In or Out of Equilibrium? How Microbial Activity Controls the Oxygen Isotopic Composition of Phosphate in Forest Organic Horizons With Low and High Phosphorus Availability

Chiara Pistocchi\textsuperscript{1,2}, Éva Mészáros\textsuperscript{1,3}, Emmanuel Frossard\textsuperscript{1}, E. K. Bünemann\textsuperscript{1,4} and Federica Tamburini\textsuperscript{1}\*  

\textsuperscript{1} Institute of Agricultural Science, ETH Zürich, Zurich, Switzerland, \textsuperscript{2} Eco&Sols, Montpellier SupAgro, Univ Montpellier, CIRAD, INRAE, IRD, Montpellier, France, \textsuperscript{3} Department of Biosystems Science and Engineering, ETH Zürich, Basel, Switzerland, \textsuperscript{4} Department of Soil Sciences, Research Institute of Organic Agriculture FiBL, Frick, Switzerland

While there are estimates of the abiotic processes contribution to soil phosphorus (P) availability, less is known about the contribution of biological processes. Two main enzymatic processes involved in soil P cycling are known to alter the oxygen isotopic composition of phosphate ($\delta^{18}$O-P), each in a different way, through the cleavage of the P–O bond: the intracellular P turnover and the organic P hydrolysis. The former induces isotopic equilibration between phosphate and water and is considered the major process affecting soil available P via microbial P release. The latter induces depleted $\delta^{18}$O-P in the phosphate released from the mineralization of organic P. We studied P dynamics in organic horizons of two contrasting soils (low- and high-P availability) from temperate beech forests. We labeled the soil with $^{18}$O-enriched water and followed changes in the $\delta^{18}$O-P of different soil P pools in the presence or absence of added leaf litter during 3 months of incubation. $\delta^{18}$O-P values of almost all P pools progressively increased indicating oxygen incorporation from the enriched soil water into phosphate via the above-mentioned enzymatic processes. $\delta^{18}$O-P of available P increased more in the P-rich soil than in the P-poor soil and approached the isotopic equilibrium between phosphate and water, revealing the impact of microbial P release into the available P pool. However, in the P-poor soil, the available P brought the isotopic signature induced by phosphatase enzymes, indicating that it was mostly originated from the hydrolysis of organic P. Therefore, under P-limiting conditions, the isotopic effect of organic P hydrolysis can outweigh the isotopic equilibrium effect. Finally, two independent isotopic approaches with $^{33}$P and $\delta^{18}$O-P provided very similar estimates of P exchanged between the available P and other inorganic soil pools. This suggests that $\delta^{18}$O-P can be successfully used to trace P fluxes, provided that the underlying processes do not break the P–O bonds of the phosphate molecule.

Keywords: soil, isotopic labeling, phosphatase, microbial phosphorus, mineralization
INTRODUCTION

In forest ecosystems, plants and their mycorrhizae take up most of their phosphorus (P) as dissolved orthophosphate (PO$_4^{3-}$, hereafter phosphate) from the soil solution, which represents often a small proportion of the total P in soil. The soil solution is continuously replenished by abiotic processes, such as the release of P from sorbed phases, by the dissolution of P containing minerals, and by biological processes, namely mineralization of organic P (Po). Microbes mineralize Po from plant litter and soil organic matter via enzymatic hydrolysis. The newly released P is taken up by microbes (immobilization), sorbed to the soil solid phases or replenishes the soil solution. Upon cell death, microbial P ultimately enters the non-living Po and inorganic P pools, thus constituting a potentially available P pool.

While there are estimates of the contribution of abiotic inorganic P pools to P availability (Helfenstein et al., 2020), less is known about the contribution of biological processes. This contribution varies widely, depending on factors such as land-use and inorganic P availability (Becquer et al., 2014; Bünemann, 2015; Pistocchi et al., 2018). Integrating this knowledge into soil P cycling models is crucial to predict the effects of changing environmental conditions, such as the ones induced by climate change, or of global P resources scarcity on net primary production.

Assessing soil P dynamics is challenging, because exchanges between P pools often occur without net changes in pool size. The use of radioactive tracers $^{32}$P and $^{33}$P is to date the sole option to quantify gross P fluxes, such as gross P immobilization and mineralization (Bünemann, 2015 and references therein), the transfer from the soil to the plant (Frossard et al., 2011 and references therein) and the fate of P added with plant residues or fertilizers (Fardeau et al., 1995; Daroub et al., 2000; Pistocchi et al., 2018). Studies on various forest soils have highlighted the impact of Po mineralization on P fluxes notably when inorganic P availability is low (Achat et al., 2009; Mooshammer et al., 2012; Spohn et al., 2013; Bünemann et al., 2016; Pistocchi et al., 2018).

Although radioisotopes have several advantages (e.g., negligible P mass addition, direct P tracing, simple to analyze), they also have several drawbacks related to safety issues, short half-lives, and low sensitivity to biological P fluxes when the baseline of isotopic dilution, i.e., the flux due to abiotic processes, is high (Pistocchi et al., 2018; Siegenthaler et al., 2020).

In the last two decades, many studies have shown that biological processes involved in soil P cycling alter the isotopic composition of oxygen in phosphate ($^{18}$O-P) through the cleavage of the P–O bond and the incorporation of O atoms from water (Tamburini et al., 2012 and references therein). Whereas in the absence of biological activity, the P–O bond is stable and exchanges of oxygen (O) between phosphate and water are negligible. Two main enzyme-mediated processes have an impact on $^{18}$O-P. Firstly, intracellular P turnover controlled by the enzyme inorganic pyrophosphatase leads to the complete exchange of the four O atoms in phosphate with O from water. This reaction produces a temperature-dependent isotopic equilibrium between phosphate and water (Longinelli and Nuti, 1973; Chang and Blake, 2015).

Secondly, the intra- and extracellular phosphatase enzymes incorporate one or two O atoms from water into phosphate released by the breakdown of Po compounds (P mono- and diesters). One O atom is incorporated during the hydrolysis of a phosphomonoester and two O atoms are incorporated during the hydrolysis of a phosphodiester. These transfers are associated with an enzyme-dependent fractionation. Most phosphatase enzymes (e.g., alkaline and acid phosphatase and phytase) have a negative fractionation factor, meaning they release phosphate with a depleted $^{18}$O-P (Liang and Blake, 2006b; von Sperber et al., 2014, 2015).

The $^{18}$O-P of available P in the soil can be affected by these processes through various mechanisms. Phosphate ions released by microorganisms to the soil solution potentially contribute a $^{18}$O-P close to isotopic equilibrium, as influenced by the intracellular inorganic pyrophosphatase (Zohar et al., 2010; Poll et al., 2006). Negative offsets from isotopic equilibrium may be caused by the hydrolysis of Po compounds by extracellular or intracellular phosphatase enzymes (Helfenstein et al., 2018). Positive offsets might result from the uptake of phosphate by organisms, as they preferentially take up lighter phosphate isotopologues, thus increasing the $^{18}$O-P of the extracellular phosphate pool (Blake et al., 2005; Lis et al., 2019).

An approach used to detect the main biological processes affecting soil P cycling consists of labeling the soil solution with $^{18}$O-enriched water (Bauke et al., 2017; Siebers et al., 2018; Siegenthaler et al., 2020). Enzyme-mediated processes inducing the P–O bond cleavage are therefore detected by tracing the incorporation of O from water into specific phosphate pools.

Tracing P fluxes with this approach is possible provided that the process generating the P flux does not induce a cleavage of the P–O bond, i.e., phosphate is transferred as intact molecule. Abiotic processes, such as sorption/desorption and precipitation/dissolution meet this condition. Additionally, phosphate ions are sorbed or precipitate with negligible isotopologues discrimination (Liang and Blake, 2006b; Jaisi et al., 2010). Through these abiotic processes, the $^{18}$O-P of phosphate acquired via biological processes can be transferred to other inorganic P pools present in the solid phase. This was observed in a study on Andosols along a rainfall gradient, where P bound to calcium was found to carry an equilibrium isotopic signature after losing the $^{18}$O-P of the original parent material (Helfenstein et al., 2018).

Within the German Priority Program SPP1685 “Ecosystem Nutrition: Forest Strategies for Limited Phosphorus Resources” we studied P dynamics and the biological contribution to P availability in organic horizons (Oe) of two contrasting (low- and high-P availability) and well-characterized soils from temperate forests. We chose organic horizons, as they are essential for the recycling of nutrients in forests, supplying up to 99% of plant P demand where inorganic P availability is low (Brändle et al., 2004; Jonard et al., 2009; Hauenstein et al., 2018).
We labeled the soil with \(^{18}\text{O}\)-enriched water and measured the \(\Delta^{18}\text{O}-\text{P}\) from different soil P pools in the presence or absence of added leaf litter at several time points during 3 months of incubation. In parallel, we conducted an identical incubation with \(33\text{P}\) to quantify P fluxes, as reported in Pistocchi et al. (2018). The results of the present study will be discussed also in relation to this parallel study and the analysis of microbial community composition of Mészáros et al. (2020).

Our hypothesis was that under low P availability, the prevalence of Po mineralization on P fluxes would cause a negative offset in the \(\Delta^{18}\text{O}-\text{P}\) of the available P due to the negative fractionation of phosphatase enzymes. Under high P availability conditions, we expected the \(\Delta^{18}\text{O}-\text{P}\) of available P pool to be affected by either (i) abiotic exchanges with mineral phases: in this case, we would observe no incorporation of oxygen from water into phosphate; or (ii) microbial P turnover: in this case, the \(\Delta^{18}\text{O}-\text{P}\) of the available P would approach isotopic equilibrium. Additionally, we measured the \(\Delta^{18}\text{O}-\text{P}\) in alkaline and acid-extractable P to trace abiotic exchanges of these pools with the available P. Finally, we made the hypothesis that O incorporation from labeled water into P pools is proportional to microbial activity as suggested by Melby et al. (2013).

**MATERIALS AND METHODS**

**Sites, Soil Sampling and Preparation**

The soil horizons come from two European Beech forest sites in Germany (Fagus sylvatica L., 100–120 years old). The site Bad Brückenau (BBR) is located at about 800 m above sea level (asl) in Northern Bavaria (50°21′7.26″N, 9°55′44.53″E). The site Lüss (LUE) is located at 100 m asl in Lower Saxony (52°30′21.77″N, 10°16′2.37″E). The soils are classified as Dystric Skeletic Cambisol (Hyperhumic, Loamic; IUSS Working Group WRB, 2006) and developed on basalt bedrock, and as a Hyperdystric Folic Cambisol (Arenic, Loamic, Nchlic, Protosporodic), developed on Pleistocene sand, respectively. The texture of the mineral topsoil in BBR is silty clay loam, while in LUE is loamy sand. A detailed description of the two sites is reported in Lang et al. (2017). At each site, we collected samples from the leaf litter deposited during the previous autumn and from the organic horizons, in April, 2015 at LUE and May, 2015 at BBR. Litter was collected from the soil surface, and then five to six subsamples were taken from the Oe horizon at each site and bulked into a composite sample (hereafter referred to as BBR and LUE soil, respectively). The soil was sieved while moist to < 5 mm. The litter was dried at 35°C, manually crushed and sieved twice to collect the fraction between 20 and 5 mm. Soil and litter samples were stored at 4°C for a period of 2 weeks (BBR) to 1 month (LUE) before the experiment. The gravimetric water content of field moist soil was determined by drying for 20 h at 105°C. The water holding capacity (WHC) of the field moist soils was determined by saturating the soils with water and then allowing gravitational water to drain by putting them in a sand bath for 4 h.

**Experimental Design and Incubation Experiment**

The soils were split in two and two incubations were undertaken. The first was the labeling experiment with \(18\text{O}\) enriched water. The second was a soil respiration experiment to measure CO\(_2\) produced as an indicator of microbial activity under the same condition of the labeling experiment. The two incubations lasted 93 and 97 days, respectively.

In both cases, the experiment design had two factors: the soil (BBR and LUE) and the litter treatment, which included the absence [non-amended treatment (NIL)] or presence [leaf-litter amended treatment (L)] of leaf litter amendment. All treatments were replicated three times for the labeling experiment and four times for the respiration experiment.

Before splitting the soil, a 3-week pre-incubation at approximately 40% of the maximum WHC was undertaken, during which we monitored the respiration to obtain constant soil respiration rates (Oehl et al., 2001).

For the labeling experiment, soils were weighed in polyethylene zip lock bags (equivalent of 108 g dry soil each) and randomly assigned to the treatments. The labeling solution was prepared with 98% \(18\text{O}\)-enriched water (Sercon Limited, Crewe, United Kingdom, the final \(\Delta^{18}\text{O}\) in the labeling water was = 34.30‰). Three ml of the labeling solution was added to each of the three replicate bags, spread on the top of the soil by pipetting and then mixed for 1 min by hand through agitating the bag. This process was then repeated to add a total of nine and 12 ml of labeling solution per bag, for BBR and LUE soils, respectively. The added volumes increased the BBR and LUE soils water content to approximately 50% of their WHC. Finally, litter was added to half of the bags, in the ratio of 10 mg per g of dry soil, equivalent to 4.6 mg C g\(^{-1}\) soil, which corresponds approximately to natural leaf litter inputs at the two sites (Lang et al., 2017). The bags were left slightly open and arranged in a completely randomized design, in plastic trays which were covered and incubated in the dark at 19°C. To reduce evaporation, which affects the \(\Delta^{18}\text{O}\) of soil water, the air moisture was kept approximately constant by placing a beaker with water in each tray.

The labeling experiment was combined with sequential extractions. Concentrations of P were measured in resin-extractable P (inorganic available P, hereafter referred to as P\(_{\text{res}}\)) and hexanol-labile P pools (P\(_{\text{hex}}\)) at days 0, 4, 11, 29, and 93 after labeling. Additionally, at days 0, 4, and 93 we performed a modified Hedley sequential extraction (Tiessen and Moir, 1993; Tamburini et al., 2018) to follow the fate of \(18\text{O}\) into other P pools (see section “Soil Phosphorus Pools Concentrations”).

For the soil respiration experiment, a set of subsamples (10 g dry weight equivalent) including all soil × litter treatment combinations was prepared on day 0 adding ultrapure H\(_2\)O instead of the labeling solution. Each sample was placed in an air-tight jar (1 L volume) with an alkaline trap made of 20 ml 0.2 M NaOH solution, including four blanks without soil. The
overnight and filtering using glass fiber filters (0.8 
µm), and purification for isotopes analyses.
The filtrates were collected for Pi
15 min), filtered through Millipore nylon filters (0.8 
µm), and extracted with NaOH/EDTA, targeting the inorganic and 
organic P bound to Fe and Al oxides (hereafter Pi
). It was extracted with NaOH/EDTA, targeting the inorganic and 
low-molecular weight (mostly inorganic) compounds using size 
exclusion gel chromatography with a Sephadex G25 Medium, 
preventing a 5 KDa cut-off (ÄKTApure plus, GE Healthcare 
Bio-Sciences AB, Uppsala, Sweden). The inorganic pool was 
purified following the standard protocol, after a precipitation 
of magnesium ammonium phosphate, which targets inorganic 
P. The organic pool was hydrolysed by UV radiation, with 
one split containing 18O-enriched water to check for possible 
corporation of O into the newly formed inorganic phosphate.

P concentration in NaOH–EDTA extracts from LUE was 
not high enough for the separation–purification procedure and 
therefore only the 18O-P values of the initial Po
NaOH (18O-Po
NaOH) are presented.

Purified phosphate in the form of Ag3PO4 was weighted in 
silver capsules in two or three analytical replicates each 
consisting in 300–600 µg of Ag3PO4 and a small amount of 
glassy carbon powder. Samples were analyzed on a 
thermal conversion elemental analyzer (vario PYRO cube, 
Elementar Analysensysteme GmbH, Langenselbold, Germany), 
coupled to an IsoPrime 100 isotopic ratio mass spectrometer 
(IRMS) at the Laboratory of the Plant Nutrition Group 
(ETH Zürich). In each run, repeated measurements of internal 
standards was better than 0.03‰.

The Pres, Phex, and Pi
HCl extracts were purified following the 
protocol of Tamburini et al. (2010) and modified by Pistocchi 
et al. (2014). In NaOH–EDTA extracts, both Po
NaOH and Pi
NaOH are present and a separation prior the purification is needed. 
According to Tamburini et al. (2018), the NaOH–EDTA pool 
was divided into high-molecular weight (mostly organic) and 
low-molecular weight (mostly inorganic) compounds using size 
exclusion gel chromatography with a Sephadex G25 Medium, 
preventing a 5 KDa cut-off (ÄKTApure plus, GE Healthcare 
Bio-Sciences AB, Uppsala, Sweden). The inorganic pool was 
purified following the standard protocol, after a precipitation 
of magnesium ammonium phosphate, which targets inorganic 
P. The organic pool was hydrolysed by UV radiation, with 
one split containing 18O-enriched water to check for possible 
corporation of O into the newly formed inorganic phosphate.

P concentration in NaOH–EDTA extracts from LUE was 
not high enough for the separation–purification procedure and 
therefore only the 18O-P values of the initial Po
NaOH (18O-Po
NaOH) are presented.

The P concentration in NaOH–EDTA extracts from LUE was 
not high enough for the separation–purification procedure and 
therefore only the 18O-P values of the initial Po
NaOH (18O-Po
NaOH) are presented.

The P concentration in NaOH–EDTA extracts from LUE was 
not high enough for the separation–purification procedure and 
therefore only the 18O-P values of the initial Po
NaOH (18O-Po
NaOH) are presented.
and calibration. The $\delta^{18}$O composition is expressed in the delta notation with respect to VSMOW (Vienna Standard Mean Ocean Water). The $\delta^{18}$O-P of a specific P pool is referred to as $\delta^{18}$O-P$_{pool}$. Analytical uncertainties, as determined from the replicate analysis of the standards, were of 0.4%.

### Oxygen isotopic values in leaf litter

Samples of the LUE and BBR leaf litter used in the incubation were extracted and purified according to the protocol of Tamburini et al. (2018). The protocol targets two pools extracted sequentially: the metabolizable Pi with trichloroacetic acid (TCA) and the organic P with NaOH–EDTA. In detail, plant material weighed in duplicate (6 g each) was first crushed with the help of liquid N$_2$ and then extracted with either $^{18}$O-labeled or unlabeled 0.3 M TCA. The supernatants were separated after homogenization with a Polytron (Kinematica AG, Lucerne, Switzerland) and shaking (0.5 h) via filtration with glass fiber filters (APFF04700 Merck Millipore). The residue was further extracted with 0.25 M NaOH – with glass fiber filters (APFF04700 Merck Millipore).

The TCA and NaOH–EDTA supernatants were measured for Pi (P$_{TCA}$, P$_{NaOH,litter}$) and Po (P$_{TCA}$, P$_{NaOH,litter}$) and purified for $\delta^{18}$O-P analysis, following the protocol described in Noack et al. (2014). The TCA and NaOH–EDTA supernatants were measured for Pi (P$_{TCA}$, P$_{NaOH,litter}$) and Po (P$_{TCA}$, P$_{NaOH,litter}$) and purified for $\delta^{18}$O-P analysis, following the procedure described by Pfahler et al. (2013) and Tamburini et al. (2018), respectively.

### Data Analyses

#### Expected $\delta^{18}$O-P at Isotopic Equilibrium and From Organic P Hydrolysis

The expected $\delta^{18}$O-P of phosphate in equilibrium with soil water ($\delta^{18}$O-P$_{eq}$) was calculated for each time point rearranging the equation from Chang and Blake (2015) as follows:

$$\delta^{18}O-P_{eq} = e^c(14.43/T + 26.54/1000)$$

* $\delta^{18}Ow + 1000 - 1000$ (1)

where $T$ is the temperature in K during the incubation and $\delta^{18}Ow$ ($\%$) is the measured oxygen isotopic composition of soil water.

The expected $\delta^{18}$O-P of phosphate released from hydrolysis of phosphoesters ($\delta^{18}$O-P$_{Pase}$) via phosphatase enzymes was calculated according to this equation:

$$\delta^{18}O-P_{Pase} = x \times (\delta^{18}O-Po) + (1 - x) \times (\delta^{18}Ow + \epsilon)$$

where $x$ is the proportion of O atoms inherited from the phosphate group in the Po compound (0.75 for phosphomonoester and 0.5 for phosphodiester), $\delta^{18}O$-Po ($\%$) is the O isotopic composition of the phosphate group in the Po compound, $\delta^{18}Ow$ ($\%$) is the measured O isotopic composition of soil water, and $\epsilon$ ($\%$) is the enzyme-specific fractionation factor. Here we estimated $\delta^{18}$O-P as the measured isotopic composition of Po extracted by NaOH–EDTA ($\delta^{18}$O-P$_{NaOH}$) either from soil or leaf litter (see Table 1 and sections “Oxygen isotopic values in soil water and phosphate” and “Oxygen isotopic values in leaf litter”).

#### Calculation of $\delta^{18}$O-P

The oxygen isotopic composition of phosphate in the P$_{mic}$ pool ($\delta^{18}$O-P$_{mic}$), was calculated with a mass balance equation (Tamburini et al., 2012):

$$\delta^{18}O-P_{mic} = (\delta^{18}O-P_{hex} \times P_{hex} - \delta^{18}O-P_{res} \times P_{res})/ (P_{hex} - P_{res})$$

where $\delta^{18}$O-P$_{hex}$ ($\%$) and $\delta^{18}$O-P$_{res}$ ($\%$) are the oxygen isotopic composition of phosphate in the hexanol and resin extracts, respectively, and $P_{hex}$ (mg P kg$^{-1}$) and $P_{res}$ (mg P kg$^{-1}$) are the P concentrations in the corresponding extracts. When $\delta^{18}$O-P$_{hex}$ and $\delta^{18}$O-P$_{res}$ were close, i.e., a difference of less than twice the standard deviation of analytical replicates (< 0.8%), the $\delta^{18}$O-P$_{hex}$ was taken directly as $\delta^{18}$O-P$_{mic}$. We did not apply any conversion factor (Kp) to correct for incomplete recovery of P$_{mic}$ due to possible ineffectiveness of the fumigant. Indeed, we cannot assume that all P$_{mic}$ compartments, including the ones non-extractable with hexanol, have the same isotopic composition.

Some extractions were done in duplicate with $^{18}$O labeled and unlabeled reagents, i.e., TCA, UV digestion of the organic pool of NaOH–EDTA and HCl, to track the incorporation of labeled oxygen into the $\delta^{18}$O-P via inorganic hydrolysis (Tamburini et al., 2010). If incorporation was detected, the actual $\delta^{18}$O-P was calculated according to Pistocchi et al. (2017):

$$\delta^{18}O-P = (\delta^{18}O-P_{l} \times \delta^{18}Ow_{nl} - \delta^{18}O-P_{nl} \times \delta^{18}Ow_{l})/ (\delta^{18}O-P_{l} - \delta^{18}O-P_{nl} - \delta^{18}Ow_{l} + \delta^{18}Ow_{nl})$$

where $\delta^{18}$O-P$_{l}$ ($\%$) and $\delta^{18}$O-P$_{nl}$ ($\%$) are the oxygen isotopic composition of phosphate for the labeled and unlabeled samples, respectively, and accordingly $\delta^{18}$Ow$_{l}$ ($\%$) and $\delta^{18}$Ow$_{nl}$ ($\%$) the oxygen isotopic composition of labeled and unlabeled extraction solution. When hydrolysis was negligible, as in most cases, the subsamples were considered as duplicates.

#### Incorporation of O From Water Into Phosphate

Through the process of labeling with $^{18}$O-enriched water, it is possible to calculate how much O from soil water was incorporated into phosphate molecules of a specific P pool over time. Oxygen incorporation ($\%$) was calculated as the slope of the straight line between two points in the $\delta^{18}$Ow-$\delta^{18}$O-P plot (Liang and Blake, 2006a):

$$O\text{ incorporation} = (\delta^{18}O-P_{l} - \delta^{18}O-P_{o})/ (\delta^{18}Ow_{l} - \delta^{18}Ow_{o}) \times 100$$

Where $\delta^{18}$Ow$_{l}$ and $\delta^{18}$Ow$_{o}$ are the oxygen isotopic compositions of soil water and $\delta^{18}$O-P$_{l}$ and $\delta^{18}$O-P$_{o}$ are the oxygen isotopic compositions of phosphate in a given pool at time point t and at day 0, respectively.

Incorporations of 25 or 50% indicate that overall one out of four or two out of four O atoms, respectively, are incorporated from water into phosphate. 100% O incorporation indicates all four O of phosphate derived from water.

For $\delta^{18}$O-P values obtained with the Equations 3, 4, and 5 the mean and standard deviation per sample were obtained with a Monte Carlo error propagation simulation (Anderson, 1976).
Calculations were repeated 10 million times by varying the $\delta^{18}$O signatures according to their mean and standard deviations. For the $\delta^{18}$OWw, we assumed a standard deviation of 0.03‰ corresponding to the analytical error.

Statistical Analyses
A two-way ANOVA (1st factor = litter application, 2nd factor = date) was applied to analyze the variables measured during the incubation for each soil separately except the respiration rates. These latter were analyzed with a mixed model, where the litter amendment was the fixed factor and the time the measurement (weekly) a random factor with the replicate nested in it. Model simplification (one-way ANOVA) was done when possible or when data were missing, e.g., $\delta^{18}$O-PoNaOH in LUE soil. In some cases, there were not enough replicates for statistical tests. These results are discussed qualitatively. The Tukey test was used for post hoc comparison. The Student’s paired t-test was used when comparing single dates and cumulative values, after checking for homogeneity of variances. The Shapiro–Wilk test was used to assess normality of the data. All analyses were performed in R 3.1.1 (R version 3.1.1, R Core Team).

RESULTS

Soil Respiration
The soil respiration declined during the first 3–4 weeks by approximately 10 and 25% for LUE and BBR soils, respectively. After the first month, the respiration remained approximately constant (Figure 1). The soil respiration of LUE soil was almost double that of the one measured for BBR soil. Similarly, the metabolic quotient cCO$_2$, calculated with the average respiration rates over the last 8 weeks, was almost double in LUE soil (Table 1).

During the first 4 weeks, differences between treatments were detectable, with leaf litter-amended soils showing higher respiration rates (Figure 1). This resulted in a cumulative additional carbon (C) release for the litter-amended soils of 3.4 and 11.1% for LUE and BBR soils, respectively, significant only for the latter (Supplementary Table 1 SI).

Soil General Characteristics and Potential Phosphatases Activity
The two soils were acidic (3.77 and 3.65 for BBR and LUE soil, respectively) but differed in almost all considered variables (Table 1). In particular, concentration of total, organic and available P was much higher in BBR than in LUE soil.

Potential acid phosphatase activity was similar for LUE and BBR soils, while potential alkaline phosphatase activity was double in LUE soil, although much lower than the acid phosphatase activity (Table 1 and Supplementary Figure 1SI). Therefore, for the calculation of the expected value from Po hydrolysis by phosphatases with Equation 2 we used the fractionation factor ($\epsilon$) of –10‰ attributed to acid phosphatases (von Sperber et al., 2014). No differences were found between litter treatments.

The Concentration and $\delta^{18}$O-P of Resin-Extractable and Microbial P Pools
In both LUE and BBR soils the concentration of P$_{res}$ increased, while P$_{mic}$ remained largely stable over the study time with small
fluctuation in the low P LUE soil and decreased significantly in the high P BBR soil (Supplementary Table 2 SI). In the litter-amended BBR soil the $P_{\text{res}}$ was slightly higher, with a mean effect size of $+ 2.1 \, \text{mg kg}^{-1}$. The litter addition increased the $P_{\text{mic}}$ slightly in both soils, with a mean effect size of $+ 4.9$ and $+ 4.4 \, \text{mg kg}^{-1}$ for LUE and BBR, respectively (Supplementary Table 2 SI).

The $\delta^{18}$O-P$_{\text{res}}$ and $\delta^{18}$O-P$_{\text{mic}}$ increased by several units during the incubation in both LUE and BBR soils (Figure 2). Therefore, incorporation of O from labeled soil water into phosphate occurred.

For the low P LUE soil, both $\delta^{18}$O-P$_{\text{res}}$ and $\delta^{18}$O-P$_{\text{mic}}$ had a similar initial value (13.9 and 12.7%, respectively) and both followed a slight increasing trend over the duration irrespective of the application of leaf litter. This change in value was similar to that predicted by the $\delta^{18}$O-P$_{\text{Pase}}$, i.e., expected from the hydrolysis of Po via acid phosphatases (Equation 2; Figure 2A).

For the high P BBR soil, however, the initial values of $\delta^{18}$O-P$_{\text{res}}$ and $\delta^{18}$O-P$_{\text{mic}}$ were different: 19.9% vs. 14.0%, respectively. The $\delta^{18}$O-P$_{\text{mic}}$ increased slightly over time and remained below the expected equilibrium between phosphate and water ($\delta^{18}$O-P$_{\text{eq}}$ in Equation 1), whereas the $\delta^{18}$O-P$_{\text{res}}$ approached and exceeded the $\delta^{18}$O-P$_{\text{eq}}$ by day 93 (Figure 2B). The $\delta^{18}$O-P$_{\text{mic}}$ values were in average lower for the non-amended treatment ($p < 0.05$).
For both soils, the picture does not change if we consider the δ¹⁸O-P\textsubscript{P_{as}e} values calculated using the Po in the litter, as they are similar to the ones from soil Po (see δ¹⁸O-P\textsubscript{P_{as}e} litter, Supplementary Table 2 SI).

**The Concentration and δ¹⁸O-P of NaOH- and HCl-Extractable Pools**

In the LUE soil incubation, concentrations of the inorganic pools P\textsubscript{NaOH} and P\textsubscript{HCl} remained approximately stable during the incubation. The organic pool, P\textsubscript{NaOH}, increased slightly (+ 36.8 mg kg\(^{-1}\) at day 93). No differences were detected between the litter amended and non-amended treatments (Table 3).

In BBR, a slight increase (mean effect size of + 49.0 mg kg\(^{-1}\) at day 93) of the P\textsubscript{NaOH} was registered over the incubation time. The P\textsubscript{HCl} and P\textsubscript{NaOH} remained stable over the incubation time. No differences due to the leaf litter addition were observed (Table 3).

The δ¹⁸O-P\textsubscript{HCl} in the LUE soil incubation had a slight increase (+ 1‰). We could not analyze the δ¹⁸O-P\textsubscript{NaOH} or δ¹⁸O-P\textsubscript{P_{as}e} in LUE soil because of the insufficient amount of P extracted. In the BBR soil, we detected a significant increase of the δ¹⁸O-P of both inorganic and organic pools. The δ¹⁸O-P\textsubscript{NaOH} increased by 4.4‰, as an average of the L and NL treatments. A similar increase was measured for the δ¹⁸O-P\textsubscript{HCl} (Table 3). Despite those increases neither δ¹⁸O-P\textsubscript{NaOH} nor δ¹⁸O-P\textsubscript{HCl} reached the corresponding value of the available P (P\textsubscript{res}) at day 93, meaning that they did not completely equilibrate with the P\textsubscript{res} at the end of the incubation. Similarly, the δ¹⁸O-P\textsubscript{NaOH} also increased slightly, but by only approximately 1‰ at the end of the incubation.

Differences due to the litter addition were found in the BBR soil for the δ¹⁸O-P\textsubscript{NaOH} (+ 1‰ for the L treatment at day 4) and for the δ¹⁸O-P\textsubscript{HCl} (−2.3‰ for the L treatment at day 93).

**¹⁸O Incorporation Into P Pools**

The addition of ¹⁸O-enriched water successfully increased O isotopic ratio of soil water (Supplementary Table 3 SI) and resulted in incorporation of ¹⁸O from water into major soil P pools. The trend, however, differed in the two soils. In LUE, both the P\textsubscript{res} and P\textsubscript{mic} incorporated O at similar rate: around one out of four O atoms per phosphate (25%) were exchanged in the first 20–30 days of incubation and up to two out of four (50%) were exchanged after 93 days (Figure 3A). In BBR instead, the ¹⁸O from water was incorporated faster, but it did not exceed 50% in the P\textsubscript{mic} by day 93, while it had reached 90% in the P\textsubscript{res}, suggesting an almost complete exchange of the four O of phosphate with water (Figure 3B).

A decline of the O incorporation in P\textsubscript{mic} in the BBR soil was observed between the second-last and the last sampling. This decline was more pronounced in the treatment without litter. A stronger relationship was found between O incorporation in P\textsubscript{res} and cumulated respiration in BBR soil as compared to LUE (Figure 4A).

**DISCUSSION**

The increase in δ¹⁸O-P value of almost all P pools indicates that biological processes were involved in P cycling in both soils, as the exchange of oxygen between soil phosphate and water involves enzymatic reactions (Tudge, 1960).

**Biological Processes Affecting Available P in the Low P Availability LUE Soil**

Under low P availability, δ¹⁸O-P\textsubscript{mic} and δ¹⁸O-P\textsubscript{res} were very similar and followed the same temporal trend (Figure 2A). There are three potential explanations for this pattern: (i) There was phosphate efflux from P\textsubscript{mic} to P\textsubscript{res}, so the P\textsubscript{res} pool was strongly influenced by the P\textsubscript{mic} pool as observed in other P-poor soils (Tamburini et al., 2012; Weiner et al., 2018); (ii) There was no phosphate efflux from P\textsubscript{mic} to P\textsubscript{res}, but the dominant enzymatic processes were the same in both pools, thus imprinting the same isotopic signature; finally (iii) There was no phosphate efflux from P\textsubscript{mic} to P\textsubscript{res}, and the two pools were impacted by different enzymatic processes which resulted in similar δ¹⁸O-P just by chance.

![Figure 3](https://example.com/f3.png) **Figure 3** Oxygen incorporation from water into available P (P\textsubscript{res}) and microbial P (P\textsubscript{mic}) pools according to Equation 5 in Lüss (LUE: A) and Bad Brückenau (BBR: B) soils during the incubation. Error bars indicate standard deviation from Monte Carlo error propagation. NL, non-amended; L, leaf litter-amended.
The parallel $^{33}$P dilution experiment showed that gross mineralization strongly contributed to the $P_{\text{res}}$ pool. It also highlighted a fast and important P immobilization into the microbial biomass (around 95% of gross mineralization, see Pistocchi et al., 2018). The $^{33}$P experiment, however, gave no direct evidence of an efflux from $P_{\text{mic}}$ to $P_{\text{res}}$. This may point to the first or to the second explanation. Lis et al. (2019) observed in an in vitro experiment that cyanobacteria growing under P-depleted conditions took up P through phosphate-specific transporters (Pst) without leaking it. Therefore, the second explanation, i.e., no or little phosphate efflux from $P_{\text{mic}}$ to $P_{\text{res}}$ and same dominant enzymatic processes, is more likely (see Figure 2A modified after Lis et al., 2019). However, compared to their study we did not observe isotopic equilibrium in the intracellular and extracellular P.

Of further consideration is that both $\delta^{18}$O-$P_{\text{mic}}$ and $\delta^{18}$O-$P_{\text{res}}$ were similar to the value expected for phosphomonoester hydrolysis over the first 30 days of incubation (one out of four O was exchanged with water, see Figure 2A). According to a two-end-members mass balance including the isotopic equilibrium ($\delta^{18}$O-$P_{\text{eq}}$, Equation 1) and the $\delta^{18}$O-$P_{\text{Pase}}$ (Equation 2), this contribution represented almost 100% of the $P_{\text{res}}$ over this period.

By the end of the incubation, the O incorporation into $P_{\text{res}}$ had attained 50% (two out of four O atoms exchanged with water; Figure 3A), suggesting the contribution of other processes. There are two possible explanations: (i) that a single enzymatic process which incorporates two O atoms from water into phosphate was prevailing; or (ii) two or more processes contributed, resulting in 50% average O incorporation. The first explanation would be that the extracellular hydrolysis of phosphodiesterases was occurring. However, Lang et al. (2017) found that the potential activity of phosphodiesterases over phosphomonoesterases was very low at this site. The contribution of multiple processes is, therefore, more likely. A two end-members balance including $\delta^{18}$O-$P_{\text{eq}}$ and $\delta^{18}$O-$P_{\text{Pase}}$ as previously hypothesized, suggests that phosphate derived from monoester hydrolysis constitutes about 80% of the $P_{\text{res}}$ at the end of the incubation. Interestingly, this value agrees with the results of parallel $^{33}$P incubation, where 94% of P exchanged with the soil solution came from gross mineralization (Pistocchi et al., 2018).

We conclude that under low P availability, both the $P_{\text{res}}$ and the $P_{\text{mic}}$ pools were dominated by phosphomonoesterase catalyzed reactions.

The attainment of the isotopic equilibrium was until now considered the main biological effect influencing the available P, corresponding to $P_{\text{res}}$ as defined in this study, via the release of intracellular phosphate ions (Zohar et al., 2010; Tamburini et al., 2012; Stout et al., 2014; Weiner et al., 2018; Lis et al., 2019). Our results support the hypothesis that under P-limiting conditions, the isotopic effect of P hydrolysis can outweigh the isotopic equilibrium effect driven by inorganic pyrophosphatase in both $P_{\text{mic}}$ and $P_{\text{res}}$ pools (Figure 5A).

**Biological Processes Affecting Available P in the High P Availability BBR Soil**

Under high P availability, the $P_{\text{res}}$ pool carried a very distinct isotopic signature from the $P_{\text{mic}}$ pool (Figure 2B). The $\delta^{18}$O-$P_{\text{res}}$ was close to or higher than the $\delta^{18}$O-$P_{\text{eq}}$ (isotopic equilibrium between phosphate and water, Equation 1), while $\delta^{18}$O-$P_{\text{mic}}$ was in between $\delta^{18}$O-$P_{\text{eq}}$ and the $\delta^{18}$O-$P_{\text{Pase}}$ (Equation 2).

The contribution of P from leaf litter does not explain the $\delta^{18}$O-$P_{\text{res}}$ values, as we did not find significant differences between litter treatments. The exchange with other soil P pools via sorption/desorption or precipitation/dissolution does not explain them either, as the $P_{\text{NaOH}}$ and $P_{\text{HCl}}$ all had lower $\delta^{18}$O-P than $\delta^{18}$O-$P_{\text{res}}$ (see Table 2), although these P pools likely exchanged phosphate ions with the $P_{\text{res}}$ pool (see section “Exchanges Between Available P and Other Inorganic and Organic P Pools”).

The incorporation of O atoms from labeled water was around 90% in the $P_{\text{res}}$ pool by day 93, suggesting the prevalence of a process that leads to an exchange of all four O atoms. The
only known enzyme which could cause a complete exchange of O atoms with water is the inorganic pyrophosphatase (Cohn, 1958), which is mostly intracellular (Poll et al., 2006). However, the \( P_{\text{mic}} \) pool in the BBR soil, showed consistently lower O incorporation when compared to the \( P_{\text{res}} \). The presence of different microorganisms in either active or dormant states, all extracted with hexanol but carrying different isotopic signatures, could possibly explain the difference in \( \delta^{18}O\)-P between \( P_{\text{res}} \) and \( P_{\text{mic}} \) pools. However, only the active microbial community would carry a signature close to \( \delta^{18}O\)-P\(_{\text{eq}} \) and contribute to the \( P_{\text{res}} \) via P uptake, intracellular turnover and release (Siegenthaler et al., 2020). Indeed, inorganic pyrophosphatase is involved in DNA synthesis, amongst other processes, and therefore is more active when cells are growing (Kottur and Nair, 2018). The microorganisms in a dormant state would interact poorly with the \( P_{\text{res}} \) pool and incorporate less O from water (see section “\( \delta^{18}O\)-P and Microbial Activity” for more discussion).

The O incorporation of close to 90% (Figure 2B) implies that almost all the phosphate in the final \( P_{\text{res}} \) pool (i.e., 45.8 and 49.8 mg kg\(^{-1}\))
underwent intracellular P turnover catalyzed by inorganic pyrophosphatases over the investigated period. Part of this efflux from P\textsubscript{mic} corresponded to a net loss, more pronounced in the NL treatment, which in turn translated in a net increase of P\textsubscript{res} (Supplementary Table 2 SI) and a transfer to other Pi or Po pools (see section “Exchanges Between Available P and Other Inorganic and Organic P Pools”).

At the end of the incubation, the $\delta^{18}$O-P\textsubscript{res} was higher than the expected equilibrium by $+3.0\%$. Recent studies on temperate forest soils reported enriched isotopic ratios compared to expected equilibrium after a period of incubation (Gross and Angert, 2017; Weiner et al., 2018). They attributed this positive offset to the steady-state between the efflux of intracellular P at isotopic equilibrium and the P uptake by microbes inducing isotopic enrichment of the extracellular phosphate ions (Blake et al., 2005; Lis et al., 2019). Although this effect could explain our finding, this explanation appears unlikely. Firstly, in our system P\textsubscript{res} increased during the incubation period, which does not fit into a Rayleigh fractionation model, which describes the kinetic fractionation induced by P uptake (Blake et al., 2005). Secondly, the mentioned studies observed the effect of P uptake by cells mostly under low environmental P conditions, which is not the case in the BBR soil.

It can also not be ruled out that a possible contribution from an unknown extracellular process occurred, leading to the exchange of four O atoms between phosphate and water, and/or the partial contribution of mineralization of Po compounds with heavy $\delta^{18}$O-P. $\delta^{18}$O-P and Microbial Activity

Saaby Johansen et al. (1991) and Melby et al. (2013) showed a positive relationship between $\delta^{18}$O-P de-labeling and respiration in incubation experiments with addition of $^{18}$O-labeled phosphate. Siegenthaler et al. (2020) suggested that a high incorporation of O from labeled water into the P\textsubscript{mic} pool reflects a high microbial activity.

In this study, we observed a higher respiration rate, therefore a higher microbial activity, in the low P LUE soil when compared to the high P BBR soil. Similarly, the microbial respiration per unit microbial biomass (metabolic quotient, cCO\textsubscript{2}) of the LUE soil was twice the value recorded for the BBR soil (Table 1). In contrast, the O incorporation into the P\textsubscript{res} per unit CO\textsubscript{2} respired was lower in LUE soil (Figure 4A).

The dominance of phosphomonoesterase catalyzed reactions in the P\textsubscript{res} pool of the LUE soil (see section “Biological Processes Affecting Available P in the Low P Availability LUE Soil”)

### TABLE 2 | Concentration and $\delta^{18}$O-P values of inorganic and organic P pools over the incubation period.

| Treatment | Days | $\delta^{18}$O-P\textsubscript{NaOH} | $\delta^{18}$O-P\textsubscript{NaOH} | $\delta^{18}$O-P\textsubscript{HCl} | P\textsubscript{NaOH} | P\textsubscript{NaOH} | P\textsubscript{HCl} |
|-----------|------|----------------|----------------|----------------|----------------|----------------|----------------|
|           | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n | Mean | SD | n |
| LUE       |      |    |   |      |    |   |      |    |   |      |    |   |      |    |   |
| NL        | 0    | nm | nm | –    | 22.47 nm | – | 22.47 | 0.12 | 2 | 95    | 3  | 3 | 168  | 37  | 3 |
| L         | 4    | nm | nm | –    | 19.30 nm | 0.29 | 19.30 | 2 | 218 | 2    | 105 | 3 | 3 | 176  | 17  | 3 |
| NL        | 93   | nm | nm | –    | 22.68 nm | 0.51 | 22.68 | 2 | 106 | 2    | 106 | 3 | 3 | 150  | 32  | 3 |
| L         | 93   | nm | nm | –    | 22.55 nm | 0.24 | 22.55 | 2 | 105 | 4    | 105 | 3 | 3 | 206  | 45  | 3 |
| BBR       |      |    |   |      |    |   |      |    |   |      |    |   |      |    |   |
| NL        | 0    | 16.52 | 0.85 | 2   | 17.22 | 0.81 | 17.22 | 2 | 658 | 9    | 3  | 1320 | 68  | 3 |
| L         | 4    | 18.65 | 0.80 | 2   | 16.90 | 0.29 | 16.90 | 2 | 621 | 39   | 3  | 1426 | 118 | 3 |
| NL        | 93   | 21.12 | 0.63 | 2   | 18.34 | 0.38 | 18.34 | 2 | 720 | 50   | 3  | 203  | 7   | 3 |
| L         | 93   | 22.38 | 0.63 | 2   | 18.55 | 0.37 | 18.55 | 2 | 699 | 13   | 3  | 206  | 45  | 3 |

$P_{NaOH}$, NaOH–EDTA-extractable inorganic P; $P_{NaOH}$, NaOH–EDTA-extractable organic P; $P_{HCl}$, HCl-extractable inorganic P; L, leaf litter-amended; NL, non-amended. Significance levels according to two-way ANOVA, where not differently specified: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, non-significant; nm, not measurable. (1) Significance value from a 1-way ANOVA.
explains the lower O incorporation. This together with the higher cCO2 might indicate differences in the microbial communities composition and/or a stronger investment of the LUE microbial community into Po mining via the production of extracellular phosphatase enzymes (Manzioni et al., 2010; Spohn and Chodak, 2015). The community composition analyses of Mészáros et al. (2020) and the δ18O-P data of the present study suggest that both factors possibly contribute simultaneously.

The O incorporation into the Pmic pool was similar in the two soils during the first month of incubation, then lower in the BBR soil, which showed a strong decrease in the non-amended treatment (Figures 3B, 4B).

We assumed earlier (section “Biological Processes Affecting Available P in the High P Availability BBR Soil”) that in the BBR soil only part of the microbial community was actively turning over P and imprinting an isotopic equilibrium signature on it. The below-equilibrium δ18O-Pmic and corresponding O incorporation resulted, therefore, from the relative contribution of δ18O-Pmic at the isotopic equilibrium and δ18O-Pmic below isotopic equilibrium. Lower δ18O-Pmic would either occur because of the dominance of intracellular phosphomonoesterase reactions or because of little exchange of O between intracellular phosphate and labeled water (Chen et al., 2019). Since the δ18O-Pmic was lower than isotopic equilibrium already before the labeling, the first explanation seems more likely. The two typologies outlined in Figures 3B,C would, therefore, contribute to the δ18O-Pmic in the BBR soil.

The decrease in the O incorporation in the δ18O-Pmic of the non-amended BBR soil by the end of the incubation was concomitant with a strong decrease in Pmic concentration (Supplementary Table 2 SI). These effects could be determined by a decrease in the activity of microbial cells or a shift in the microbial community composition in response to less favorable environmental conditions (Fanin et al., 2013; Mooshammer et al., 2014). The respiration rates were very similar between treatments over the last 2 months of incubation (Figure 1). However, the analysis of microbial community composition showed a shift in both the fungal and bacterial communities. Additionally, the bacterial community changed differently in the amended and non-amended treatments over the last incubation period (Mészáros et al., 2020). The second explanation seems, therefore, more likely.

In LUE the incorporation of O from labeled water into Pmic was driven by phosphomonoesterase catalyzed reactions as for Pres (see section “Biological Processes Affecting Available P in the Low P Availability LUE Soil”). Pfahler et al. (2013) reported δ18O-P of soybean leaves decreased under P-limiting conditions and suggested this was the result of increased recycling of Po within plant tissue. Therefore, P-limiting conditions might induce a tighter intracellular Po recycling and translate in below-equilibrium δ18O-Pmic values (Figure 5A). Interestingly, we found below-equilibrium δ18O-Pmic also in non-incubated samples taken at the site LUE at two different dates (unpublished results), which suggests this effect is not an artifact of the incubation.

In summary, despite similar below-equilibrium δ18O-Pmic in LUE and BBR soils, data such as δ18O-Pres, respiration rates and microbial communities composition, suggest a tighter P cycling in LUE. Interestingly, such differences were not reflected in different potential acid phosphatases activities (Table 1 and Supplementary Figure 1 in SI). Our data also suggest that the relationship between microbial activity and O incorporation into phosphate is not simply proportional as we initially hypothesized but likely modulated by P availability.

### Exchanges Between Available P and Other Inorganic and Organic P Pools

Due to the low extractable P concentration in LUE, we can only discuss results from the BBR soil.

In the parallel 33P experiment the Pres and the PiNaOH were shown to exchange rapidly in the BBR soil, with the tracer found in the PiNaOH pool after 4 days. This suggests a fast exchange process, such as sorption/desorption. However, 3-months of incubation were not sufficient to attain a complete exchange between these two pools (Pistocchi et al., 2018).

As these exchanges are abiotic, i.e., without P–O bond cleavage, it is possible to trace P fluxes also with the δ31P-P, provided that the two exchanging pools have different initial isotopic signatures.

### TABLE 3 | Estimation of P exchange fluxes in the Bad Brückenau (BBR) soil between the available and other inorganic P pools over 93 days according to a mass balance (δ18O-P data from this study) or to an isotopic dilution approach (33P data from Pistocchi et al., 2018).

| P pool | Treatment | δ18O-P Mass Balance | 33P Isotopic Dilution |
|--------|-----------|----------------------|-----------------------|
|        |           | Proportion of the P pool exchanged (1) | mg kg⁻¹ day⁻¹ P exchanged | Proportion of the P pool exchanged (2) | mg kg⁻¹ day⁻¹ P exchanged |
| PiNaOH | NL        | 0.47                 | 3.5                   | 0.47                      | 3.5 |
|        | L         | 0.53                 | 3.9                   | 0.54                      | 4.0 |
| PiHCl  | NL        | 0.51                 | 0.5                   | 0.29                      | 0.8 |
|        | L         | 0.22                 | 0.3                   | 0.51                      | 0.9 |

L, leaf litter-amended; NL, non-amended. (1) proportion of the P pool exchanged = (δ18O-P (target pool)₀ – δ18O-P (target pool)ₜ)/ (δ18O-P(pres)₀ – δ18O-P (target pool)₀), where 0 and t represent the day 0 and a time t (93 days), respectively, and δ18O-P(pres)₀ is the oxygen isotopes ratio of the available P at time t. (2) According to the equation of Fardeau et al. (1995) qₜ = Q (rₜ/R), where qₜ is the proportion of P exchanged in the target pool at time t, r is the radioactive in the target pool, R is the total radioactivity introduced, and Q is the mass of the labeled pool (in this case the available P).
The increase of the $\delta^{18}$O-P$_{\text{NaOH}}$ suggests that phosphate ions were transferred from an enriched P pool, presumably P$_{\text{res}}$. As was found with the $^{33}$P, the P$_{\text{NaOH}}$ and the P$_{\text{res}}$ did not attain similar isotopic compositions by the end of the incubation. On the contrary, their isotopic signature diverged, due to a large increase of the $\delta^{18}$O-P$_{\text{res}}$ (see Table 3 and Supplementary Table 2 SI). It would appear that over the time scale of this incubation study, the biological processes that led to the isotopic enrichment of the P$_{\text{res}}$ had a stronger effect than the ions exchange with the P$_{\text{NaOH}}$ pool.

A mass balance, assuming constant P concentrations over the incubation, yields an exchange flux between P$_{\text{res}}$ and P$_{\text{NaOH}}$ of approximately 3.5–3.9 mg kg$^{-1}$ day$^{-1}$, which is similar to estimates provided by the $^{33}$P tracing experiment (Table 3).

The $\delta^{18}$O-P$_{\text{HCl}}$ also increased over time, suggesting an exchange with an isotopically enriched P pool. Similarly, we assume that the P$_{\text{HCl}}$ exchanged phosphate ions with the P$_{\text{res}}$ pool via abiotic reactions, allowing us to trace P fluxes via the $\delta^{18}$O-P. Through a mass balance calculation, we estimated 0.3–0.5 mg kg$^{-1}$ day$^{-1}$ of P was exchanged on average between these two P pools (Table 3).

The addition of leaf litter resulted in a slightly lower $\delta^{18}$O-P$_{\text{HCl}}$ at the end of the incubation (Table 2). To explain this, we have to assume that an abiotic transfer of depleted phosphate from the litter to the P$_{\text{HCl}}$ has occurred. Although the inorganic P in the litter had a slightly depleted $\delta^{18}$O-P (17.06‰ for the TCA extract, see Table 1), the amount of inorganic P contained within the litter was small compared to the P$_{\text{HCl}}$ pool. Furthermore, no other differences in other inorganic P pools of the leaf litter-amended soils were observed. Therefore, we cannot exclude the lower $\delta^{18}$O-P$_{\text{HCl}}$ was not simply a processing error.

In summary, the P$_{\text{res}}$ exchanged mostly with the P$_{\text{NaOH}}$ pool, which is in agreement with previous studies on BBR (Lang et al., 2017; Pistocchi et al., 2018) or on low-pH and Fe/Al oxides-rich soils (Buehler et al., 2002; Helfenstein et al., 2020).

The $\delta^{18}$O-P$_{\text{NaOH}}$ increased slightly in the BBR soil (+ 1.1–1.3‰ in the NL and L treatment, respectively). The effect of leaf litter on the $\delta^{18}$O-P$_{\text{NaOH}}$ was minor, as a difference between the treatments was only observed at day 4, and this subsequently leveled out. The increase in microbial activity observed in the first few weeks after the addition of the litter could have determined a faster turnover of Po derived from microbes. If the microbial Po was isotopically enriched as a consequence of the incorporation of O from labeled water, it would enrich the $\delta^{18}$O-P$_{\text{NaOH}}$ once released from cells. Unfortunately, the $\delta^{18}$O-P of microbial Po is unknown, which prevents the calculation of its contribution to the P$_{\text{NaOH}}$ pool. Measuring the isotopic signature of microbial Po or single microbial metabolites constitutes a major research gap that needs to be investigated in order to better clarify Po dynamics (Tamburini et al., 2018).

CONCLUSION

Until now the isotopic equilibrium between phosphate and water was believed to be the main biological effect on microbial and available P. Here, we show that a below-equilibrium signature can still be an indicator of control of microbial P on the available P via Po hydrolysis reactions. This effect is possibly induced by P limiting conditions for microbes.

Labeling with $^{18}$O-water allowed the identification of the major biological process contributing to available P where radioisotope tracers fail because of low sensitivity, i.e., high baseline of isotopic dilution, as in the case of P-rich soils. Two independent isotopic approaches ($^{33}$P and $\delta^{18}$O-P) provided very similar estimates of P exchanged between the P$_{\text{res}}$ and P$_{\text{NaOH}}$ pools. This suggests that $\delta^{18}$O-P can be successfully used to trace P fluxes, provided that the underlying processes do not break the P – O bonds of the phosphate molecule.

DATA AVAILABILITY STATEMENT

The dataset on fungal and bacterial community composition discussed in this study can be found in the Portail Data INRAE VI repository (https://doi.org/10.15454/XOFCHY).

AUTHOR CONTRIBUTIONS

CP wrote the manuscript with the inputs from all the co-authors. CP and EM conducted the experiments and performed the analyses. EF, FT, EB, and CP contributed to the experiment design. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Swiss National Science Foundation (SNF project 200021E-149130). The ETH Library funded the publication fee.

ACKNOWLEDGMENTS

We want to acknowledge Friedericke Lang and Jaane Krueger for the project coordination, their support in sampling organization, and the many information they provided. We are also very grateful to the two reviewers for providing valuable and detailed comments to the manuscript. We also want to thank Laurie Schönholzer for her support in the laboratory analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.564778/full#supplementary-material
REFERENCES

Achat, D. L., Bakker, M. R., Augusto, L., Saur, E., Dousserlon, L., Morel, C., et al. (2009). Evaluation of the phosphorus status of P-deficient podzols in temperate pine stands: combining isotopic dilution and extraction methods. *Biogeochemistry* 92, 183–200. doi: 10.1007/s10533-008-9283-7

Alef, K. (1995). “Soil respiration,” in *Soil Microbiology and Biochemistry*, eds K. Alef, and P. Nannipieri (San Diego, CA: Aelf K. and Nannipieri P).

Anderson, G. M. (1976). Error propagation by the Monte Carlo method in chemical geochemistry. *Soil Biol. Biochem.* 8, 425–432.

Alef, K. (1995). “Soil respiration,” in *Soil Microbiology and Biochemistry*, eds K. Alef, and P. Nannipieri (San Diego, CA: Aelf K. and Nannipieri P).

Becquer, A., Trap, J., Irshad, U., Ali, M. A., and Claude, P. (2014). From soil to plant, the journey of P through trophic relationships and ectomycorrhizal association. *Front. Plant Sci.* 5:548. doi: 10.3389/fpls.2014.00548

Blagodatskaya, E., and Kuzyakov, Y. (2013). Active microorganisms in soil: critical review of estimation criteria and approaches. *Soil Biol. Biochem.* 67, 192–211. doi: 10.1016/j.soilbio.2013.08.024

Blake, R. E., O’Neil, J. R., and Surkov, A. V. (2005). Biogeochemical cycling of phosphorus: insights from oxygen isotope effects of phosphoenzymes. *Am. J. Sci.* 305, 596–620. doi: 10.2475/ajs.305.6-8.596

Brandtberg, P.-O., Bengtsson, J., and Lundkvist, H. (2004). Distributions of the critical step of the DNA synthesis reaction. *Nucleic Acids Res.* 46, 5875–5885. doi: 10.1093/nar/gkq402

Koutou, K., Tuchiya, Y., and Ando, T. (1993). Measurement of soil microbial biomass phosphorus by an anion exchange membrane method. *Soil Biochem.* 27, 1353–1357. doi: 10.1016/0038-0717(93)00057-1

Lang, F., Krüger, J., Amelung, W., Willbold, S., Frossard, E., Bünemann, E. K., et al. (2017). Soil phosphorus supply controls P nutrition strategies of beech forest ecosystems in Central Europe. *Biogeochemistry* 136, 5–29. doi: 10.1007/s10533-017-0375-0

Liang, Y., and Blake, R. E. (2006a). Oxygen isotope composition of phosphate in organic compounds: isotope effects of extraction methods. *Organic Geochem.* 37, 1263–1277. doi: 10.1016/j.orggeochem.2006.03.009

Liang, Y., and Blake, R. E. (2006b). Oxygen isotope signature of P regeneration from organic compounds by phosphomonoesterases and photooxidation. *Geochim. Cosmochim. Acta* 70, 3957–3969. doi: 10.1016/j.gca.2006.04.036

Lis, H., Weiner, T., Pitt, F. D., Keren, N., and Angert, A. (2019). Phosphate Uptake by Cyanobacteria Is Associated with Kinetic Fractionation of Phosphate Oxygen Isotopes. *ACS Earth Space Chem.* 3, 233–239. doi: 10.1021/acsearthspacechem.8b00099

Longinelli, A., Tamburini, F., and Nuti, S. (2020). Revised phosphate-water isotopic temperature scale. *Earth Planet. Sci. Lett.* 19, 373–376. doi: 10.1016/j.epsl.2019.07.037

Mészáros, É, Pistocchi, C., Frossard, E., Buenemann, E. K., and Tamburini, F. (2011). “The use of tracers to investigate phosphate cycling in soil–plant systems,” in *Phosphorus in Action*. (Berlin: Bunemann E. K.).

Pistocchi et al. (2020). Microbial Activity Impact on δ18O-P of Phosphate.
Pistocchi, M., Mészáros, É, Tamburini, F., Frossard, E., and Bünemann, E. K. (2018). Microbial processes dominate phosphorus dynamics under low phosphorus availability in organic horizons of temperate forest soils. Soil Biol. Biochem. 126, 64–75.

Pistocchi, C., Tamburini, F., Grau, G., Ferhi, A., Trevisan, D., Dorioz, J. M., et al. (2017). Tracing the sources and cycling of phosphorus in river sediments using oxygen isotopes: methodological adaptations and first results from a case study in France. Water Res. 111, 346–356.

Pistocchi, C., Tamburini, F., Savoye, L., Sebilo, M., Baneschi, L., Lacroix, D., et al. (2014). Développement d'une méthode d'extraction et purification des phosphates à partir de matières sédimentaires pour l'analyse isotopique de leur oxygène. Le cahier des techniques de l'INRA 81, 1–23.

Poll, C., Ingwersen, J., Stemmer, M., Gerzabek, H., and Kandeler, E. (2006). Mechanisms of solute transport affect small-scale abundance and function of soil microorganisms in the detritusphere. Eur. J. Soil Sci. 57, 583–595. doi:10.1111/j.1365-2389.2006.00835.x

Saaby Johansen, H., Middelboe, V., and Larsen, S. (1991). Delabeling of 18O Enriched Phosphate Added to Soil as a Function of Biological Activity in the Soil. Vienna: IAEA.

Seth, B., Schneider, C., and Storck, F. (2006). Improved reliability of oxygen isotopic analysis of water using the Finnigan GasBench II periphery of a continuous flow isotope ratio mass spectrometer by backflushing of the sampling line. Rapid Commun. Mass Spectrom. 20, 1049–1051. doi:10.10111/j.1365-2389.2006.00835.x

Siebers, N., Bauke, S. L., Tamburini, F., and Amelung, W. (2018). Short-term impacts of forest clear-cut on P accessibility in soil microaggregates: an oxygen isotope study. Geoderma 315, 59–64. doi:10.1016/j.geoderma.2017.11.024

Siegenthaler, M. B., Tamburini, F., Frossard, E., Chadwick, O. A., Vitousek, P., Chiara, P., et al. (2020). A dual isotopic (32P and 18O) incubation study to disentangle mechanisms controlling phosphorus cycling in soils from a climatic gradient (Kohala, Hawaii). Soil Biol. Biochem. 149:107920. doi:10.1016/j.soilbio.2020.107920

Spohn, M., and Chodak, M. (2015). Microbial respiration per unit biomass increases with carbon-to-nutrient ratios in forest soils. Soil Biol. Biochem. 81, 128–133. doi:10.1016/j.soilbio.2014.11.008

Spohn, M., Ermak, A., and Kuziyakov, Y. (2013). Microbial gross organic phosphorus mineralization can be stimulated by root exudates – A 33P isotopic dilution study. Soil Biol. Biochem. 65, 254–263. doi: 10.1016/j.soilbio.2013.05.028

Stout, L. M., Joshi, S. R., Kana, T. M., and Jaisi, D. P. (2014). Microbial activities and phosphorus cycling: an application of oxygen isotope ratios in phosphate. Geochim. Cosmochim. Acta 138, 101–116. doi:10.1016/j.gca.2014.04.020

Tamburini, F., Bernasconi, S. M., Angert, A., and Frossard, E. (2010). A method for the analysis of the 818O of inorganic phosphate extracted from soils with HCl. Eur. J. Soil Sci. 61, 1025–1032. doi:10.1111/j.1365-2389.2010.01290.x

Tamburini, F., Pfahler, V., Bünnemann, E. K., Guelland, K., Bernasconi, S. M., Frossard, E., et al. (2012). Oxygen isotopes unravel the role of microorganisms in phosphate cycling in soils. Environ. Sci. Technol. 46, 5956–5962. doi: 10.1021/es300311h

Tamburini, F., Pistocchi, C., Helfenstein, J., and Frossard, E. (2018). A method to analyse the isotopic composition of oxygen associated with organic phosphorus in soil and plant material. Eur. J. Soil Sci. 69, 816–826. doi:10.1111/ejs.12693

Tjissen, H., and Moir, J. O. (1993). “Characterization of Available P by Sequential Extraction,” in Soil Sampling and Methods of Analysis, eds M. R. Carter, and E. G. Gregorich (Ann Arbor, MI: CARTER M.R.

Tudge, A. P. (1960). A method of analysis of oxygen isotopes in orthophosphate—its use in the measurement of paleotemperatures. Geochim. Cosmochim. Acta 18, 81–93. doi:10.1016/0016-7037(60)90019-3

von Sperber, C., Kries, H., Tamburini, F., Bernasconi, S. M., and Frossard, E. (2014). The effect of phosphomonoesterases on the oxygen isotope composition of phosphate. Geochim. Cosmochim. Acta 125, 519–527. doi:10.1016/j.gca.2013.10.010

von Sperber, C., Tamburini, F., Brunner, B., Bernasconi, S. M., and Frossard, E. (2015). The oxygen isotope composition of phosphate released from phytic acid by the activity of wheat and Aspergillus niger phytase. Biogeosciences 12, 4175–4184. doi:10.5194/bg-12-4175-2015

Weiner, T., Gross, A., Moreno, G., Migliavacca, M., Schrumpf, M., Reichstein, M., et al. (2018). Following the turnover of soil bioavailable phosphate in mediterranean savanna by oxygen stable isotopes. J. Geophys. Res. 123, 1850–1862. doi:10.1029/2017JG004086

Werner, F., Mueller, C. W., Thieme, J., Gianoncelli, A., Rivard, C., Höschen, C., et al. (2017). Micro-scale heterogeneity of soil phosphorus depends on soil substrate and depth. Sci. Rep. 7:23003. doi:10.1038/s41598-017-03537-8

Zohar, I., Shaviv, A., Young, M., Kendall, C., Silva, S., Paytan, A., et al. (2010). Phosphorus dynamics in soils irrigated with reclaimed waste water or fresh water — A study using oxygen isotopic composition of phosphate. Geoderma 159, 109–121. doi:10.1016/j.geoderma.2010.07.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Pistocchi, Mészáros, Frossard, Bünnemann and Tamburini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.