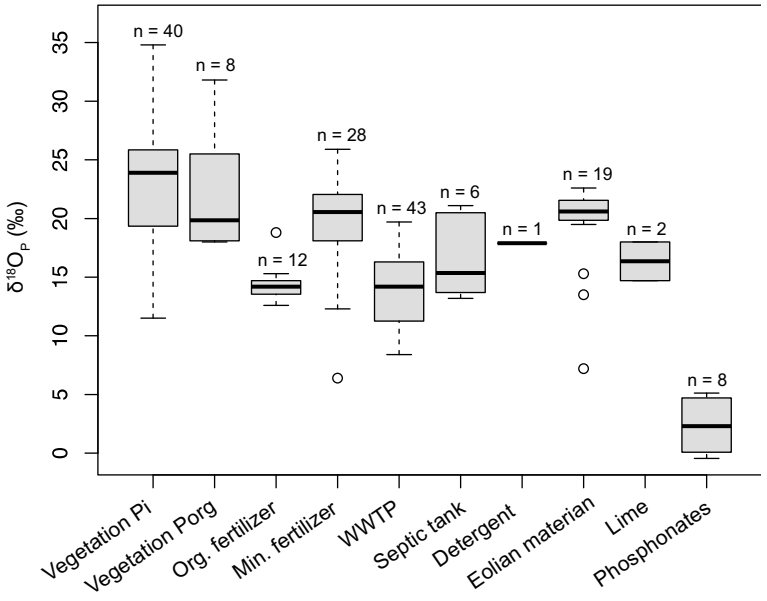

Fig. 1.1 Examples for $\delta^{18}O_P$ values of potential phosphorus (P) inputs into soils and aquatic systems. Vegetation P_i = inorganic P in vegetation; vegetation P_{org} = organic P in vegetation; org. fertilizer = organic fertilizer; min. fertilizer = mineral fertilizer; WWTP = wastewater treatment plant

for $\delta^{18}O_P$ values of potential P inputs like animal faeces and mineral P fertilizers into soils and aquatic systems (see Annex Table 1.2 for references).

The $\delta^{18}O_P$ method was also considered ideal to proof life on other planets, for example, on Mars (Greenwood et al. 2003). To be able to analyse the $\delta^{18}O_P$ from such a diverse range of substrates, several obstacles had to be overcome. The main obstacles for the $\delta^{18}O_P$ method are low P concentrations and/or high concentrations of interfering compounds like organic matter (Tamburini et al. 2010; Goldhammer et al. 2011b; Nisbeth et al. 2019). Therefore, modern protocols of the $\delta^{18}O_P$ method involve several purification steps before the final precipitation of silver phosphate (Ag_3PO_4) which is then used to determine the $\delta^{18}O_P$ via TC/EA-IRMS. McLaughlin et al. (2004) adapted the method for seawater samples adding a MAGIC step (precipitation of Mg $(OH)_2$) and purifying the samples using cerium phosphate. Tamburini et al. (2010) applied this protocol to soil extracts but found that the Ag_3PO_4 could be still contaminated with organic matter. It was therefore suggested to use precipitation of ammonium phosphomolybdate and magnesium ammonium phosphate to purify extracts (Kolodny et al. 1983), adding an additional purification step (DAX-resin) at the beginning of the protocol.

Nowadays, the $\delta^{18}O_P$ method is more and more used to investigate P cycling in the environment, including sediments (Jaisi and Blake 2010; Goldhammer et al. 2011a; Pistocchi et al. 2017; Liu et al. 2019), soils (Amelung et al. 2015; Gross and Angert 2015; Helfenstein et al. 2018; Pistocchi et al. 2020; Siegenthaler et al. 2020; Pfahler et al. 2020) and plants/algae (Pfahler et al. 2013; Mellett et al. 2018). The focus in case of aquatic systems is often on source apportionment (McLaughlin et al. 2006a; Gross et al. 2013; Granger et al. 2017). Other studies characterise the $\delta^{18}O_P$ of different P inputs into the environment like mineral fertilizers and farm-yard manure (Granger et al. 2018) or investigate the effect of enzymes on $\delta^{18}O_P$ values (Blake et al. 1998; Liang and Blake 2006b; von Sperber et al. 2014).

1.3 Examples of $\delta^{18}O_P$ Studies

1.4 Sediments

Goldhammer et al. (2011a) analysed dissolved P extracted from sediment pore water, using marine sediments from the Benguela upwelling system of the coast of Namibia in Africa. They found dissolved P $\delta^{18}O_P$ values in and out of equilibrium, ranging between 12.8 and 26.6‰, and attributed these values to different P uptake strategies of microorganisms at low and high inorganic P availability.

Investigating river sediments from the Redon River in France, Pistocchi et al. (2017) showed that the $\delta^{18}O_P$ can potentially be used to trace P sources and study P cycling in river sediments. They also adapted the method by Tamburini et al. (2010) successfully to river sediments. Liu et al. (2019) explored a pre-treatment method for

the $\delta^{18}O_P$ of different P fractions in sediments. They also concluded that the $\delta^{18}O_P$ can be a promising tool to trace P and study P cycling in freshwater sediments.

1.5 Soils

With the $\delta^{18}O_P$ approach, Tamburini et al. (2012) investigated the importance of microorganisms for soil P cycling. They extracted available, microbial, vegetation and mineral P and analysed the corresponding $\delta^{18}O_P$ values from soils taken along a soil chronosequence at the Damma glacier fore field in Switzerland. Regardless of the contribution of vegetation, mineral or P released by organic P hydrolysis, available P $\delta^{18}O_P$ values were always close to microbial P $\delta^{18}O_P$ values. This showed, for the first time under field conditions, the importance of microbial P cycling for the available P.

Recently, Bi et al. (2018) combined the $\delta^{18}O_P$ method with measuring the abundance of genes related to the P cycle like *phoX* (acid phosphatase) and *phoD* (alkaline phosphatase D) to investigate P cycling in agricultural soil from the Fengqiu State Key Experimental Station for Ecological Agriculture (Henan, China). Like Tamburini et al. (2012), they measured acid phosphatase activity and additionally also alkaline phosphatase, phosphodiesterase and dehydrogenase activity. They showed the importance of microbial P cycling in agricultural soil with $\delta^{18}O_P$ values of the more labile P pools (water, sodium bicarbonate and sodium hydroxide extractable inorganic P) tending towards equilibrium.

1.6 Salt and Fresh Water

After McLaughlin et al. (2004) successfully developed a method to analyse the $\delta^{18}O_P$ of phosphate in seawater samples, Elsbury et al. (2009) applied this method to investigate P cycling and inputs into Lake Erie. Along with water samples from different locations at Lake Erie, they also analysed water samples from seven rivers feeding into Lake Erie. The $\delta^{18}O_P$ values ranged between 10 and 17‰, with an expected equilibrium value of 14‰. The $\delta^{18}O_P$ values of the rivers were around 11‰ and could thus be one source of P, however, they did not find a P source with higher $\delta^{18}O_P$ values (Elsbury et al. 2009). They proposed that P released from sediments might cause the higher $\delta^{18}O_P$ values found in Lake Erie. Gooddy et al. (2016) used the $\delta^{18}O_P$ along with $\delta^{15}N$ and $\delta^{18}O$ of nitrate and $\delta^{15}N$ of ammonium to investigate eutrophication. They took water samples from River Beult (Kent, UK) twice within six months and at seven sampling sites along the river. One of their conclusions was that abiotic processes in the river cause the P concentration changes as the isotopic values did not change dramatically along the river.

1.7 Dust

The main goal of the study by Gross et al. (2013) was to trace the input of P into Lake Kinneret in Israel through atmospheric deposition of dust. They analysed the $\delta^{18}O_P$ values of available P from soil and dust samples and found that in natural soils, the $\delta^{18}O_P$ values were between 17.4 and 18.2‰ and between 19.3 and 22.1‰ in agricultural soils. Resin P extracted from dust samples had $\delta^{18}O_P$ values of around 22‰, indicating that agricultural soils were the main source for dust in the region around Lake Kinneret. Following this study, Gross et al. (2016) found that P associated with Saharan dust can be traced to South America.

1.8 Mineral Fertilizers, Manures and Rocks

The $\delta^{18}O_P$ values of mineral fertilizers range from 6.4 to 25.9‰ (Fig. 1.1). This wide range is most likely caused by the bedrock material used to produce the mineral fertilizers (Gruau et al. 2005), which $\delta^{18}O_P$ values vary similarly to the ones of mineral fertilizers (Smith et al. 2021). Phosphorus leached from fresh cattle faeces can be an important P input into aquatic systems, but is sometimes hard to quantify (Bond et al. 2014). Granger et al. (2018) investigated how variable faeces $\delta^{18}O_P$ values are. They collected faeces samples from seven different cows, grazing on different pastures in Devon (UK) and differing in gender, race and age. Values ranged between 13.2 and 15.3‰ without correlation with any of the variables (pastures, gender, race and age), but they were within equilibrium range calculated with the $\delta^{18}O_P$ of groundwater and cattle body temperature (Granger et al. 2018).

In one of the early $\delta^{18}O_P$ studies, Mizota et al. (1992) determined the $\delta^{18}O_P$ of a range of lithogenic material and soil samples from the Great Rift Valley in Africa and Java (Indonesia). Volcanic ashes had $\delta^{18}O_P$ values between 5.3 and 6.2‰, apatite from hydrothermal deposits had $\delta^{18}O_P$ values between 2.4 and 12.2‰, and apatite from carbonatites $\delta^{18}O_P$ values between 0.2 and 10‰. The soil samples were extracted sequentially, first with 2.5% acetic acid and then with 1M ammonium fluoride (NH4F). Their soil $\delta^{18}O_P$ values ranged between 10.1 and 20.6‰ (acetic acid-extractable P) and between 12.7 and 24.8‰ (NH4F-extractable P). Their conclusion was that available soil P $\delta^{18}O_P$ values are due to biogenic and volcanic inputs.

1.9 Plants

Inorganic P in plants tends to have higher $\delta^{18}O_P$ values compared to the soil due to the 18O-enriched plant water compared to the soil water (Pfahler et al. 2013). However, as Pfahler et al. (2017) revealed, P limitation lowers inorganic P $\delta^{18}O_P$ values in soybean leaves. Using ^{33}P along with measurement of the $\delta^{18}O_P$, the authors could

also calculate P fluxes to and from different plant parts. Qin et al. (2018) used the $\delta^{18}O_P$ method to investigate P uptake by maize plants. They found that the $\delta^{18}O_P$ value of the P applied to the soil and taken up via the roots directly was found in maize shoots, whereas the $\delta^{18}O_P$ value had changed if P was taken up via arbuscular mycorrhizal fungi (Qin et al. 2018).

Glossary

Fractionation (isotope) Isotopes of the same element have slightly different chemical and physical properties. During processes like enzymatic reactions those differences lead to changes in the relative abundance of the isotopes, i.e. to an isotope fractionation.

Fractionation factor A fractionation factor describes the change of, e.g. the $^{18}O/^{16}O$ ratio in a substrate to the $^{18}O/^{16}O$ ratio of a product due to a fractionation process.

Isotopologue Molecules which differ in their isotopic composition are called isotopologues. For example, a phosphate molecule with only ^{18}O and a phosphate molecule with only ^{16}O are two isotopologues of the same molecule.

Per mil (‰) Parts per thousand. $\delta^{18}O$ values are usually expressed in ‰.

TC/EA-IRMS Thermal conversion elemental analyser (TC/EA) coupled to an isotope ratio mass spectrometer (IRMS) is commonly used to determine the $\delta^{18}O_P$. The oxygen in silver phosphate is converted, via pyrolysis, into carbon monoxide, which isotopic composition is then measured in the IRMS.

$\delta^{18}O$ The oxygen isotope ratio is conventionally given in the delta notation: $\delta^{18}O = (R_{sample}/R_{standard}) - 1$, where R_{sample} is the $^{18}O/^{16}O$ ratio of a sample and $R_{standard}$ is the $^{18}O/^{16}O$ ratio of the Vienna Standard Mean Ocean Water (V-SMOW). $\delta^{18}O_P$ is the $\delta^{18}O$ of oxygen bound to P. $\delta^{18}O_W$ is the $\delta^{18}O$ of water.

Annex

Table 1.2 References included in Fig. 1.1

Phosphorus input/source	References
Inorganic P vegetation	Tamburini et al. 2012; Pfahler et al. 2017, 2020; Helfenstein et al. 2018
Organic P vegetation	Helfenstein et al. 2018
Organic fertilizer	McLaughlin et al. 2006a; Amelung et al. 2015; Granger et al. 2017, 2018

(continued)

(continued)

Phosphorus input/source	References
Mineral fertilizer	Gruau et al. 2005; McLaughlin et al. 2006a; Gross et al. 2013; Amelung et al. 2015; Gross et al. 2015b; Tian et al. 2016; Granger et al. 2017; Bi et al. 2018; Ide et al. 2020; Pfahler et al. 2020
Wastewater treatment plant	Gruau et al. 2005; McLaughlin et al. 2006b; Elsbury et al. 2009; Young et al. 2009; Gooddy et al. 2016, 2018; Granger et al. 2017; Ide et al. 2020
Septic tank	Granger et al. 2017; Tonderski et al. 2017
Detergent	Gruau et al. 2005
Aeolian material	Gross et al. 2013, 2015a
Lime	Pfahler et al. 2020
Phosphonates	Sandy et al. 2013

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Chapter 2
Extraction Protocol

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Abstract Studies showed that the $\delta^{18}O_P$ is a useful tool to study P in the environment. Adequate extraction protocols for the targeted P pools of the study are a prerequisite for a successful study. Likewise, for most environmental samples, including water, soil, sediment and plant samples, it is crucial that the samples are processed as soon as possible after they have been taken to avoid any alterations of the original $\delta^{18}O_P$ signature. This is especially true when more bioavailable P pools, like soluble reactive P (SRP) in water samples, are extracted and analysed. Brucite precipitation of water samples should be directly done in the field, fresh soil and sediment samples have to be extracted within 7 days (if microbial P is targeted, on the day of sampling), and plant samples have to be extracted within a few hours of sampling or be frozen. The chapter briefly describes the P cycle in aquatic and terrestrial ecosystems and give an overview about extracting the most common P pools for $\delta^{18}O_P$ analysis: soluble reactive P in water samples, sequentially extracted P pools of soil, sediment, fertilizer and plant samples.

2.1 An Overview of the Extraction Protocols

Studies showed that the $\delta^{18}O_P$ is a useful tool to study P in the environment (Helfenstein et al. 2018). Adequate extraction protocols for the targeted P pools of the study

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are a prerequisite for a successful study. Likewise, for most environmental samples, including water, soil, sediment and plant samples, it is crucial that the samples are processed as soon as possible after they have been taken in order to avoid any alterations of the original $\delta^{18}O_P$ signature. This is especially true when more bioavailable P pools, like soluble reactive P (SRP) in water samples, are extracted and analysed. Brucite precipitation of water samples should be directly done in the field (see Sect. 2.2), fresh soil (see Sect. 2.3) and sediment (see Sect. 2.4) samples have to be extracted within 7 days (if microbial P is targeted, on the day of sampling), and plant samples (see Sect. 2.5) have to be extracted within a few hours of sampling or be frozen.

The following chapters briefly describe the P cycle in aquatic and terrestrial ecosystems and give an overview about extracting the most common P pools for $\delta^{18}O_P$ analysis: soluble reactive P in water samples, sequentially extracted P pools of soil, sediment, fertilizer and plant samples. In general, P occurs in microbial, organic and inorganic forms in the environment (Condron and Newman 2011). These forms are interlinked with each other through inorganic and biological processes and are often clustered together according to their extractability with different chemicals. Which of these pools needs to be extracted and analysed for its $\delta^{18}O_P$ value depends on the research question.

2.2 Aquatic Systems

Phosphorus inputs into aquatic systems are mainly from non-point sources like runoff from roads and fields or point sources, for example, from wastewater treatment plants. Direct disposal of untreated wastewater, effluents and solid wastes to water ways from households and small-scale industries, and leachates from septic tanks also contributed to the enrichment of P in water ways. Atmospheric inputs of P into aquatic systems are often low but can still negatively affect water quality in some cases. Since bioavailability of P in aquatic systems is often low and hence aquatic biota like plants are efficient in taking up P (Reynolds and Davies 2001), those P inputs can have a drastic effect on aquatic ecosystems like eutrophication (Schindler et al. 2016). In marine ecosystems, P is often considered as the nutrient which is limiting primary production (Filippelli 2008).

Soluble reactive P (SRP) is considered most bioavailable and many studies and water quality guidelines focus on this pool (Fig. 2.1) (Directive 2000).

Particulate P in water is also important as it can contribute significantly to the bioavailable P in aquatic systems (Ellison and Brett 2006). Both particulate P and SRP contribute to sedimentary P, either by deposition or adsorption onto particles. Through resuspension and desorption processes their bioavailability for aquatic organisms increases again. Once taken up by the biota, inorganic P can be incorporated into organic P (P_{org}). The P bond in organic forms becomes available through the hydrolysis by enzymes. Organic P like organic matter, in general, can also be buried into the sediment.

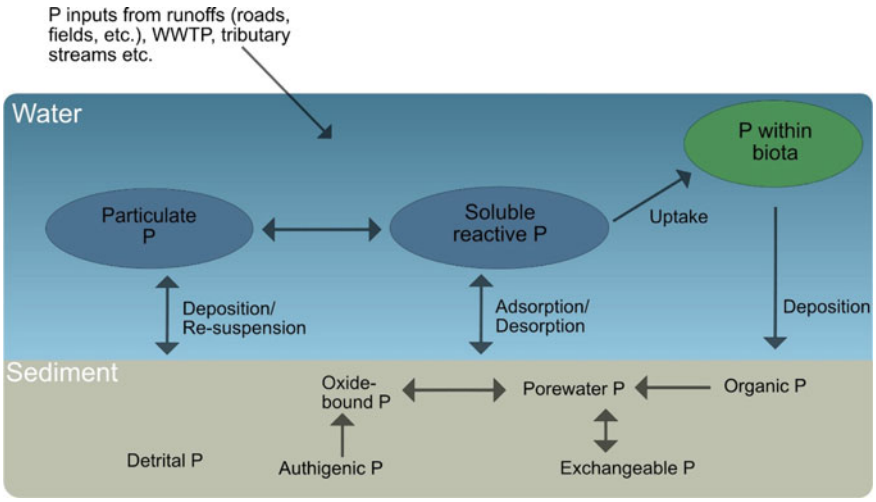

Fig. 2.1 Simplified phosphorus (P) cycling in aquatic systems. WWTP = wastewater treatment plant

2.2.1 Extraction of Soluble Reactive Phosphorus (SRP)

Concentrations of SRP can be very low in water bodies and thus it might be necessary to collect several litres of water for the determination of the $\delta^{18}O_P$ as 20–30 µmol P (= 0.65–0.97 µg P) which are necessary for the purification protocol. Granger et al. (2017b) sampled, for example, between 25 and 50 L of water. It is therefore recommended to measure the SRP concentration in the water before the actual sampling. The below-described sampling protocol is based on the method by Nisbeth et al. (2019) (Fig. 2.2).

As SRP concentrations are often low, it is recommended to thoroughly clean all equipment/consumables like polypropylene bottles with P-free detergent and rinse them with diluted HCl and ultrapure water before using.

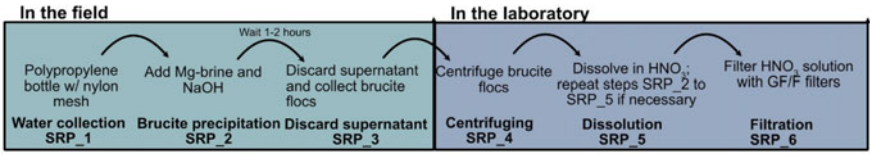

Fig. 2.2 Scheme of water collection and processing for $\delta^{18}O_P$ analysis

2.2.2 Equipment and Consumables

- Standard lab glassware and equipment.
- Polypropylene bottles, different sizes.
- Nylon mesh.
- Vacutainers.
- Plastic tubes.
- GF/F filters.

2.2.3 Reagents

1. **3 M magnesium brine**

   Weigh out 1.6 kg of $MgCl_2$ (hexahydrate; MW: 203.3 g/mol). Add 2.5 L of ultrapure water. After the salt has dissolved, filter the brine on a GF/F filter.
2. **1 M NaOH**

   Weigh out 40 g of NaOH pellets. Dissolve it in 1 L of ultrapure water.
3. **1 M HNO_3**

   In a volumetric flask add 66 mL concentrated HNO_3 to 800 mL ultrapure water and make up to 1 L.

Step SRP_1—Water collection (field)

- Choose two different sizes of polypropylene bottles: a smaller one (<5 L) to take the water samples and a larger one (<50 L) for collecting the water.
- In shallow water ways and ponds, samples need to be collected carefully without disturbing the sediments deposited in bed of the water ways.
- Attach a nylon mesh on top of the larger bottle to remove any coarse material like leaves and small branches from the water samples.
- Fill up the larger bottle using the smaller one. Depending on the SRP concentration, more than one large bottle might be necessary.
- In a small bottle, e.g. 125 mL, collect water to measure phosphate concentration. Fill up a vacutainer for the determination of the $\delta^{18}O$ of water.

Step SRP_2—First Brucite precipitation (field)

- Measure out 1 L of the Mg brine and pour it into the 50 L bottle. Shake or stir using a rod well. Measure out 250 mL of 1 M NaOH and add it. Shake again.

Step SRP_3—Discarding supernatant (field)

- If it is cold, leave the 50 L bottle in the field and let the $Mg(OH)_2$ (brucite) floc settle. After about 1 h, siphon out the supernatant by using the plastic tube. Thick pipes might be faster; however, thinner tubes can reduce losses and are therefore preferable. About 1/10 of the initial volume will remain. If it is warm, the bottle

should be transferred to the lab, put into a fridge and shaken. After a couple of hours, the floc is settled and no brucite remains in suspension, the supernatant is discarded.

Step SRP_4—Centrifuging brucite flocs (laboratory)

- The remaining brucite floc should be centrifuged at about 3000 rpm for 15 min. The supernatant is then eliminated.

Step SRP_5—Dissolution of brucite flocs (laboratory)

- Brucite is removed from the centrifuging bottles by dissolving it with 1 M HNO_3. Use the minimum amount of acid to dissolve the brucite from each bottle. Combine the solutions. pH should be around 1. If needed, the volume can be further reduced by performing additional MAGIC (steps SRP_2 to SRP_5) steps. This is done only by raising the pH to about 10–11 (e.g. by adding NaOH). Then, repeat step SRP_2–5. Generally, after three MAGIC steps, the volume is reduced from 50 L to about 250–300 mL.

Step SRP_6—Filtration

- Once the desired volume is reached (generally around 100–150 mL), the solutions are filtered using GF/F filters.

2.3 Soils

Phosphorus occurs in inorganic and organic forms in soils (Fig. 2.3). The concentration of inorganic P in the soil solution (available P) is often relatively low, around 0.1 mg P L^{-1}, and is controlled by several biotic and abiotic processes (Fig. 2.3).

Immobilization of inorganic P by microorganisms, precipitation of P minerals, and sorption of P onto iron oxides or clay minerals reduce the concentration of inorganic P in the soil solution (Arai and Sparks 2007). Mineralization of organic P, dissolution of P minerals and desorption of Pi from soil particles increase the concentration of P in the soil solution (Shen et al. 2011). Organic P in soils typically makes up 30–65% of total P (Harrison 1987) and includes phosphomonoesters and diesters, such as DNA and RNA. These forms of organic P cannot directly be taken up by plants or microorganisms. Plants and microorganisms developed several strategies to make organic P and other immobilized P available again, such as exudation of organic acids and phosphoenzymes like acid phosphatase. Association with mycorrhizal fungi is also a common strategy among plants to increase P availability (Shen et al. 2011).

The extraction methods for different soil P pools vary greatly from country to country and soil to soil (Nawara et al. 2017). Sodium bicarbonate, anion exchange resin membranes and water are only some of the extractants used for available P. Not all existing methods for extraction different soil P pools are suitable for the analysis of the $\delta^{18}O_P$. In some cases, P concentrations in the extracts would be too low so that a large amount of soil would be necessary, or the extraction would

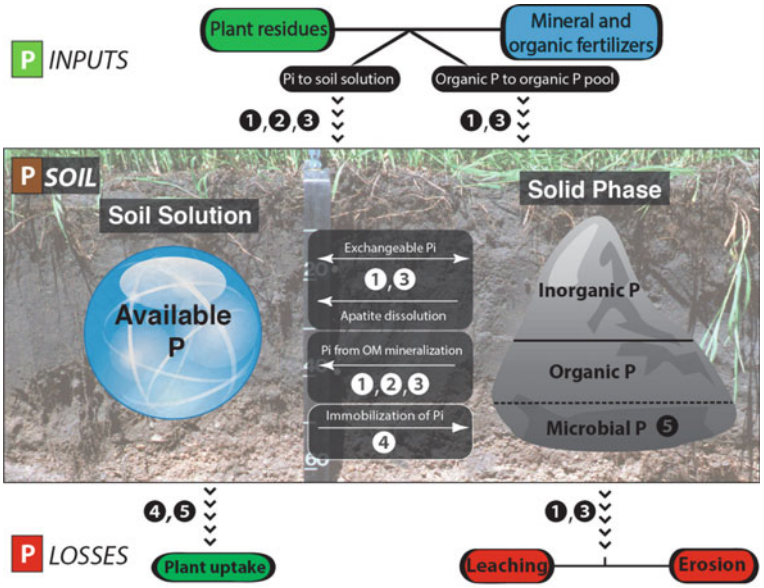

Fig. 2.3 Abiotic processes with no/little fractionation 2. Inorganic hydrolysis of condensed phosphates 3. Preferential uptake of P by, for example, microorganisms 4. Intracellular cycling of P leading to equilibrium between O in phosphate and water, mediated my inorganic pyrophosphatase 5. Hydrolysis of organic P by phosphoenzymes like acid and alkaline phosphatase

lead to an uncontrolled O exchange between phosphate and the extracting reagent. Other extracts might cause issues during the purification protocol. A new extraction method should therefore first be tested for its suitability for the $\delta^{18}O_P$ analysis. The most tested and used method is the extraction with 1 M HCl (Tamburini et al. 2010; Amelung et al. 2015; Granger et al. 2017a). It is also often the easiest when it comes to sample handling as the samples do not need to be fresh but can be dried. It might, however, not always be the most meaningful method, depending on the research hypothesis. When investigating the impact of biological processes on soil P cycling, the available (and microbial) P pool is better suited (see also Chap. 5). The following sequential extraction protocol is based on Tamburini et al. (2018) and consists of the extraction of resin and hexanol P, NaOH-EDTA inorganic and organic P and HCl P (Fig. 2.4). Tamburini et al. (2018) developed the method to purify inorganic and organic P in NaOH-EDTA extracts and follows the protocols by Weiner et al. (2011) and Kouno et al. (1995) for resin and hexanol P and Tamburini et al. (2010) for HCl P. The microbial P is calculated with a simple mass balance using the resin and hexanol P concentrations and $\delta^{18}O_P$ values (Tamburini et al. 2012).

Equipment and consumables

- Polypropylene bottles, different sizes.
- Vacutainers.

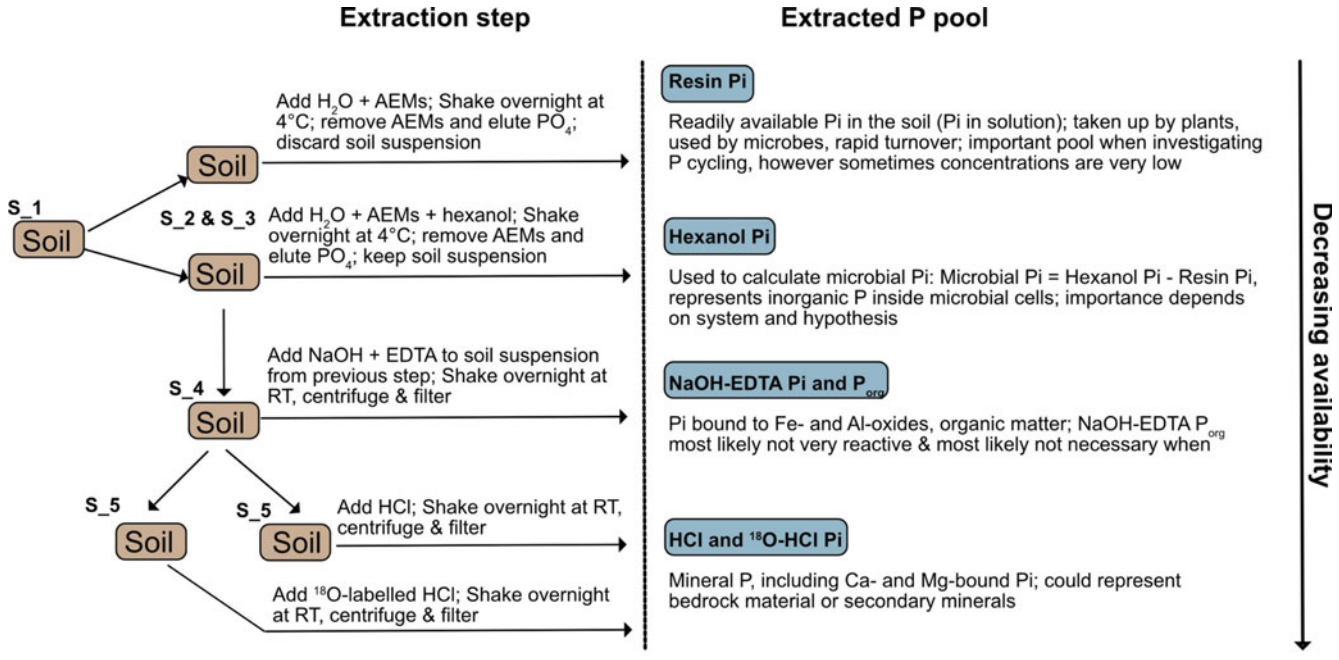

Fig. 2.4 Extraction scheme for sequential soil extraction for $\delta^{18}O_P$ analysis; modified after Tamburini et al. (2018). $P_{i\,=}$ inorganic P; $P_{org\,=}$ organic P; H_2O = here: ultrapure water; AEMs = anion exchange resin membranes; RT = room temperature

- Standard lab glassware and equipment.
- GF/F filters.

Reagents

1. **Anion exchange resin membranes**

   The resin membranes are stored in 1 M HNO_3. Take out the number of membranes you need and wash with ultrapure water. Shake for 1 h in 0.5 M $NaHCO_3$. Wash with ultrapure water. Shake for 1 h in 0.5 M $NaHCO_3$. Wash three times with ultrapure water and store in ultrapure water before use.
2. **0.5 M $NaHCO_3$**

   Weigh out 42.3 g of sodium bicarbonate ($NaHCO_3$; MW = 84.01 g/mol). Add 1 L of ultrapure water. Dissolve well by stirring on magnetic plate. Prepare fresh.
3. **Hexanol**
4. **0.2 M HNO_3**

   Measure 986 mL ultrapure water and add 14 mL concentrated HNO_3 (65% HNO_3).
5. **1 M HNO_3**

   In a volumetric flask add 66 mL concentrated HNO_3 to 800 mL ultrapure water and make up to 1 L.
6. **0.25 M NaOH–0.05 M EDTA solution**

   Weigh out 10 g NaOH and 18.612 g EDTA disodium salt and dissolve in 1 L ultrapure water.
7. **1 M HCl**

   In a volumetric flask add 82.7 mL concentrated HCl (37%) in 900 mL ultrapure water and make up to 1 L. Prepare two batches of 1 M HCl (^{18}O-labelled and unlabelled HCl solutions). Take a subsample from each batch for the determination of the $\delta^{18}O_w$ value of each batch.

Use a soil: solution ratio of 1:10 throughout the protocol.

Step S_1 Sample preparation

- After sampling the soil, sieve it to 2 mm and store at 4 °C. Put a subsample into a vacutainer for the extraction of soil water and store it in the freezer. The soil sample should be processed as soon as possible.
- Weigh fresh soil samples in duplicates into plastic bottles. Amounts weighed depend on P concentrations, since for each extract to be purified, 20 μmoles of P (0.65 μg of P) are needed:
  - Sample 1: Resin P.
  - Sample 2: Hexanol P.

Step S_2 Resin and hexanol P extraction

- Add ultrapure water to sample 1 and 2.

- Add hexanol to the sample 2, e.g. 20 mL hexanol to 600 mL ultrapure water.
- Add previously conditioned anion exchange resin membranes to each sample and shake overnight at 4 °C.

Step S_3 Recovering resin strips

- Recover the resin membranes from the samples, rinse them with ultrapure water to remove any adhering particles.
- Put the resin membranes in 1 L plastic bottles. Add about 100 mL ultrapure water and shake the resin membranes for 1 h. Discard the water. Add 75 mL 0.2 M HNO_3 to each sample. Place on a horizontal shaker and shake for 16 h at 160 rpm. Collect the 0.2 M HNO_3 elution solution. Recover the resin membranes and store them in 1 M HNO_3.
- Use soil + solution from sample 2 for the remaining extractions, discard soil + solution from sample 1.

Step S_4 NaOH-EDTA extraction

- Add NaOH and EDTA to the soil-solution mix of sample 2 to obtain a final concentration of 0.25 M NaOH and 0.05 M EDTA.
- Put on a shaker at room temperature and shake overnight.
- The following day remove the samples from the shaker. Centrifuge for 15 min at 5000 rpm. Filter the supernatant through a GF/F filter. Recover soil from each of the centrifuge bottles and dry at 40 °C.
- The supernatant is stored in the freezer and freeze-dried ASAP after the extraction.

Step S_5 HCl extraction

- Split the recovered soil from step S_4 into two equal parts.
- Add ^{18}O-labelled 1 M HCl to one part and unlabelled 1 M HCl to the second part. Put on the shaker at 160 rpm at room temperature for 16 h.
- The following day remove the samples from the shaker. Centrifuge for 15 min at 5000 rpm. Filter the supernatant through a GF/F filter. Collect supernatant.

2.4 Extraction of P from Sediments

Extracting P from sediments for $\delta^{18}O_P$ analysis is similar to the extraction of P from soils (see Sect. 2.3; Fig. 2.4). Sediment samples can be extracted sequentially including extraction with a citrate-dithionite-bicarbonate (CDB) solution and a sodium acetate buffer prior to the extraction with 1 M HCl (Ruttenberg 1992; Ruttenberg et al. 2009). The CDB and acetate fractions can be discarded and the 1 M HCl fraction purified is described in Chap. 3.

2.5 Plants and Fertilizers

Plants and fertilizers are a source of P into aquatic and terrestrial ecosystems. They are therefore important endmembers in $\delta^{18}O_P$ studies. Both are relatively easy to extract since concentrations are usually higher than in other samples like soils and thus less material is necessary.

2.5.1 Fertilizers

Phosphorus fertilizers are divided into mineral (e.g. triple superphosphate (TSP)) and organic fertilizers (e.g. manure). In mineral P fertilizers most of the P is present as inorganic P and is readily available. Organic P fertilizers also contain less readily available P forms. It is thus useful to extract especially organic P fertilizers sequentially. ^{18}O-labelled and unlabelled 1 M HCl have to be used for the extraction as 1 M HCl could hydrolyse some organic P and/or condensed phosphate, which can be present especially in organic fertilizers.

Equipment and consumables

- Standard lab glassware and equipment.
- Polypropylene bottles.
- Pestle and mortar.
- GF/F filters.

Reagents

1. **1 M HCl**

   In a volumetric flask add 82.7 mL concentrated HCl (37%) in 900 mL ultrapure water and make up to 1 L. Prepare two batches of 1 M HCl (^{18}O-labelled and unlabelled HCl solutions). Take a subsample from each batch for the determination of the $\delta^{18}O_w$ value of each batch.

Step Fert_1—Sample preparation

- Homogenize fertilizer with a pestle and mortar. It might be useful to freeze dry organic fertilizer samples to obtain a homogeneous sample.
- Weigh fertilizer sample into plastic bottle.

Step Fert_2—Water extraction

- Add ultrapure water and shake overnight.

Step Fert_3—Filtration

- The next day, filter extract using GF/F filters.

This will yield the readily available P of a fertilizer. For less available P, extract the fertilizer samples also with HCl. Ideally this is done sequentially; however, some fertilizers will almost completely dissolve during the water extraction. In that case, HCl extraction needs to be done on a separate subsample without prior water extraction.

Step Fert_4—HCl extraction

- Either split the sample remaining on the filter in step Fert_3 into two equal parts or weigh fertilizer sample in duplicate.
- Add ^{18}O-labelled 1 M HCl to one part and unlabelled 1 M HCl to the second part. Put on the shaker at room temperature at 160 rpm for 16 h.
- The following day remove the samples from the shaker. Centrifuge for 15 min at 5000 rpm. Filter the supernatant through a GF/F filter. Collect supernatant.

2.5.2 Plants

Plants contain inorganic and organic P. Inorganic P can be leached from plant material and contributes directly to the available P pool in soils. Organic P needs to be mineralized by enzymes first. Especially in low P soils with high biomass production like tropical rainforest, P from plants can contribute significantly to the P nutrition of the rainforest. Therefore, both inorganic and organic P from plants are important pools in $\delta^{18}O_P$ studies. The main challenge for extracting P from plant material is to stop enzymatic activity during the extraction (Bieleski 1964; Adu-Gyamfi et al. 1990). As enzymatic activity leads to O exchange between phosphate and water, it is essential to choose extraction conditions which minimize it. Trichloroacetic acid (TCA) is efficient in halting enzymatic activity and it has been successfully used to analyse the $\delta^{18}O_P$ in plant material (Pfahler et al. 2013). TCA could also extract some organic P and therefore ^{18}O-labelled and unlabelled TCA solutions are used, similar to extracting samples with 1 ^{M} HCl. After the extraction with TCA, plant material can be extracted with NaOH-EDTA for the determination of the $\delta^{18}O_P$ of structural P (mainly organic P) (Helfenstein et al. 2018; Tamburini et al. 2018).

Equipment and consumables

- Standard lab glassware and equipment.
- Polypropylene bottles, 125 mL.
- Vacutainers.
- Scissors.
- Liquid nitrogen.
- Pestle and mortar.
- GF/F filters.
- Tweezers.

Reagents

1. **0.3 M Trichloroacetic acid**

   Weigh 49.02 g TCA (MW 163.39 g/mol) in a volumetric flask and fill it up to 1000 mL with ultrapure water. Prepare two batches of 0.3 M TCA (^{18}O-labelled and unlabelled TCA solutions). Take a subsample from each batch for the determination of the $\delta^{18}O_w$ value of each batch.
2. **0.25 M NaOH—0.05 M EDTA**

   Weigh out 10 g NaOH and 18.612 g EDTA disodium salt and dissolve in 1 L ultrapure water.

Step Pl_1—Sample preparation

- After sampling, carefully wash the plant material with ultrapure water in case any soil/dust/sediment particles are attached to the plant material. Plot dry with paper towels.
- Cut out middle vein from plant leaves with scissors, if they are relatively big compared to the whole leaf. This step is necessary only if the $\delta^{18}O_P$ is used to investigate P cycling within plants.
- Cut plant material in small pieces using scissor. Put a subsample into a vacutainer for the extraction of plant water (only necessary if the $\delta^{18}O_P$ is used to investigate P cycling within plants). Freeze the plant material and the subsample as soon as possible at −20 °C and store in the freezer until used.
- Prior to the extraction, the plant material is homogenized using liquid nitrogen and a pestle and mortar. Grasses could be cut in small pieces using scissors while still frozen.

Step Pl_2—Extraction

- Weigh between 1 and 2 g of the plant material in duplicates into two 125 mL plastic bottles for the extraction with ^{18}O-labelled and unlabelled 0.3 M TCA.
- Between 20 and 40 mL 0.3 M TCA is added to each sample.
- The TCA-plant mixture is then treated with a homogenizer for about 30 s. Wash the probe/generator of the homogenizer with ultrapure water after each sample to avoid cross contamination. The samples are then put on a shaker at 4 °C for 1 h.

Step Pl_3

- The samples are filtered through GF/F filters and the supernatant is collected.
- Take a subsample of the supernatant for the determination of the P concentration and purify the remaining extract as soon as possible.

If the $\delta^{18}O_P$ of the organic P in the plants needs to be analysed, proceed with steps Pl-4 and Pl_5.

Step Pl_4

- Carefully remove the plant material remaining on the GF/F filter with tweezers and extract it with NaOH-EDTA (see step S_4 for more details).

Step Pl_5

- Shake sample overnight at room temperature.
- Filter through GF/F filter and collect supernatant.
- Take a subsample of the supernatant for the determination of the P concentration and purify the remaining extract as soon as possible.

Glossary

^{18}O-labelled/unlabelled solutions A set of ^{18}O-labelled and unlabelled solutions is used whenever P is extracted with acids. A small amount of ^{18}O-enriched water is added to the ^{18}O-labelled solution (prepared with ultrapure water), whereas only ultrapure water is used for the unlabelled solution.

δ^{18}O The oxygen isotope ratio is conventionally given in the delta notation: $\delta^{18}O = (R_{sample}/R_{standard}) - 1$, where R_{sample} is the $^{18}O/^{16}O$ ratio of a sample and $R_{standard}$ is the $^{18}O/^{16}O$ ratio of the Vienna Standard Mean Ocean Water (V-SMOW). $\delta^{18}O_P$ is the $\delta^{18}O$ of oxygen bound to P. $\delta^{18}O_W$ is the $\delta^{18}O$ of water.

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Chapter 3
Purification Protocol

V. Pfahler, J. Adu-Gyamfi, D. O'Connell, and F. Tamburini

Abstract The five stepwise purification of extracts and final precipitation of silver phosphate (A1–A5) are described. The first two steps (A1 and A2) are removing organic matter and are concentrating the phosphate in the extract by reducing the volume. Certain cations could interfere with the precipitation of silver phosphate and are removed in step A3. Silver chloride, which, if not removed, could co-precipitate with silver phosphate, is removed in step A4. The final analyte is then precipitated in step A5. The filtration steps can be quite tedious, using vacuum filtration equipment is therefore recommended. Following step A5, the silver phosphate samples need to be weighed in for the measurement with a thermal conversion elemental analyser (TC/EA) coupled to a continuous-flow isotope-ratio mass spectrometer (IRMS).

3.1 Introduction

The main difference between the protocol by Tamburini et al. (2010) and other methods is the precipitation of cerium phosphate to purify extracts (McLaughlin et al. 2004). An issue with the precipitation of cerium phosphate is that it precipitates around pH 5.5, which is the same range where iron oxides flocculate. While flocculating iron oxides can strip out other components like dissolved organic matter (DOM) from the solution (Hiradate et al. 2006). This combination of iron oxides

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and OM stays until the end and the obtained silver phosphate will be contaminated (Tamburini et al. 2010).

The following purification protocol is based on the original protocol published by Tamburini et al. (2010) and the protocol described in Chap. 2 of the IAEA-TECDOC-1783 (IAEA 2016). The original protocol was developed for 1 M HCl extracts of soils and fertilizers but has been adapted since its publication to a range of other extracts including NaOH-EDTA from soils (Tamburini et al. 2018) and trichloroacetic acid (TCA) extracts from plants (Pfahler et al. 2013). Those modifications are described in Chap. 4.

3.2 Reagents

(1) **Ammonium nitrate solution, 35% (w/v) (= 4.2 M)**

Weigh out 538.5 g of ammonium nitrate salt (NH_4NO_3; molecular weight (MW) 80.052 g/mol). Add 1000 mL of ultrapure water and dissolve the salt, stirring well. Store at room temperature.

(2) **Ammonium nitrate solution, 5% (w/v) (= 0.6 M)**

Weigh out 105.3 g of NH_4NO_3 salt. Add 2000 mL of ultrapure water. Stir well to dissolve salt. Store at room temperature.

(3) **Ammonium heptamolybdate solution, 10% (w/v)**

Weigh out 53.3 g of ammonium molybdate salt ($NH_4Mo\ x7H_2O$; tetrahydrate form; MW 1235.86 g/mol). Add 480 mL of ultrapure water. Dissolve well. Enough for 12 samples. Prepare fresh before each use.

(4) **Ammonium-citrate solution**

Weigh out 10 g of citric acid. Add 300 mL of ultrapure water and 140 mL of concentrated ammonia solution (NH_4OH). Prepare and use under hood. Stable at room temperature.

(5) **Magnesia solution**

Weigh out 50 g of magnesium chloride ($MgCl_2 \times 6H_2O$; hexa-hydrate salt; MW 203.3 g/mol) and 100 g of ammonium chloride (NH_4Cl; MW 53.49 g/mol). Dissolve them in 500 ml of ultrapure water. Acidify to pH 1 with conc. HCl. Volume is then adjusted to 1 L with ultrapure water. Stable indefinitely at room temperature. Caution: $MgCl_2$ can be contaminated with P.

(6) **1:1 and 1:20 ammonia solutions**

Measure in a volumetric cylinder the concentrated ammonia (NH_4OH; 50 mL for the 1:1 and 100 mL for the 1:20). Pour into an appropriate glass bottle and dilute with ultrapure water (50 mL for the 1:1 and 1900 mL for the 1:20). Store at room temperature.

(7) **1 M HCl**

In a volumetric flask add 82.7 mL concentrated hydrochloric acid (HCl; 37%) in 900 mL ultrapure water and make up to 1 L.

(8) **0.5 M HNO_3**

Measure 967 mL of ultrapure water and add 33 mL of concentrated nitric acid (HNO_3).

(9) **1 M HNO_3**

In a volumetric flask add 66 mL concentrated HNO_3 to 800 mL ultrapure water and make up to 1 L.

(10) **7 M HNO_3**

Add 463 mL concentrated HNO_3 to 537 mL ultrapure water.

(11) **AG 50 × 8 cation resin**

Take the equivalent of 6 mL of resin per sample and place it in a plastic bottle. Add 1.5 BV of 7 M HNO_3 and shake well and let rest overnight. The next day, discard the acid and wash it thoroughly using ultrapure water till pH is close to neutrality. After use, collect the resin and store it in $1_{\,M}$ HNO_3 ($66_{\,mL}$ concentrated HNO_3 + 934 mL ultrapure water).

(12) **Silver ammine solution**

Weigh out 10.2 g of silver nitrate ($AgNO_3$; MW 169.87 g/mol) and 9.6 g of NH_4NO_3. Dissolve in 81.5 mL of ultrapure water and add 18.5 mL of concentrated NH_4OH. Store in amber bottle in the dark.

Some of the above-mentioned chemicals are quite hazardous. According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), concentrated HCl, for example, has the following hazardous statements: H290 may be corrosive to metals, H314 causes severe skin burns and eye damage and H335 may cause respiratory irritation. It is therefore advised to check the GHS hazardous and precautionary statements and the safety data sheets for the chemicals before using the protocol. It is also recommended to follow the good laboratory practice which includes wearing personal protective equipment (PPE) like lab coats, safety glasses and gloves.

3.3 Equipment and Consumables

(1) Standard lab glassware and equipment.
(2) 50 mL polypropylene tubes.
(3) GF/F filters.
(4) Cellulose acetate filters (pores 0.2 μm).
(5) Cellulose nitrate filters (pores 0.2 μm).
(6) Items 4 and 5 could be substituted by the GPWP filters by Millipore or equivalent, resistant to a larger pH range.

(7) Polycarbonate filters (pores 0.2 μm).
(8) Parafilm.
(9) Silver capsules, pressed, 4 × 3.2 mm.
(10) Lamp black (Gasruß; glassy carbon), conditioned, 10 mL.
(11) Fume hood.
(12) Fridge (max. +8 °C).
(13) Water bath shaker set at 50 °C.
(14) Multiplate magnetic stirrer.
(15) Drying oven (no ventilation).
(16) IAEA-601 and 602 benzoic acid standards.
(17) Silver phosphate standards.

3.4 Procedure

The following describes the ideal case, i.e. 1 M HCl extract with around 20 μmol P, low in organic matter and no other compounds which might interfere. How to deal with other extracts and potential problems during the purification is described in Chap. 5. The extractants are first purified in four consecutive steps (A1–A4; Fig. 3.1), before the precipitation of the final analyte in Step A5 (Fig. 3.1).

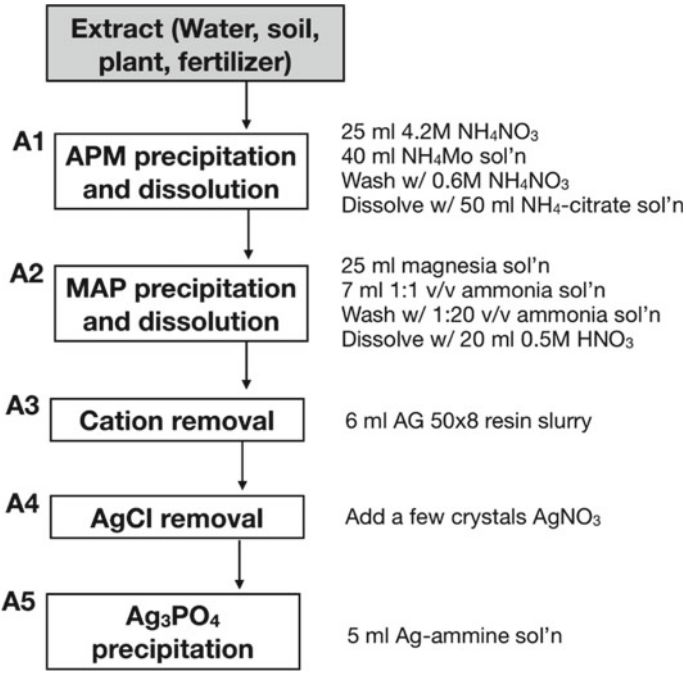

Fig. 3.1 Stepwise purification of extracts and final precipitation of silver phosphate

The first two steps (A1 and A2) are removing, for example, organic matter and are also concentrating the phosphate in the extract by reducing the volume. Certain cations could interfere with the precipitation of silver phosphate and are removed in Step A3. Silver chloride, which, if not removed, could co-precipitate with silver phosphate is removed in Step A4. The final analyte is then precipitated in Step A5. The filtration steps can be quite tedious, and using vacuum filtration equipment is therefore recommended.

Step A1: Ammonium phosphor-molybdate (APM) mineral precipitation and dissolution

- Pour supernatant (about 100–150 mL) into 200 mL Erlenmeyer flasks and place the flasks into the water bath set at 50 °C.
- Add 25 mL of 35% ammonium nitrate solution and 40 mL of the 10% NH_4-Mo solution. Shake gently overnight in the warm water bath.
- The following day filter the formed crystals (generally bright yellow, but variations might happen) by using cellulose acetate filters (or filters resistant to low pH). Wash thoroughly with the 5% ammonium nitrate solution (about 200–300 mL). Collect crystals and discard supernatant.
- Place the filter with the crystals into 100 mL Erlenmeyer flasks and add about 50 mL of the NH_4-citrate solution (work under the fume hood!). Gently swirl to dissolve the crystals. Remove the filter.

Step A2: Magnesium ammonium phosphate (MAP) mineral precipitation and dissolution

- Place the Erlenmeyer flasks onto the multiplate magnetic stirrer. Add 25 mL of the Mg solution, while stirring. Then add slowly about 7 mL of the 1:1 ammonia solution. Cover with parafilm and make small holes to vent. Leave overnight.
- The following day filter the white crystals by using the cellulose nitrate filters (or filters resistant to high pH). Wash thoroughly with the 1:20 ammonia solution. Discard supernatant.
- Collect the filter and white, fine crystals into 50 mL polypropylene tubes. Add about 20 mL of 0.5 M HNO_3 and shake to dissolve the crystals.

Step A3: Cation removal

- Add about 6 mL of the cation resin slurry which was brought to pH 7. Seal with parafilm, shake overnight.
- The following day filter the samples by using the 0.2 μm polycarbonate filters. Rinse the resin with 1–2 mL ultrapure water.
- Collect supernatant and place the resin to be reconditioned.

Step A4: Silver chloride (AgCl) removal

- Check if Cl^- is still present in solution by adding a little amount of $AgNO_3$. If a white cloud forms (AgCl crystals), wait 10 min and filter again.

Step A5: Ag_3PO_4 precipitation

- Once that the supernatant is Cl free, add 5 mL of the Ag-ammine solution. Place the tubes open into the oven set at 50 °C. Add ultrapure water to keep volume as constant as possible.
- If yellow crystals are not formed in the next 24–48 h, check the pH of the solution and bring it to 7, using either HNO_3 or NH_4OH (**absolutely no HCl or NaOH**). When NH_4OH is used to adjust the pH value, the pH value of the solution changes sharply, so NH_4OH should be added slowly drop by drop.
- Once that the crystals are formed, filter them using the 0.2 μm polycarbonate filters. Wash thoroughly with ultrapure water. Collect filters and crystals, and discard supernatant.
- Place the filters on Petri dishes and put them into the oven set at 50 °C for at least 1 day. Cover the Petri dishes to prevent filters (and crystals) from flying away!
- By gently scraping the filter, collect the dried crystals and put them into little vials. Store possibly inside a desiccator. Homogenize well before weighing, for example, by using a small pestle.

3.5 Preparation for TC/EA-IRMS and Data Analysis

Following Step A5, the silver phosphate samples need to be weighed in for the measurement with a thermal conversion elemental analyser (TC/EA) coupled to a continuous flow isotope ratio mass spectrometer (IRMS). In the TC/EA the silver capsules containing the samples and standards are pyrolysed at 1450 °C and the oxygen is converted into CO. The CO is then, via a helium stream, transported through a GC column which is separating CO from any nitrogen (N_2) that might be present to the IRMS. In the IRMS the isotopic composition of CO is then measured. Before each run, the linearity of the IRMS should be checked and this will inform us about the range of weight that should be used for the silver phosphate standards and samples. In general, most instruments are linear between 250 and 350 μg of silver phosphate; however, linearity should still be checked (Carter and Barwick 2011). Likewise, a stability check of the IRMS should be done before each run (Carter and Barwick 2011). Further information about normalization and selection of reference materials can also be found in Skrzypek (2013) and Skrzypek and Sadler (2011).

- Store all isotope reference standards, laboratory standards, samples and the capsule in desiccator with a drying material to avoid condensation of water. After weighing in, transfer the samples and standards back to the desiccator and dry for a minimum of 24 h.
- Weigh the silver phosphate crystals into silver capsules in triplicates in the previously determined linearity range. Depending on the instrument preconditioned addition of glassy carbon powder can be added to improve the pyrolysis. Close tightly to avoid trapping air by forming little balls using tweezers.
- Weigh 100 μg of the two benzoic acid standards IAEA-601 and IAEA-602 into silver capsules. Together with the silver phosphate standards these have to cover the range of the $\delta^{18}O_P$ values of the samples. There is no need to add glassy carbon

even if it was added to the silver phosphate samples and standards. Close tightly by forming little balls using tweezers and gloves (Werner and Brand 2001).

- Weigh in the silver phosphate standard, which was calibrated against international certified reference material (Halas et al. 2011; Watzinger et al. n.d.) into silver capsules in triplicates. If glassy carbon was added to the samples, it should also be added to the standards (Werner and Brand 2001). Close tightly by forming little balls using tweezers.
- Weigh different amounts of pure silver phosphate obtained from a chemical company or of the internal standards (Lécuyer et al. 2007) into silver capsules. If glassy carbon was added to the samples, it should also be added to the standards (Werner and Brand 2001). They should cover the range of weights used for the samples. Close tightly by forming little balls using tweezers and gloves.
- Prepare 5–6 blanks (adding glassy carbon if added to silver phosphate samples and standards) to empty capsules and closing them tightly. These are placed at the beginning of the run. The blanks aid preconditioning the column and are also necessary if the IRMS does a blank correction.
- Transfer closed capsules to coded racks. Most commonly 96-well microtiter plates are used as racks.

During the whole preparation process neither the crystals nor the silver capsules should be touched with bare hands in order to avoid contamination with any O-containing compounds.

A sample sequence might look as follows (see the Annex for an example):

- Five blanks.
- Silver phosphate standard from certified source ($\times 5$ at different weights).
- Additional standards (for example, internal silver phosphate standard, IAEA-601, IAEA-602) are used for normalization, at least four of each, distributed along the run.
- Samples can be put in groups of 12, weighing each sample in triplicates.
- After each group, standards should be added to obtain a sequence like
  - 12 samples,
  - 1 silver phosphate standard,
  - 1 IAEA-601 and
  - 12 samples.
- Repeat until the run is almost full and finish with
  - $4\times$ certified silver phosphate standard and
  - Additional standard.

Once the $\delta^{18}O_P$ values are analysed with the TC/EA-IRMS, a quality check needs to be done. The first three steps of the data analysis therefore are

1. Checking the oxygen yield; 15.3% for pure silver phosphate, between 14 and 17%, is acceptable.
2. Checking for nitrogen (N_2) contamination.

3. Drift correction and normalization to international standards.
4. Calculating average $\delta^{18}O_P$ value and standard deviation.

Oxygen yield above or below expectation implicates that the silver phosphate sample was not pure silver phosphate and hence the obtained $\delta^{18}O_P$ value might be erroneous. To check for the purity of the Ag_3PO_4, we control the O% given by the TC/EA. The silver phosphate standards weighed at different amounts provide a calibration curve used to determine the O content of all samples. If the analysed Ag_3PO_4 samples are pure, their O content should be in the range of the Ag_3PO_4 standard.

Similarly, a contamination with N_2 could also lead to erroneous $\delta^{18}O_P$ values. Either due to interference during the mass spectrometer measurements or because of N compounds containing oxygen (Pederzani et al. 2020). Sometimes, if the final wash of the Ag_3PO_4 is not done properly, NO_3 could remain on the crystals. The presence of N_2 is checked by looking at the chromatogram from the TCEA and from the IRMS. The N_2 peak should be identified before the CO peak. Only when the first two checks have been passed, an average $\delta^{18}O_P$ value can be calculated.

The isotopic values of the certified standards should remain stable along the run. If this is not the case, a drift correction needs to be done (Carter and Barwick 2011). First the drift needs to be calculated:

$$\text{Drift} = \left(\text{Average}_{\text{STD10}-12} - \text{Average}_{\text{STD1}-4}\right)/\left(\text{Position}_{\text{STD10}} - \text{Position}_{\text{STD1}}\right), \tag{3.1}$$

where $\text{Average}_{\text{STD10–12}}$ is the average $\delta^{18}O_P$ value of the last three replicates of the certified silver phosphate standard in a run, $\text{Average}_{\text{STD1–4}}$ is the average $\delta^{18}O_P$ value of the first four replicates of the certified silver phosphate standard in a run, $\text{Position}_{\text{STD10}}$ is the position of the 10th certified silver phosphate standard in a run and $\text{Position}_{\text{STD1}}$ is the position on the 1st certified silver phosphate standard in a run.

The drift can then be used to perform a drift correction of the $\delta^{18}O$ values:

$$\delta^{18}O_{\text{D\_corr}} = \delta^{18}O_{\text{meas}} - \text{drift} \cdot \left(\text{Position}_{\text{sample}} - \text{Position}_{\text{STD1}}\right), \tag{3.2}$$

where $\delta^{18}O_{\text{meas}}$ is the measured $\delta^{18}O$ value of a sample/standard, drift is the previously calculated drift value, $\text{Position}_{\text{sample}}$ is the position of a sample/standard in a run and $\text{Position}_{\text{STD1}}$ is the position on the 1st certified silver phosphate standard in a run.

After drift correction of samples and standards, average measured and drift corrected values of the standards should be correlated to the reported values (Brand et al. 2009). Using the slope and intercept of this correlation, calculate the real value of the samples.

A good practice is to compare the standard deviation of each sample to the standard deviations of the silver phosphate and benzoic acid standards within the same run. Standard deviations are around ±0.3‰ (Brand et al. 2009; Halas et al. 2011;

Watzinger et al. 2021). Only if the standard deviation of a sample is equal or smaller than the standard deviations of the standards, a $\delta^{18}O_P$ value can be trusted. In case oxygen yield is outside the acceptable range and/or N_2 contamination was found, the silver phosphate sample can be treated with H_2O_2 (removing organic compounds), thoroughly rinsed again with ultrapure water (removing N-containing compounds) and/or homogenized better (in case of crystals with different sizes). If the oxygen yield and N_2 are ok, but the standard deviation (SD) of the replicates is still high (higher than SD of certified standard), then repeat analysis and possibly homogenize sample better. Vacuum roasting is also sometimes used to remove contamination with other O-bearing compounds; however, this procedure also has some issues including potential reoxidation when samples are stored too long (Mine et al. 2017).

Glossary

TC/EA-IRMS A thermal conversion elemental analyser (TC/EA) coupled to an isotope ratio mass spectrometer (IRMS) is commonly used to determine the $\delta^{18}O_P$. The oxygen in silver phosphate is converted, via pyrolysis, into carbon monoxide, which isotopic composition is then measured in the IRMS.

$\delta^{18}O$ The oxygen isotope ratio is conventionally given in the delta notation: $\delta^{18}O = (R_{sample}/R_{standard}) - 1$, where R_{sample} is the $^{18}O/^{16}O$ ratio of a sample and $R_{standard}$ is the $^{18}O/^{16}O$ ratio of the Vienna Standard Mean Ocean Water (VSMOW). $\delta^{18}O_P$ is the $\delta^{18}O$ of oxygen bound to P. $\delta^{18}O_W$ is the $\delta^{18}O$ of water.

IAEA-601 A benzoic acid standard provided by the IAEA, its $\delta^{18}O$ value is 23.14‰ (Brand et al. 2009).

IAEA-602 A benzoic acid standard provided by the IAEA, its $\delta^{18}O$ value is 71.28‰ (Brand et al. 2009).

Appendix

See Fig. 3.2 below.

Fig. 3.2 Example for a TC/EA-IRMS run. This run includes a certified silver phosphate standard (Ag_3PO_4_certified), two internal silver phosphate standards (Ag_3PO_4_internal A and B) and one benzoic acid standard (IAEA-601). Each sample is weighed in triplicates (rep1-3) and six empty silver capsules are included as blanks at the beginning of the run

Sample Number	Name	Weight (mg)
1	Blank	
2	Blank	
3	Blank	
4	Blank	
5	Blank	
6	Blank	
7	Ag3PO4_certified_ 1	
8	Ag3PO4_certified_ 2	
9	Ag3PO4_certified_ 3	
10	Ag3PO4_certified_ 4	
11	Ag3PO4_internalA_1	
12	Ag3PO4_internalA_2	
13	IAEA 601_1	
14	Ag3PO4_internalB_1	
15	Sample 1_rep1	
16	Sample 1_rep2	
17	Sample 1_rep3	
18	Sample 2_rep1	
19	Sample 2_rep2	
20	Sample 2_rep3	
21	Sample 3_rep1	
22	Sample 3_rep2	
23	Sample 3_rep3	
24	Sample 4_rep1	
25	Sample 4_rep2	
26	Sample 4_rep3	
27	Ag3PO4_certified_ 5	
28	Sample 5_rep1	
29	Sample 5_rep2	
30	Sample 5_rep3	
31	Sample 6_rep1	
32	Sample 6_rep2	
33	Sample 6_rep3	
34	Sample 7_rep1	
35	Sample 7_rep2	
36	Sample 7_rep3	
37	Sample 8_rep1	
38	Sample 8_rep2	
39	Sample 8_rep3	
40	Ag3PO4_certified_ 6	
41	IAEA 601_2	
42	Sample 9_rep1	
43	Sample 9_rep2	
44	Sample 9_rep3	
45	Sample 10_rep1	
46	Sample 10_rep2	
47	Sample 10_rep3	
48	Sample 11_rep1	
49	Sample 11_rep2	
50	Sample 11_rep3	
51	Sample 12_rep1	
52	Sample 12_rep2	
53	Sample 12_rep3	
54	Ag3PO4_internalB_2	
55	Sample 13_rep1	
56	Sample 13_rep2	
57	Sample 13_rep3	
58	Sample 14_rep1	
59	Sample 14_rep2	
60	Sample 14_rep3	
61	Sample 15_rep1	
62	Sample 15_rep2	
63	Sample 15_rep3	
64	Sample 16_rep1	
65	Sample 16_rep2	
66	Sample 16_rep3	
67	Ag3PO4_certified_ 7	
68	Ag3PO4_internalA_3	
69	Sample 17_rep1	
70	Sample 17_rep2	
71	Sample 17_rep3	
72	Sample 18_rep1	
73	Sample 18_rep2	
74	Sample 18_rep3	
75	Sample 19_rep1	
76	Sample 19_rep2	
77	Sample 19_rep3	
78	Sample 20_rep1	
79	Sample 20_rep2	
80	Sample 20_rep3	
81	Ag3PO4_certified_ 8	
82	IAEA 601_3	
83	Sample 21_rep1	
84	Sample 21_rep2	
85	Sample 21_rep3	
86	Sample 22_rep1	
87	Sample 22_rep2	
88	Sample 22_rep3	
89	Sample 23_rep1	
90	Sample 23_rep2	
91	Sample 23_rep3	
92	Sample 24_rep1	
93	Sample 24_rep2	
94	Sample 24_rep3	
95	Ag3PO4_internalB_3	
96	Sample 25_rep1	
97	Sample 25_rep2	
98	Sample 25_rep3	
99	Sample 26_rep1	
100	Sample 26_rep2	
101	Sample 26_rep3	
102	Sample 27_rep1	
103	Sample 27_rep2	
104	Sample 27_rep3	
105	Sample 28_rep1	
106	Sample 28_rep2	
107	Sample 28_rep3	
108	Ag3PO4_certified_ 9	
109	Ag3PO4_internalA_4	
110	Sample 29_rep1	
111	Sample 29_rep2	
112	Sample 29_rep3	
113	Sample 30_rep1	
114	Sample 30_rep2	
115	Sample 30_rep3	
116	Ag3PO4_certified_ 10	
117	Ag3PO4_certified_ 11	
118	Ag3PO4_certified_ 12	
119	Ag3PO4_internalB_4	
120	IAEA 601_4	

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Chapter 4
Modifications and Issues During Purification

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Abstract Depending on the extract, it is necessary to modify the purification protocol slightly. Each sample is different and despite a thorough testing of the purification protocol, issues might occur. The three modifications suggested include (1) adjustments in pH, (2) magnesium ammonium phosphate (MAP) precipitation and (3) reductions, prior to A1, of cations like iron (Fe), silica (Si) and calcium (Ca) which could cause interferences during the purification process. Some of the major issues often encountered are (1) no APM precipitation due to the presence of high carbonate concentrations, (2) the presence of high organic matter that requires additional steps in the protocol, (3) crystals not dissolving and (4) discoloration of solution.

4.1 Method Modifications During the Purification Process

The original purification protocol is using 1 M HCl extracts but it is nowadays used for a diverse set of extracts (Tamburini et al. 2010, 2018; Pistocchi et al. 2017; Granger et al. 2018; Pfahler et al. 2020). Depending on the extract it is necessary to modify the purification protocol slightly. Also, each sample is different and despite thorough testing of the purification protocol, issues might occur.

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4.1.1 Modification 1

The ideal pH for the precipitation of ammonium phosphomolybdate (APM; step A1 of purification protocol) is 1. When using acidic reagents that are weaker than 1 M HCl, for example, 0.2 M HNO_3 for eluting resin P, it is therefore necessary to add concentrated sulphuric acid (H_2SO_4). Concentrated H_2SO_4 is a strong acid that could potentially hydrolyse any organic or condensed P present in the extract. Add concentrated H_2SO_4 slowly to the sample only after ammonium molybdate and ammonium nitrate are added to the extract in step A1 of the purification protocol. Usually, 1 ml concentrated H_2SO_4 is sufficient to facilitate the precipitation of APM.

4.1.2 Modification 2

If the initial extract, e.g., 0.5 M $NaHCO_3$, has an alkaline pH, start with step A2 to avoid unnecessary pH adjustment. Indeed, step A2 of the purification protocol (magnesium ammonium phosphate (MAP) precipitation) requires an alkaline pH (>7). In these cases, the crystals obtained from the MAP step are never properly clean. So, after the dissolution of the MAP crystals, proceed with step A1, repeat step A2 and continue with the remaining steps.

4.1.3 Modification 3

Certain cations like iron (Fe), silica (Si) and calcium (Ca) can interfere with the purification protocol and therefore their concentrations need to be reduced prior to Step A1. High concentrations of Si can be present in volcanic soils and can interfere with the molybdate complexation in step A1 of the purification protocol. Ca can also interfere in step A1 due to the formation of crystals with molybdate. Fe could form colloids and co-precipitated with organic P. Brucite precipitation should be added before step A1 to reduce the concentrations of interfering cations. Alternatively, cation exchange resins could help in reducing the cation concentrations; however, this has, to the best of our knowledge, not yet been tested in case of the purification protocol.

4.2 Major Issues

4.2.1 Issue 1: No APM Precipitation

This is often the case when the initial extract is not acidic enough. In most cases modification 1 will help. In the presence of high carbonate concentrations, even the 1 M HCl extract might not be acidic enough (Pistocchi et al. 2017). Pistocchi et al. (2017) therefore adjusted the liquid-to-solid ratio for their samples during the extraction to 100:1 instead of 50:1. Too low P concentrations (<10 μmol) can also be a reason for no APM precipitation. In that case, the sample needs to be extracted again and a higher amount of material needs to be used. In this case, it is possible that multiple subsamples are extracted and then the extracts are combined. A brucite step is most likely needed in order to reduce the extract volume prior to APM precipitation.

4.2.2 Issue 2: High Organic Matter

In most cases, for example with low organic matter but high inorganic P concentrations, steps A1 and A2 are sufficient to remove organic matter from the initial extracts. Sometimes steps A1 and A2 are not sufficient and it is necessary to use the DAX-8 resin or brucite precipitation before proceeding with the purification protocol. Organic matter or colouration is also an issue in Olsen P extracts and charcoal is used to deal with this issue. However, charcoal is often contaminated with P and hence not ideal for the $\delta^{18}O_P$ method, unless acid cleaned and checked for P.

For conditioning the DAX-8 resin, proceed as follows: Take the equivalent of 10 ml of resin per sample and place it in a 500 ml plastic bottle. To condition the new resin, use 1.5 bed volumes (BV) of methanol, shake well, and let rest for 15 min. Discard methanol, rinse with 1.5 BV of ultrapure water, shake and let rest for 15 min. Carefully discard water. Add ultrapure water just to cover the surface of the resin. After use, the resin should be collected and washed with 1 M HCl + methanol. Store in methanol and at room temperature.

4.2.3 Issue 3: Crystals not Dissolving and Discolouration of Solution

Sometimes the ammonium phosphomolybdate (APM), which formed during step A1, does not dissolve immediately in the ammonium citrate solution (step A2). In this case, leave the solution for about 1 h and if it did not dissolve by then, filter it before continuing with the remaining parts of step A2. Likewise, magnesium ammonium phosphate (MAP), which formed during step A2, does not dissolve immediately

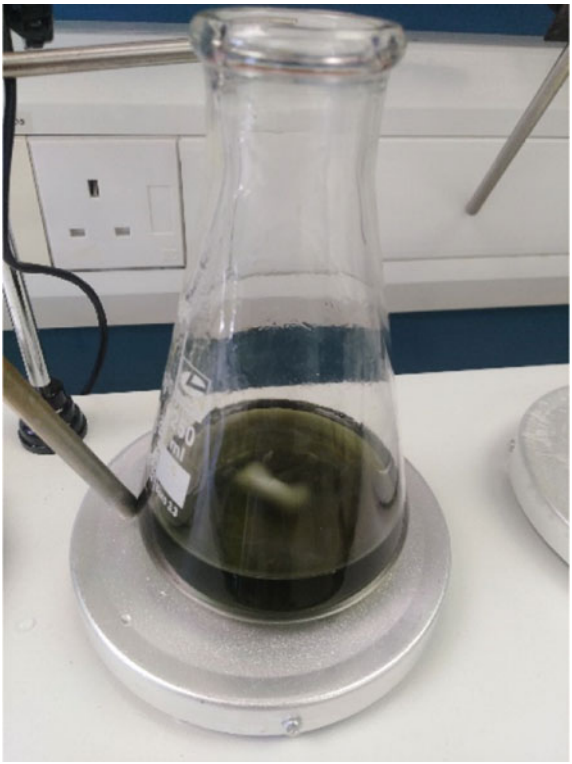

Fig. 4.1 Dissolved, but decoloured ammonium phosphomolybdate (APM)

when adding 0.5 M HNO_3. Leave the solution for one hour; if not dissolved by then, filter the solution.

Another issue, which might occur, is the discolouration of the solution (Fig. 4.1). Dissolved APM and MAP should yield clear solutions. Filtering the solutions prior to continuing with the respective step (A2 or A3), usually reduces the discolouration.

Glossary

Brucite precipitation Brucite precipitation, also referred to as MAGIC (magnesium induced co-precipitation), is used to concentrate P in a solution, e.g., water sample. By adding magnesium chloride and sodium hydroxide to a solution brucite flocs ($Mg(OH)_2$) precipitate and thereby P is stripped out from a solution.

Olsen P The Olsen P method is one of the extraction methods for available P in soils. 0.5 M sodium bicarbonate ($NaHCO_3$) is used as an extractant.

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Chapter 5
How to Design a Study Including the Analysis of $\delta^{18}O_P$

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Abstract To plan a research study, one needs to (1) establish a research question, (2) make a set of observations, (3) form a hypothesis in an attempt to explain the observations and (4) test the hypothesis based on the data collected. The following questions should be addressed when designing a study including the analysis of δ^{18}OP: (i) what is the research hypothesis? (ii) what is the main objective of the study? (iii) what are the aims to address these objectives? and (iv) which techniques are appropriate to address such research question. In addition, one needs to consider (1) which kind of samples needs to be collected, e.g. soil, vegetation or water? (2) in case of soil and sediment samples, which sampling depths and increments need to be sampled? (3) which P pools need to be extracted and analysed for the corresponding δ^{18}OP values? (4) when and how often should samples be taken and (5) how many samples can be processed per week?

5.1 Planning or Designing a Study

To plan a research study, one needs to (1) establish a research question, (2) make a set of observations, (3) form a hypothesis in an attempt to explain the observations and (4) test the hypothesis based on the data collection.

The following questions should be addressed when designing a study including the analysis of $\delta^{18}O_P$:

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1. What is the research hypothesis? (i) What is the main objective of the study? (ii) What are the aims to address these objectives? (iii) Which techniques are appropriate to address such research question?
2. Which kind of samples needs to be collected, e.g., soil, vegetation or water?
3. In the case of soil and sediment samples, which sampling depths and increments need to be sampled?
4. Which P pools need to be extracted and analysed for the corresponding $\delta^{18}O_P$ values?
5. When and how often should samples be taken?
6. How many samples can be processed per week?

In addition to the above questions, (1) monitoring of progress towards results, resources consumed and budget, (2) a reflection and a shared experience and lessons drawn from success and failure, should be considered a set of observations, (3) form a hypothesis in an attempt to explain the observations and (4) test the hypothesis based on the data collection.

5.2 Comments to the Questions

- Question 2: For example, when investigating P cycling in a lake, collecting samples from all potential P inputs into the lake is necessary.
- Question 3: For soils, the top 20 cm are often the most biologically active and hence most studies focus on this layer. When investigating the fate of fertilizer P, taking samples at different depths might be necessary.
- Question 4: This depends a lot on the research hypothesis. When investigating inorganic P for example, purifying the organic P in NaOH-EDTA extract is probably not necessary.
- Question 5: As mentioned by Pistocchi et al. (2017), timing is crucial when working with river (water and sediment) samples. Fast flow in streams, for example after storm events, can re-suspend a large amount of river sediments, which is making it more challenging to determine other sources of particulate P. Low flow in streams, could on the other hand lead to an overprinting of the original $\delta^{18}O_P$ values due to a longer residence time, and hence more time for the equilibrium to be reached.
- Question 6: Especially question 6 should not be underestimated because ideally samples should not be stored for an extended period due to the potential alteration of the $\delta^{18}O_P$ value during storage. In most laboratories, places on the stirring plate and/or in the water bath are limiting the number of extracts that can be purified at once. Realistically, in most cases, only 15 samples can be processed for the purification within one week.

Once questions 1 to 6 are answered, one could think about analyses that complement the $\delta^{18}O_P$ analysis. Essential analyses which should always be included are:

A. P concentrations (inorganic and total) in all extracts and water samples from aquatic systems.
B. The $\delta^{18}O_w$ of soil, plant or river water.
C. The ambient temperature of the air, water or soil.

Depending on the hypothesis, it might also be useful to include further analyses. The $\delta^{18}O_P$ has been successfully combined, in incubation and glasshouse studies, with ^{33}P in soils (Helfenstein et al. 2018; Siegenthaler et al. 2020) and plants (Pfahler et al. 2017). Also, the combination with metagenomics and other microbial analyses becomes more popular (Bi et al. 2018; Shen et al. 2020). Table 5.1 shows examples of analyses and data typically used along the $\delta^{18}O_P$ for investigating P cycling in the environment.

5.3 External Quality Assurance/Control

Quality assurance of isotope measurements by TC/EA-IRMS is based on the trueness and precision of the values from external standards that are analysed along with the samples in daily sequences (Watzinger et al. 2021). Most commonly, researchers use two benzoic acid standards provided by the IAEA (IAEA-601 with a $\delta^{18}O$ value of 23.14‰ and IAEA-602 with a $\delta^{18}O$ value of 71.28‰), a commercially bought silver phosphate, and sometimes also silver phosphate produced in-house. Before a new batch of silver phosphate, bought or produced in-house, is used as a standard, it should be sent to at least one additional laboratory for cross-validation of the $\delta^{18}O_P$ value.

An inter-laboratory study for silver phosphate standards was conducted by Watzinger et al. (2021). A silver phosphate reference material was produced by the University of Natural Resources and Life Science (Austria) and sent to four other laboratories: The University of Western Australia (Australia), the ETH Zurich (Switzerland), the University of Helsinki (Finland) and the Helmholtz Centre for Environmental Research (Germany). This new reference material has an $\delta^{18}O_P$ value of 13.8 ± 0.3‰ and is available for research laboratories.

In addition, the purification protocol should be tested in an inter-laboratory study using different materials: pure potassium dihydrogen phosphate and silver phosphate as a control and a set of different dried soil and sediment samples. A water sample would also be nice; however as water samples should be processed right away after sampling, storing and sending water samples to different laboratories might alter the $\delta^{18}O_P$ signature due to an extended storage period.

Table 5.1 Examples of studies using the isotopic composition of oxygen bound to phosphorus ($\delta^{18}O_P$) to investigate P cycling in different environments

References	Hypothesis/subject of study	Environmental samples	Sampling depths/increments	P pool for $\delta^{18}O_P$ analysis	Analyses/data
Amelung et al. (2015)	Effect of different P fertilizers on soil $\delta^{18}O_P$ values, difference between surface and subsurface soil	Fertilizers, soils	0–30 cm, diagnostic soil horizons of whole soil profiles	HCl P (after sequential extraction)	Sequential extraction of soil, P concentrations
Jaisi and Blake (2010)	Partitioning of sedimentary P phases	Marine sediments	0–200 m	Authigenic and detrital phosphates	Sequential extraction; P, Fe, Cl, Ca, and sulphate concentrations
Angert et al. (2012)	Variability of $\delta^{18}O_P$ values across a climate and rainfall gradient	Soils, sampling up to four times per year	0–10/20 cm	Resin and HCl P	–
Tian et al. (2016)	Identification of processes affecting the $\delta^{18}O_P$ values of inorganic soil P pools, better understanding the sources, migration, and transformation of P	Soils, four sampling locations	0–20 cm	Inorganic P in water, $NaHCO_3$, NaOH, and HCl extracts	Sequential extraction, P concentrations

5.4 Interpretation of Isotopic Data from the TC/EA-IRMS and Its Applications

There is no one-fits-all approach when it comes to the interpretation of $\delta^{18}O_P$ data especially since not all variables influencing $\delta^{18}O_P$ values are known. How to interpret $\delta^{18}O_P$ data strongly depends on the research question, the research subject (soil, lake water, marine sediments etc.), and the data itself. However, there are some general steps/rules which can help to interpret the $\delta^{18}O_P$ data:

- The first step in the interpretation of the obtained $\delta^{18}O_P$ values is the calculation of the temperature-dependent equilibrium $\delta^{18}O_P$ value. This value is an indication for intracellular cycling of P via the enzyme inorganic pyrophosphatase (PPase) and is thus assumed to be a good approximation for the expected $\delta^{18}O_P$ of microbial P. As a general rule of thumb, a difference of 1‰ between a measured $\delta^{18}O_P$ value and a calculated equilibrium value is often not very relevant due to the uncertainties associated with the calculation of the equilibrium value.
- The next step is the calculation of theoretical $\delta^{18}O_P$ values of inorganic P released by the hydrolysis of organic P via enzymes. If organic P $\delta^{18}O_P$ values are unknown, already published organic P $\delta^{18}O_P$ values could be used as an approximation.

A good starting point for the interpretation of $\delta^{18}O_P$ data is the comparison with the theoretical equilibrium value. Values lower than the equilibrium value are often an indication for hydrolysis of organic P but could also be due to other P inputs with a lower $\delta^{18}O_P$ value like P from igneous rocks. Values higher than the equilibrium value could for example be caused by inorganic P leached from plants as plant inorganic P $\delta^{18}O_P$ values tend to be enriched in ^{18}O compared to other P pools (Table 5.1).

The following examples are very simple case studies and only show the general workflow when designing a $\delta^{18}O_P$ study.

5.5 Example Research Study—Small Lake

A small lake, surrounded by five agricultural fields, is suffering from eutrophication during the summer. Each of the agricultural fields has a drainage didge which flows into the lake. Three of those agricultural fields are fertilized with mineral P fertilizers, the other two with farm-yard manure. One river, with a wastewater treatment plant (WWTP) upstream, is fed into the lake.

1. Hypothesis/objective: Identifying the main P sources into a lake
2. Samples: Water samples from the lake, the river, the drainage didges and the WWTP; fertilizers applied to the fields; and soil samples from the agricultural fields
    a. Eight water samples
    b. Five fertilizer samples

   c. One bulked soil sample from each field

3. Sampling depths/increments: only relevant for soil, topsoil (0–20 cm); if possible, samples should be taken also throughout the soil profile to determine background $\delta^{18}O_P$ values
4. P pools: soluble reactive P in water samples; water extractable and HCl P of fertilizers; resin and HCl P from soils
5. Sampling time points: winter and summer
6. Processing samples: 15 per week (based on laboratory equipment).

To calculate the total number of samples for $\delta^{18}O_P$ analysis, one needs to now consider the following:

A. **Factor 2**, because two sampling time points
B. At each time point:

   a. **Eight** water samples (SRP only)
   b. Two P pools for each fertilizer sample, including a factor 2 for the HCl P due to using ^{18}O-labelled and unlabelled HCl: **15 samples**
   c. Two P pools for each soil sample, including a factor 2 for the HCl P due to using ^{18}O-labelled and unlabelled HCl: **15 samples**.

In total, 76 samples need to be analysed and afterwards purified, with **38 samples** at each sampling time point, and 38 samples cannot be analysed within one week. It is recommended to first deal with the samples/extracts which are more susceptible to changes due to biological activities. One would therefore start with the water samples and extract the more labile P pool (water-extractable or resin P) from the soils and farm-yard manure.

5.6 Example Research Study—Agricultural Fields

To investigate P cycling at agricultural fields where rapeseed is cultivated, five agricultural fields are selected as study sites. Those five fields are in the same climatic zone, but differ in their soil properties, like soil pH and P saturation index. All five agricultural fields are fertilized with the same mineral P fertilizers.

1. Hypothesis/objective: Does P cycling change along a soil profile (100 cm) where rapeseed is grown?
2. Samples: mineral fertilizer applied to the fields; soil samples from the agricultural fields; plant samples

   a. One fertilizer sample
   b. Soil samples from each field at different depths
   c. Only above-ground plant sample

3. Sampling depths/increments: 100 cm, divided into 10 cm increments; if possible, samples should also be taken throughout the soil profile to determine background $\delta^{18}O_P$ values
4. P pools: water-extractable and HCl P of fertilizers; resin, microbial, NaOH-EDTA Pi and P_{org}, and HCl P from soils; TCA P and NaOH-EDTA P_{org} for plant samples.
5. Sampling time points: peak of plant P demand
6. Processing samples: 15 per week (based on laboratory equipment).

To calculate the total number of samples for $\delta^{18}O_P$ analysis, one needs to now consider the following:

A. **Factor 5**, because five fields
B. Two P pools for the fertilizer sample, including a factor 2 for the HCl P due to using ^{18}O-labelled and unlabelled HCl: **Three samples**
C. From each field:
    a. Five P pools for each soil sample, including a factor 2 for the HCl P due to using ^{18}O-labelled and unlabelled HCl: **60 samples**
    b. One bulked plant sample; two P pools; including a factor 2 for the TCA P due to using ^{18}O-labelled and unlabelled TCA: **Three samples**.

In total, 318 samples need to be analysed and afterwards purified, with **63 samples** at each field and three samples from the fertilizer. Those samples cannot be analysed within one week. It is recommended to first deal with the samples/extracts which are more susceptible to changes due to biological activities. One would therefore start with extracting the most labile P pool (resin and microbial P) from the soils. It might also be useful to analyse other parameters relevant for P cycling like enzyme activities.

Glossary

δ^{18}O The oxygen isotope ratio is conventionally given in the delta notation: $\delta^{18}O = (R_{sample}/R_{standard}) - 1$, where R_{sample} is the $^{18}O/^{16}O$ ratio of a sample and $R_{standard}$ is the $^{18}O/^{16}O$ ratio of the Vienna Standard Mean Ocean Water (V-SMOW). $\delta^{18}O_P$ is the $\delta^{18}O$ value of a P compound or pool.

Soluble reactive P SRP; considered the most bio-available P pool in water samples.

^{18}O-labelled/unlabelled solution ^{18}O-labelled and unlabelled solutions are used in case of acidic extractions, e.g., with 1 M HCl, to account for any oxygen exchange between phosphate and the solution during the extraction.

TC/EA-IRMS A thermal conversion elemental analyser (TC/EA) coupled to an isotope ratio mass spectrometer (IRMS) is commonly used to determine the $\delta^{18}O_P$. The oxygen in silver phosphate is converted, via pyrolysis, into carbon monoxide, whose isotopic composition is then measured in the IRMS.

^{33}P Radioisotope of phosphorus (P); half-life 25.4 days, beta emitter.

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Chapter 6
Conclusions and Way Forward

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Abstract With an increasing number of researchers using the $\delta^{18}O_P$ method to investigate P cycling in the environment, it is necessary to conduct an inter-laboratory comparison study for the purification protocol as well as the measurement of silver phosphate with the TC/EA-IRMS like Watzinger et al. (2021) did. For the $\delta^{18}O_P$ method to progress, further fundamental research as well as field and laboratory studies need to be conducted. To the best of our knowledge, the effect of synthesizing enzymes on the $\delta^{18}O_P$ has not been investigated yet, despite the importance of those enzymes in the P cycle.

6.1 Future Trends and Opportunities

With increasing fertilizer prices and the negative environmental issues such as eutrophication of aquatic systems and phosphorus contaminations in surface and groundwater that affect water quality, it is now, more than ever, vital to better understand the environmental P cycle. Studies during the last 10 years showed that the $\delta^{18}O_P$ method is a promising tool to study the environmental P cycle under field conditions and the importance of microbes for available P in soils (Tamburini et al. 2012). The oxygen isotopes in phosphates have helped in identifying P inputs in Lake Erie and Chesapeake Bay, some of the aquatic systems most struck by eutrophication (Elsbury et al. 2009; Joshi et al. 2015; Paytan et al. 2017; Depew et al. 2018). It has the potential to track P derived from glyphosate in the environment (Li et al. 2016),

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to name just a few highlights. The $\delta^{18}O_P$ could also provide parameters for environmental models (Jaisi et al. 2017). Modeling P cycling and including P in carbon and nitrogen models is becoming more important and the interest to also include P in life cycle assessments is increasing, especially the mineralization of organic P and its quantification are of interest for modelers (Vereecken et al. 2016; Helfenstein et al. 2018; Thum et al. 2019).

6.2 Need for Inter-laboratory Studies for Quality Control

With an increasing number of researchers using the $\delta^{18}O_P$ method to investigate P cycling in the environment, it is necessary to conduct an inter-laboratory comparison study for the purification protocol as well as the measurement of silver phosphate with the TC/EA-IRMS like Watzinger et al. (2021) did. While nowadays silver phosphate is the analyte of choice, there are different protocols for purifying extracts and precipitating silver phosphate (see, e.g., Paytan and McLaughlin 2011). Even when using the same protocol, every laboratory is most likely amending the protocol for their needs, using different equipment, consumables, and chemicals. For better comparability of different $\delta^{18}O_P$ studies, there is a need for an inter-laboratory study similar to other protocols, like for the extraction of microbial P (Bergkemper et al. 2016). Equally important is an international silver phosphate standard which should be easily available to most laboratories (Watzinger et al. 2021).

6.3 From Laboratory to Field Studies in Different Ecotones

For the $\delta^{18}O_P$ method to progress, further fundamental research, as well as field and laboratory studies, need to be conducted. To the best of our knowledge, the effect of synthesizing enzymes on the $\delta^{18}O_P$ has not been investigated yet, despite the importance of those enzymes in the P cycle. Furthermore, the effects of some processes like P uptake on the $\delta^{18}O_P$ were only shown in laboratory studies but are missing proof from field studies. The majority of $\delta^{18}O_P$ studies so far was conducted in countries in the northern hemisphere. An increase of studies in the southern hemisphere is therefore desirable. This will advance the knowledge about the variables influencing $\delta^{18}O_P$ values as countries in the global South tend to have different ecosystems, climates, and soil types compared to the North. Two aspects that need to be addressed in further field studies are the importance of soil P concentrations, i.e., P limitation and surplus, and microorganisms (community structure and activity) for the $\delta^{18}O_P$ of different soil P pools. Is there for example a relationship between P concentrations and $\delta^{18}O_P$ values and could thus the $\delta^{18}O_P$ be used as an indicator for P limitation? This will not only help with the interpretation of $\delta^{18}O_P$ data but will ultimately lead to a better understanding of P cycling in the environment.

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