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The $\delta^{18}O$ signatures of HCl-extractable soil phosphates: methodological challenges and evidence of the cycling of biological P in arable soil

W. Amelung[a,b], P. Antar[a], I. Kleeberg[a], Y. Oelmann[c], A. Lücke[b], F. Alt[c], H. Lewandowski[b], S. Pätzold[a] & J. A. M. Barej[a]

[a]*Institute of Crop Science and Resource Conservation (INRES) – Soil Science and Soil Ecology, University of Bonn, Nussallee 13, 53115 Bonn, Germany,* [b]*Forschungszentrum Jülich GmbH, Institute of Bio- and Geoscience, Agrosphere Institute (IBG-3), 52425 Jülich, Germany, and* [c]*Geoecology, University of Tübingen, Rümelinstr. 19-23, 72070 Tübingen, Germany*

Summary

Soil phosphates exchange oxygen atoms rapidly with soil water once recycled by intracellular enzymes, thereby approaching an equilibrium $\delta^{18}O_P$ signature that depends on ambient temperature and the $\delta^{18}O_W$ signature of soil water. We hypothesized that in the topsoil, phosphates reach this equilibrium $\delta^{18}O_P$ signature even if amended by different fertilizers. In the subsoil, however, there might be phosphates with a smaller $\delta^{18}O_P$ value than that represented by the isotopic equilibrium value, a condition that could exist in the case of limited biological P cycling only. We tested these hypotheses for the HCl-extractable P pool of the Hedley fractionation scheme of arable soil in Germany, which integrates over extended time-scales of the soil P cycle. We sampled several types of fertilizer, the surface soil that received these fertilizer types and composites from a Haplic Luvisol depth profile under long-term agricultural practice. Organic fertilizers had significantly smaller $\delta^{18}O_P$ values than mineral fertilizers. Intriguingly, the fields fertilized organically also tended to have smaller $\delta^{18}O_P$ signatures than other types of surface soil, which calls into question full isotopic equilibrium at all sites. At depths below 50 cm, the soil $\delta^{18}O_P$ values were even depleted relative to the values calculated for isotopic equilibrium. This implies that HCl-extractable phosphates in different soil horizons are of different origins. In addition, it supports the assumption that biological cycling of P by intracellular microbial enzymes might have been relatively inefficient in the deeper subsoil. At depths of 50–80 cm, there was a transition zone of declining $\delta^{18}O_P$ values, which might be regarded as the first evidence that the degree of biological P cycling changed at this depth interval.

Introduction

Phosphorus (P) is essential for all living organisms, and it is also one of the most important constituents of agricultural fertilizers. As reserves of rock phosphate are becoming scarce (Cordell *et al.*, 2009), it is important that fertilizer P added to soil is used efficiently by plants and microorganisms. However, this efficiency of P use is difficult to measure. Radioactively labelled P fertilizers may be used for tracing P uptake into plants under controlled laboratory conditions, but their use in the field is restricted by safety considerations. Furthermore, radioactively labelled P cannot be used for tracing P uptake from the subsoil, because the addition of fertilizer to subsoil would not reflect realistic field conditions.

Analysis of the isotope ratio of oxygen-18 (^{18}O) to oxygen-16 (^{16}O) in phosphate ($\delta^{18}O_P$) has emerged as a promising alternative for tracing the biological use of P in the environment. When biological cycling of phosphate is mediated by intracellular enzymes, there is a rapid exchange of oxygen atoms in the phosphate molecule with surrounding water; a process that is completed within hours (Paytan *et al.*, 2002; Blake *et al.*, 2005) to days (Melby *et al.*, 2013a). As a consequence, Middleboe & Saaby (1998) proposed use of the change in $\delta^{18}O$ signature as an indication of microbial activity in soil. The intracellular microbial turnover of phosphate is controlled mainly by pyrophosphatases and leads finally to an isotopic equilibrium with ambient water, which depends on the site temperature and isotopic signature of oxygen of the surrounding water (Longinelli & Nuti, 1973; Blake *et al.*, 2005; Colman *et al.*, 2005). In contrast to the intracellular P cycle, extracellular hydrolysis and turnover of soil P result in kinetic isotope fractionation processes with a fractionation factor of specific enzymes that ranges from +20 to −30‰ (Liang & Blake, 2006, 2009; Tamburini *et al.*, 2014). As long as the enzymatic

Correspondence: W. Amelung. E-mail: wulf.amelung@uni-bonn.de

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pattern involved in extracellular cycling of soil P remains unclear, these processes cannot be identified clearly in soil, but they might result in deviations from isotope equilibrium. Therefore, only if intracellular enzymes convert and recycle fertilizer P efficiently can it be taken for granted that the soil $\delta^{18}O_P$ values resemble these equilibrium $\delta^{18}O_P$ values. In such a case, different isotopic signatures of fertilizer source materials would not be maintained. In abiotic environments, oxygen isotope exchange in phosphates is negligibly small (Tudge, 1960; Blake *et al.*, 1997).

The equilibrium $\delta^{18}O$ values for phosphates of terrestrial systems usually range between 10 and 20‰ (Zohar *et al.*, 2010; Angert *et al.*, 2012; Tamburini *et al.*, 2012, 2014; Burmann *et al.*, 2013). Even smaller $\delta^{18}O_P$ signatures have been reported for magmatic bedrock (e.g. 8.1–9.7‰, reported by Matsuhisa *et al.*, 1973, and −1.2 to +7.5‰, Harris & Ashwal, 2002). Little is known regarding background $\delta^{18}O_P$ values in different fertilizer types or how these values might compare with those of soil at different depths. Nevertheless, it can be argued that a lack of biological recycling of mineral-bound phosphates in soil should be represented by P pools that more or less retain their original isotopic signature; that is, they do not readily approach the isotopic equilibrium with soil water. On the other hand, plant uptake of soil phosphates usually results in strong isotope enrichment because of equilibrium reactions with heavier leaf water (Pfahler *et al.*, 2013). Therefore, when this P is returned to soil with plant debris, one might observe temporarily elevated $\delta^{18}O_P$ values in the surface soil until microbial uptake and intracellular P recycling re-equilibrate the isotope signature with that of soil water.

The current work is a preliminary study to elucidate whether surface soils with different P fertilizer applications have similar $\delta^{18}O_P$ signatures, as assumed from isotopic equilibrium. We hypothesized that the $\delta^{18}O_P$ signature of the surface soil might be larger than that of the subsoil, as would be expected if the latter shows only limited cycling of soil P through microbial cells. First, we assessed the $\delta^{18}O_P$ signature for the HCl-extractable P pool of the Hedley fractionation scheme. This fraction is usually free from organic P and is not readily available to plants and microorganisms; in other words, changes in its pool size usually occur over the longer time-scales of soil P dynamics than those of more readily available P fractions (Negassa & Leinweber, 2009). We then compared the soil $\delta^{18}O_P$ values of the HCl-extractable P pool with those calculated for isotopic equilibrium. Samples were taken from Ap horizons of fields that had been fertilized differently and from different diagnostic subsoil horizons at the Klein-Altendorf experiment station, Germany. The methodology for $\delta^{18}O$ phosphate isolation was controlled by recovery experiments, and the final purity of the $\delta^{18}O_P$ isolates was controlled with Raman spectroscopy.

Materials and methods

Commercially available fertilizers served as controls for the field sites where they had been applied in the study region. The mineral fertilizers examined included triple super phosphate, super phosphate and Thomas slag, and the organic variants consisted of samples of pig slurry and dairy cattle manure.

In November 2011, we took six topsoil samples (0–30 cm) from each site with a soil auger and mixed them to form a composite sample. The sites had different histories of P fertilizer applications as follows:

1. > 30 years of mineral fertilizer application (from 1996 to 2005, Thomas slag only; since 2006, triple superphosphate – Campus Klein-Altendorf, Germany);
2. mineral P fertilizer application from 1942 (from 1942 to about 1990, Thomas slag; from then on, mainly triple super phosphate – long-term field trial at Dikopshof, Germany);
3. organic fertilizer application with cattle farmyard manure since 1991 (research station at Wiesengut, Germany);
4. organic fertilizer application since 1904 (long-term field trial at Dikopshof, Germany);
5. about 20 years without fertilizer application (wildlife food plot, near Klein-Altendorf, Germany); and
6. no P fertilizer application since 1942 (long-term field trial at Dikopshof, Germany).

All sites are near Bonn, Germany. The soil has developed from Weichselian loess, except for the site at Wiesengut where the soil has formed as part of a floodplain in sediments of the river Sieg. The soil texture at all sites ranges from silty to loamy.

We also sampled the diagnostic soil horizons in two profiles at Campus Klein-Altendorf from different sides of the profile down to the C horizon. This site was similar to site (1), but P fertilizer has not been applied there for the past 5 years. The site has been used for a crop sequence trial, and in the year of sampling the crop was winter wheat (*Triticum aestivum* L.) preceded by 2 years of chicory cultivation (*Cichorium intybus* L.). One profile was analysed completely; for the other we obtained reliable data only for the surface soil and soil at the lowest depth.

The soil near Klein-Altendorf was in the Lower Rhine Basin and is classified as a Haplic Luvisol (IUSS Working Group WRB, 2006). The soil at the site Wiesengut is classified as a Haplic Fluvisol. The texture of the soil at all sites was silt loam at the surface, and pH values were around 7 in the Luvisol and 5.5 in the Fluvisol. The Luvisol profile showed no geological layering.

All soil samples were dried at 40°C and sieved with a 2-mm mesh prior to analysis. The fertilizers were extracted directly with 1 M HCl, according to the respective extraction step in Hedley's fractionation scheme. For soil samples, sequential P extraction was carried out according to Hedley *et al.* (1982) and Tiessen & Moir (2008). The P concentration in aqueous solutions was measured by the molybdenum blue method of Murphy & Riley (1962).

For the isolation of phosphates we followed the method protocols of Tamburini *et al.* (2010) and Jaisi & Blake (2010). We combined both methods because that of Tamburini *et al.* (2010) works well for soil, but the recovery of P has not yet been reported, whereas the method of Jaisi & Blake (2010) was developed originally for sediments and has not yet been optimized for soil. Furthermore, some modifications were necessary to obtain pure Ag_3PO_4 crystals. For $\delta^{18}O_P$ analyses, the samples were extracted sequentially with 0.5 M $NaHCO_3$, followed by 0.1 M NaOH and 1 M HCl.

For subsequent purification steps, we used only samples extracted with 1 M HCl, which were free from organic P. The basic extracts were discarded.

The magnesium-induced co-precipitation method (MAGIC; Karl & Tien, 1992) was used to increase the P concentration by sample volume reduction. However, the changes in pH involved in this treatment led to oxidation and precipitation of previously dissolved iron, and these particles made it impossible to do the subsequent treatments. Therefore, we filtered the affected samples with activated charcoal filters; however, these filters contained detectable amounts of P. We eliminated this contamination successfully by washing each filter with 250 ml of 1 M HCl. Thereafter, the phosphates were isolated by precipitation first as ammonium phosphomolybdate (APM) and later as magnesium ammonium phosphate (MAP) (Jaisi & Blake, 2010; Tamburini *et al.*, 2010). Excess nitrate in the silver-phosphate salts was removed successfully by wet filtration.

At each stage in the process, we determined the recovery of phosphate in the extract as an analytical control (see Table 1); samples with little recovery of P were discarded. The final purity of the Ag_3PO_4 precipitates was controlled with Raman spectroscopy. The Raman spectra were obtained by a RFS 100/S FT-Raman spectrometer (Bruker, Billerica, USA) with a liquid nitrogen-cooled germanium diode detector and ND : YAG laser, which excited the sample at 1064 nm. All samples were analysed as solid powders. The spectra were recorded at approximately 200 mW laser power in a spectral region from 200 to 4000 cm^{-1} at a spectral resolution of 4 cm^{-1} with 300 sample scans. The $\delta^{18}O$ values of silver phosphate ($\delta^{18}O_P$) were measured with a high-temperature pyrolysis furnace (HT-O, HEKAtech, Wegberg, Germany) coupled to a continuous-flow isotope ratio mass spectrometer (Isoprime, Stockport, UK) at the Forschungszentrum Jülich, Germany. The ^{18}O:^{16}O ratios were measured against standards and are reported in the conventional $\delta^{18}O$ notation against Vienna Standard Mean Ocean Water (VSMOW) as the reference.

As an additional check on the accuracy of the extraction protocol, we monitored oxygen contamination by ensuring the correct oxygen content of Ag_3PO_4 during isotope ratio measurement. The reliability of isotope recovery for the different stages in the analytical process had been established previously by Jaisi & Blake (2010). The analytical precision of the IRMS measurements was approximately 0.2 delta units; replicate analyses of the same samples indicated an average precision of 0.72 delta units for the whole procedure.

Soil water at the sites studied was from rainwater because of a lack of access to groundwater aquifers. Bowen & Revenaugh (2003) developed a mathematical model for calculating the $\delta^{18}O$ values of meteoric water for a given location. With their method of interpolation, the $\delta^{18}O_W$ of rainwater in Bonn, Germany, was estimated at -8.4 ± 0.4‰ (95% confidence interval, VMSOW). This is somewhat less than the value at the nearest station of the Global Network of Isotopes in Precipitation (GNIP); it reported an average $\delta^{18}O_W$ in the period between 1996 and 2002 of approximately −6.7‰.

We had no access to pure soil water samples from the different soil depths. However, the HCl-extractable P pool is not readily available to plants and comprises phosphates that are likely to be used beyond one single cropping season. We decided, therefore, that for our purpose, the average $\delta^{18}O_W$ value of rainwater for the year might be more suitable than that of soil water from only one individual sampling point.

We used the interpolated $\delta^{18}O_W$ values and the equation developed by Longinelli & Nuti (1973) as a reference for the equilibrium range in the given soil environment (Equation (1)), while considering the GNIP data in the discussion of uncertainty:

$$\delta^{18}O_P = \frac{111.4 - T(°C)}{4.3} + \delta^{18}O_W. \quad (1)$$

This equation was originally derived from the shells of marine organisms and has been validated repeatedly for soil environments; for example, by Tamburini *et al.* (2012). The mean soil temperature (T) was 6.8°C. With a maximum soil temperature of 11.8°C and a minimum of 3.8°C at the time of sampling, we estimated that the temperature-dependent isotopic equilibrium might range between at least 14.8 and 16.6‰, respectively. Finally, taking into consideration that the interpolated isotopic value of rainwater has an uncertainty of 1.7‰ relative to the nearest GNIP station, and adding this uncertainty to the calculation of isotopic equilibrium, we estimated that the isotopic equilibrium value ranged from 14.8 to 18.3‰ with a mean of 16.5‰.

Statistical analyses

Data from treatments with organic and mineral fertilizers were compared with Student's *t*-test. Comparisons of the $\delta^{18}O_P$ signature for the different fertilizer trials were based on the least significant differences (LSD) procedure, with $LSD = t_{0.5,f} \cdot SED$ (f = degrees of freedom, SED = standard error of differences), following the suggestion of Webster (2007).

Results

Methodological challenges

In general, the recovery of phosphate ranged between 86 and 110% (mean of all soil and fertilizer samples) at all stages of the analytical procedure, which demonstrates that the modified fractionation scheme is viable. The greatest recovery was obtained after the first use of cation exchange resin; it even exceeded 100% for the organically fertilized sites (individual data not shown). The smallest recoveries followed the MAGIC treatment ($Mg(OH)_2$ precipitation and subsequent dissolution, Table 1). The MAGIC method is time-consuming and had to be repeated up to three times to achieve the desired volume reduction. It did not lead to a loss of phosphate concentration in the fertilizer samples (mean recovery 98 ± 6%). Furthermore, the phosphate recovered during an individual MAGIC treatment was large (86% on the average), but because of the need to perform the analysis in triplicate this resulted in P losses in some soil samples (mean recovery 80 ± 20%). The

Table 1 Mean recovery (± standard deviation) of inorganic P from samples after different phases in the analytical procedure

	n	Cation exchange resin 1 / %	Activated charcoal filter 1 / %	MAGIC / %	Activated charcoal filter 2 / %	AMP / %	MAP / %	Cation exchange resin 2 / %
Fertilizers	5	104 ± 8.0	100 ± 5.1	98 ± 6.0	81 ± 4.4	99 ± 5.3	95 ± 5.3	98 ± 6.2
Soil samples	11	113 ± 15	97 ± 12	80 ± 20	106 ± 5.4	90 ± 10	99 ± 9.5	89 ± 6.4
Mean	16	110 ± 5.2	98 ± 3.8	86 ± 5.3	95 ± 1.6	93 ± 2.9	98 ± 10	92 ± 7.4

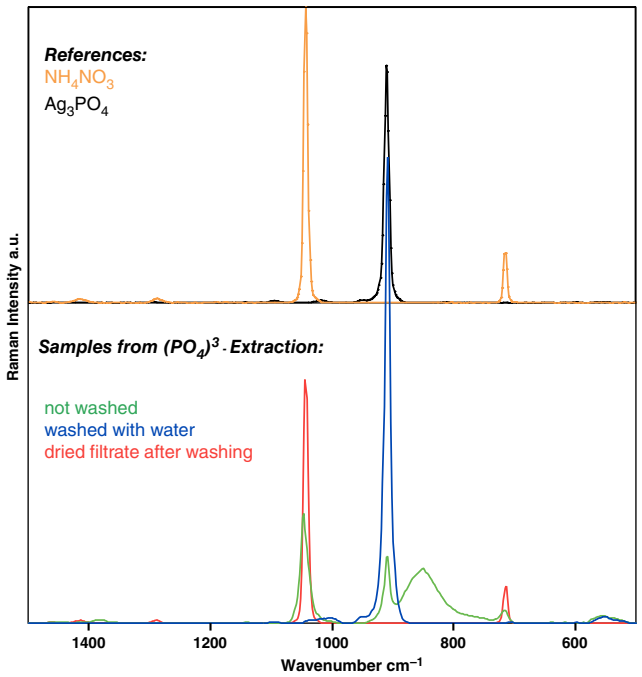

Figure 1 Raman spectroscopy to assess the purity of Ag_3PO_4 precipitates. The y-axis has arbitrary units (a.u.).

large standard deviation for soil samples suggested that recovery was complete (≫80%) for some replicates, but for others it was approximately 60% only. Individual values of the replicates showed that the $\delta^{18}O_P$ values of samples with small recovery rates differed by up to 0.34‰ only from those with large rates of recovery.

Raman spectroscopy might help to differentiate the vibrations of phosphates from those of sample impurities. In the study samples, the impurities were mainly nitrates as indicated by the similar waveband numbers of ammonium nitrates that contributed to the signals of the isolated Ag_3PO_4 (Figure 1). Dried filtrates were washed with water to remove these impurities, and finally the oxygen of the silver phosphates alone could be measured by the isotope ratio mass spectrometer.

Fertilizer and soil analyses

The $\delta^{18}O_P$ values of the three mineral fertilizers were of similar magnitude (Table 2). The organic fertilizers had smaller $\delta^{18}O_P$ values by almost eight delta units (Table 2). The smallest $\delta^{18}O_P$ values were for farmyard manure, which is in line with the range of $\delta^{18}O_P$ values reported in reviews by Young *et al.* (2009) and Tamburini *et al.* (2014) for animal faeces in general.

For the fertilizer trials, we did not observe any consistent differences in the proportion of HCl-extractable P relative to the unfertilized controls in the Ap horizon (Table 2). Overall, HCl-extractable P comprised 13–27% of total P in the topsoil, rising to 60% in subsoil horizons (Figure 2, Table 2; see also Barej *et al.*, 2014 for Hedley data of related trials). The $\delta^{18}O_P$ values of the HCl-extractable P fraction in the mineral-fertilized surface soil ranged from 18.2 to 21.3‰, which overlapped with the range of values of the non-fertilized controls ($\delta^{18}O_P = 20.3–22.8$‰, Table 2). Furthermore, the means of the mineral-fertilized soil and the controls were not significantly different from one another, and it was not possible to identify a mineral P fertilizer signal in soil after long-term fertilizer application.

The trials under organic management tended to have smaller $\delta^{18}O_P$ values (16.8–17.2‰) than the mineral-fertilized trials, and they had significantly smaller $\delta^{18}O_p$ values than the non-fertilized ones ($P < 0.05$). The difference in $\delta^{18}O_p$ reached 4.4‰ relative to that of the controls (Table 2), which is also far above the analytical precision. In addition, the organically fertilized soil did not immediately reach the same equilibrium $\delta^{18}O_P$ level as the non-fertilized soil. Therefore, these data lend support to the hypothesis that it might be possible to identify an organic P fertilizer signal in soil with $\delta^{18}O_p$ natural abundance measurements.

With increasing soil depth, there was no detectable loss of HCl-extractable P. In the subsoil, HCl-extractable P was the most abundant P pool (Figure 2). The $\delta^{18}O_P$ signature of the HCl-extractable P, however, showed a clear depth gradient. The soil near the surface showed elevated $\delta^{18}O_P$ values, larger than the means calculated with Equation (1) for the isotopic equilibrium with rainwater (16.5‰; Figure 3). As soil depth increased, the $\delta^{18}O_P$ values decreased. Below a depth of 40 cm, and at the two deepest soil horizons sampled, the δ^{18}Op value was clearly smaller than that calculated for isotopic equilibrium (Figure 3). Therefore, the oxygen of HCl-extractable soil phosphates has different origins at different soil depths.

Discussion

The methodology was adapted for soil by restricting analyses to Hedley's pool of HCl-extractable phosphates by adding an additional purification step with activated carbon and with Raman spectroscopy for purity control. For the MAGIC stage we cannot discount the possibility of isotopic fractionations that result from

Table 2 Mean $\delta^{18}O_P$ values of HCl-extractable P (‰, VSMOW) and standard deviations of mineral and organic fertilizers, and of surface soil amended with different P fertilizers

Fertilizer and soil	Mean $\delta^{18}O_P$ / ‰	SD / ‰	Difference from non-fertilized control / ‰	1 M HCl-extractable P concentration in topsoil / mg kg^{-1}	1 M HCl-extractable P (proportion of total P) / % of total P
Fertilizer					
Farmyard manure (FYM, cattle)	12.6	0.47	–	–	–
Slurry	14.2	0.37	–	–	–
Triple super phosphate	20.2	0.49	–	–	–
Superphosphate	20.5	0.77	–	–	–
Thomas slag	19.5	0.72	–	–	–
Topsoil (0–30 cm)					
No fertilizer application for > 20 years (sites 5 and 6)	21.6	1.73	0	61.4–164	17.9–26.6
Mineral fertilization for > 30 years (sites 1 and 2)	19.8	2.23	NS[a]	103.0–140	20.9–23.5
Organic fertilization for > 12 years (sites 3 and 4)	17.2	0.56	4.36[b]	87.3–107	13.3–18.9

[a]NS: not significant at $P < 0.05$.
[b]Significant at $P < 0.05$.
Estimated meteoric $\delta^{18}O_W = -8.4 \pm 0.4$‰ (VSMOW) (1 M HCl-extractable P, $n = 4$ replicate analyses).

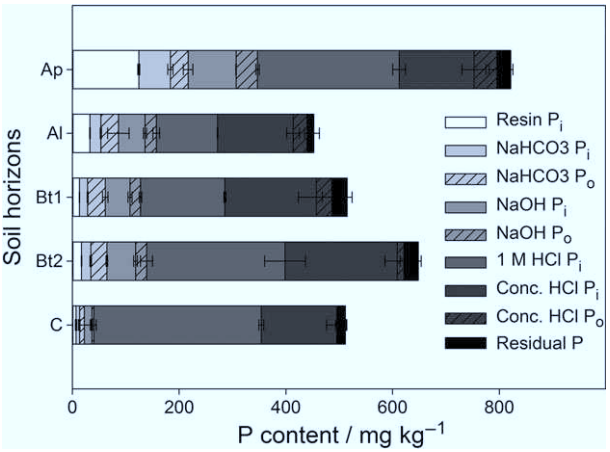

Figure 2 Phosphorus pools of the Hedley fractionation for the Haplic Luvisol depth profile at Klein-Altendorf, Germany.

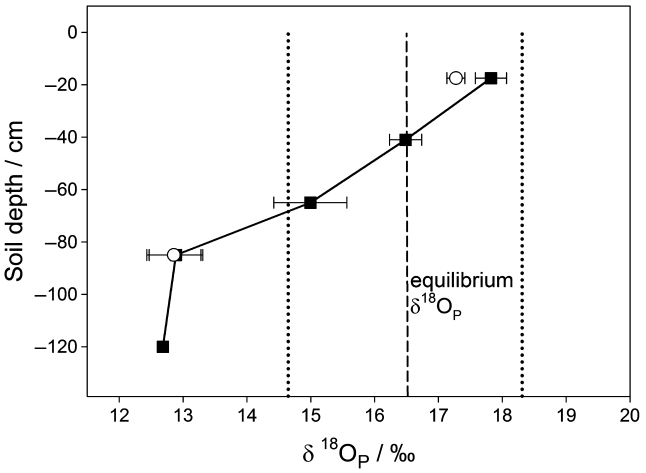

Figure 3 Depth profile of $\delta^{18}O_P$ in a Haplic Luvisol (Germany; black squares are replicate measurements for the surface soil and open circles are those for the subsoil from a neighbouring profile). Outlined sampling depths represent mean depth of the respective soil horizons. The vertical dashed line represents the $\delta^{18}O$ equilibrium in the soil; the dotted lines represent the uncertainty of this equilibrium $\delta^{18}O_p$ value according to temperature fluctuations and uncertainties in the $\delta^{18}O_W$ of rainwater. Error bars indicate standard deviations.

losses with MAGIC, but the potential error is acceptably small relative to the large overall variation of more than 10‰ in $\delta^{18}O_P$ values among samples (Table 2). Nevertheless, future methodological developments that avoid this analytical stage (as done for protocols by Tamburini *et al.*, 2010) warrant further attention.

Identification of fertilizer P with $\delta^{18}O_p$ measurements of HCl-extractable phosphates

Within the Hedley fractionation scheme, HCl-extractable P is a pool of intermediate availability to plants and microbes. However, once the HCl-extractable P had been cycled by the soil microbial community its oxygen was exchanged with the surrounding soil water. This exchange reaction might occur within hours (Blake *et al.*, 2005); therefore, fertilizer phosphates should approach a similar $\delta^{18}O_P$ value in soil when cycled efficiently by plants and microorganisms.

The similarity in the δ^{18}Op signature of mineral fertilizers and of HCl-extractable phosphates shows that we have to refute the hypothesis that using the $\delta^{18}O_P$ value of HCl-extractable P at natural isotope abundance level could be useful to trace the origin or cycling of P from mineral fertilizers.

The $\delta^{18}O_P$ value of phosphates from organic fertilizers was smaller, probably because P excreted by the animals was at least partially equilibrated with their body water. By applying equation (1) of Longinelli & Nuti (1973) to a body temperature of 37°C, we calculated smaller $\delta^{18}O_P$ values (8.9‰) when rainwater was

used as drinking water. Subsequent fractionation during storage and a larger $\delta^{18}O_W$ value in the body water of these animals (Bryant & Froelich, 1995) possibly led to the values observed and clearly differentiated the isotopic signatures of organic fertilizers from those of inorganic ones.

When added to soil, the smaller δ^{18}Op value from organic fertilizers remained in part. The differences in the soil $\delta^{18}O_p$ values between the sites indicated that the respective HCl-extractable phosphates comprise a mixture of phosphates from different origins. They stem, for example, from phosphates from dung (small $\delta^{18}O_P$, Table 2), from microbial cycles (isotopically equilibrated with soil water) and from plants (large $\delta^{18}O_P$, Pfahler *et al*., 2013). If these proportions change, the overall soil $\delta^{18}O_p$ values change. In addition, fertilizer P might not be dominant in the HCl-extractable P pool relative to other sources of P, such as inorganic P from the mineralization of organic matter that might have precipitated with Ca.

With the extrapolated $\delta^{18}O_W$ values for rainwater and uncertainties of approximately 1.7‰ because of the extrapolation procedure used for estimating the $\delta^{18}O_W$ value, we estimated that the equilibrium $\delta^{18}O_P$ value in soil ranged from 14.8 to 18.3‰ (Figure 3). The isotopic signals of the organically fertilized fields were in this range of isotopic equilibrium for the site under study. These data, in general, support the idea that a substantial portion of the original fertilizer P has been cycled by microorganisms. However, the extraction in the present study did not cover the whole pool of soil P; therefore, we cannot discount the possibility that at least some of the fertilizer P was trapped directly in more stable P pools with an isotopic signature that reflects the fertilizer origin. Therefore, we cannot prove that isotopic equilibrium has been reached completely, and there is lingering doubt that it was reached immediately because of the differences in isotopic $\delta^{18}O_P$ signatures between organically fertilized trials and the non-fertilized controls.

The $\delta^{18}O_P$ signature as an indicator of the degree of biological P cycling in the soil profile

We found large amounts of HCl-extractable P, not only in the topsoil, but particularly in the subsoil (Figure 2). Apparently, the pool of these phosphates has not yet been depleted by plant growth. It is unlikely that the elevated values of HCl-extractable P in the subsoil were caused by leaching. In general, P is immobile at near-neutral pH values (Holford & Patrick, 1979). Furthermore, phosphate fertilizers are applied to the soil surface only and are mixed subsequently within the first 30 cm of the soil by ploughing. In addition, topsoil (Ap) has the largest P concentration in the profile, whereas the horizon immediately below that has the smallest (Figure 2). These findings suggest negligible leaching of phosphates with a potentially distinct isotopic signature. Therefore, we could use these isotopic signatures as evidence of soil P cycling.

The $\delta^{18}O_P$ values nearest to the land surface were larger than those in the subsoil (Figure 3). Several processes might account for this. On the one hand, $\delta^{18}O_p$ values can be larger in the surface soil because of reactions with water that was isotopically enriched by evaporation. On the other hand, some subsoil phosphates might have had only limited isotope exchange with soil water. The following paragraphs discuss these arguments in more detail.

When calculating the equilibrium $\delta^{18}O_P$ signature, one still has to consider that evapotranspiration processes near the soil surface result in the accumulation of isotopically heavier water. The formation of such $\delta^{18}O_W$ gradients in soil has been reported by other authors; for example, Hsieh *et al.* (1998) found an inverse correlation between $\delta^{18}O_W$ values and volumetric water content at sites with different amounts of rainfall in Hawaii. The $\delta^{18}O_W$ values at the surface of soil profiles were up to 4‰ greater than in the subsoil. The $\delta^{18}O_W$ values remained constant at around a depth of 20 cm and below. Wang *et al.* (2012) observed similar discrepancies in the first 10 cm of soil profiles after artificial irrigation with approximately 110 mm of water. At the evaporation front, Rothfuss *et al.* (2010, 2012) indicated that isotopic enrichment exceeded 6‰, which resulted in even larger $\delta^{18}O_P$ values near the soil surface following ploughing of the whole Ap. We conclude that in the top 40–50 cm of the soil profile, the $\delta^{18}O_P$ signatures in the Hedley HCl fractions reflected the evaporation gradient of soil water; the soil δ^{18}Op values increased near the soil surface because of isotope equilibrium processes with heavier soil water (see also Zohar *et al*., 2010). An exact assessment of $\delta^{18}O_P$ values would require data for the $\delta^{18}O_w$ evaporation profile within the plough layer and for the whole growing season, which was beyond the scope of this study.

Further elevation of $\delta^{18}O_P$ signatures above the calculated equilibrium value can be caused by extracellular cycling of soil P, which might lead to additional isotope fractionation by RNA nucleotidases (for example, Liang & Blake, 2009; Tamburini *et al.*, 2014). However, as outlined by Tamburini *et al*. (2014), a few enzymes only have been studied in this way. Another possibility is that the elevated $\delta^{18}O_P$ signals reflect recent inputs of inorganic P from plant debris, which might be enriched in $\delta^{18}O_P$. Pfahler *et al.* (2013) reported an isotope enrichment of $\delta^{18}O_P$ in soya bean plants of 16.9 delta units (‰) and more relative to hydroponic solution, which is attributable to equilibrium isotope exchange with ^{18}O-enriched leaf water during structural P_o formation. When such plant-derived $^{18}O_P$ reaches the soil, it will raise the soil's $\delta^{18}O_P$ value. Here, we were unable to differentiate between intra- and extra-cellular cycling of soil P and to quantify the amount of $^{18}O_P$ that originated from plant debris. However, all of these processes can be considered as pathways of biological P cycling.

Melby *et al*. (2013b) stated that heavier phosphates might be bound preferably by soil minerals. If this had taken place in our study, it could reinforce the enrichment of heavier phosphates in the surface soil until they were cycled again by soil microorganisms.

As soil depth increases, parts of the recently added plant debris decrease and soil water is no longer affected by ^{18}O-enrichment by evaporation. Therefore, soil $\delta^{18}O_P$ values also decrease, as shown in Figure 3. Bioturbation might contribute to smoothing the gradient in between the surface soil and subsoil; however, bioturbation adds heavy $^{18}O_P$ primarily from the surface soil and cannot account for

the small $\delta^{18}O_P$ values in the deeper subsoil. There were no clear morphological signs of bioturbation in the C horizon (> 120-cm soil depth; Luvisol). It is assumed, therefore, that the small $\delta^{18}O_P$ values in the subsoil are due to increasing amounts of mineral-bound P. The $\delta^{18}O_P$ values of bedrock were not measured directly, but we can assume that the C horizon (Figure 2) provides a good approximation of the loess $\delta^{18}O_P$ value. The C horizon is still calcareous, and much of its P is assumed to be bound in apatite, which can be dissolved in HCl (Figure 2).

Notably, the $\delta^{18}O_P$ values of the deeper subsoil were smaller than the equilibrium value of 16.5‰, and they were even smaller when the range of uncertainty of 1.7‰ was subtracted from this value (Figure 2). Therefore, large amounts of subsoil phosphates did not equilibrate with soil water for these samples. This does not discount the possibility that some of the subsoil P had been in contact with extracellular enzymes, which might have resulted in deviations from full isotopic equilibration. The latter process can shift the $\delta^{18}O_P$ values not only in a negative direction (Liang & Blake, 2006) but also in a positive one (Liang & Blake, 2009; Tamburini *et al.*, 2014). Nevertheless, the small $\delta^{18}O_P$ signature of subsoil P suggests that biological cycling of the deeper subsoil P within microbial cells was inefficient.

In contrast to the topsoil, soil microbial biomass in subsoil is generally less abundant; therefore, small amounts only of total organic C (Fontaine *et al.*, 2007) and P are used by the subsoil microbial community. Analysis of the $\delta^{18}O_P$ depth profile revealed that our data matched the expected equilibrium only at about 40 cm depth. Within the 0–40-cm depth interval, the measured values exceeded the equilibrium because of biological cycling of this soil P pool with heavier soil water as described, but in the subsoil values were clearly below the $\delta^{18}O_P$ equilibrium value (Figure 3). These subsoil phosphates had not equilibrated with soil water because they had not been released by or cycled by intracellular enzymes in detectable amounts. As the HCl-extractable P fraction comprises a moderately labile P fraction (Negassa & Leinweber, 2009), it is difficult to imagine that the limited use of subsoil P by microorganisms was a consequence of its limited bioavailability to them. It is likely that either subsoil P was not used by the soil microbial community because it was not bioaccessible or the lack of additional carbon sources did not motivate soil microorganisms to consume additional mineral P. Regardless of the reason, the agronomically interesting zone is that where decreasing $\delta^{18}O_P$ values with depth indicate decreasing use of the subsoil phosphates by microorganisms. From the soil $\delta^{18}O_P$ values, we suggest that the HCl-extractable P pool can be assigned to three zones: a zone characterized by efficient biological cycling of P (bioaccessible HCl-P, 0–50 cm), a transition zone in which only parts of the HCl-P have been used by microorganisms (partly bioaccessible HCl-P at approximately 50–80 cm) and a zone characterized by P that has not been used by intracellular enzymes (non-bioaccessible HCl-P at ≥ 80 cm soil depth, Figure 2). Nevertheless, the exact delineation of the boundaries of these zones remains difficult to determine because of uncertainties in the exact calculation of $\delta^{18}O_P$ values at equilibrium (see above).

Conclusions

This study indicates that the $\delta^{18}O_P$ values of mineral fertilizer P might be too similar to be distinguished from soil-inherent P. However, organic fertilizer phosphates have smaller $\delta^{18}O_P$ values that tend to reduce soil $\delta^{18}O_P$ values of HCl-extractable phosphates over an unknown period of time. The data show clearly that the $\delta^{18}O_P$ signature of the surface soil can be larger than that of the subsoil, which would be expected if the latter shows only limited cycling of soil P through microbial cells.

The data from several topsoil samples and one soil profile with two independent field replicates supported the hypothesis that certain subsoil phosphates did not equilibrate isotopically with soil water. More data are needed, however, to validate the depth of such P pools at other sites and under other management treatments, and also for other P fractions. Nevertheless, the current data support the potential of this methodology not only to track microbial transformations but also to identify P that has not yet been transformed by soil microorganisms. The observed paucity of isotope exchange could be interpreted as a sign of low bioaccessibility or as a sign of the limited bioavailability of P in certain bonding forms.

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References

Angert, A., Weiner, T., Mazeh, S. & Sternberg, M. 2012. Soil phosphates stable oxygen isotopes across rainfall and bedrock gradients. *Environmental Science & Technology*, **46**, 2156–2162.

Barej, J.A.M., Pätzold, S., Perkons, U. & Amelung, W. 2014. Phosphorus fractions in bulk subsoil and its biopore systems. *European Journal of Soil Science*, **65**, 553–561.

Blake, R.E., O'Neil, J.R. & Garcia, G.A. 1997. Oxygen isotope systematics of biologically mediated reactions of phosphate: I. Microbial degradation of organophosphorus compounds. *Geochimica et Cosmochimica Acta*, **61**, 4411–4422.

Blake, R.E., O'Neil, J.R. & Surkov, A.V. 2005. Biogeochemical cycling of phosphorus: insights from oxygen isotope effects of phosphoenzymes. *American Journal of Science*, **305**, 596–620.

Bowen, G. & Revenaugh, J. 2003. Interpolating the isotopic composition of modern meteoric precipitation. *Water Resources Research*, **39**, 9-1–9-13.

Bryant, J.D. & Froelich, P.N. 1995. A model of oxygen isotope fractionation in body water of large mammals. *Geochimica et Cosmochimica Acta*, **59**, 4523–4537.

Burmann, F., Keim, M.F., Oelmann, Y., Teiber, H., Marks, M.A.W. & Markl, G. 2013. The source of phosphate in the oxidation zone of ore deposits: evidence from oxygen isotope compositions of pyromorphite. *Geochimica et Cosmochimica Acta*, **123**, 427–439.

Colman, A.S., Blake, R.E., Karl, D.M., Fogel, M.L. & Turekian, K.K. 2005. Marine phosphate oxygen isotopes and organic matter remineralization

in the oceans. *Proceedings of the National Academy of Sciences*, **102**, 13023–13028.

Cordell, D., Drangert, J.O. & White, S. 2009. The story of phosphorus: global food security and food for thought. *Global Environmental Change*, **19**, 292–305.

Fontaine, S., Barot, S., Barré, P., Bdioui, N., Mary, B. & Rumpel, C. 2007. Stability of organic carbon in deep soil layers controlled by fresh carbon supply. *Nature*, **450**, 277–280.

Harris, C. & Ashwal, L.D. 2002. The origin of low δ^{18}O granites and related rocks from the Seychelles. *Contributions to Mineralogy and Petrology*, **143**, 366–376.

Hedley, M.J., Stewart, J.W.B. & Chauhan, B.S. 1982. Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. *Soil Science Society of America Journal*, **46**, 970–976.

Holford, I.C.R. & Patrick, W.H. 1979. Effects of reduction and pH changes on phosphate sorption and mobility in an acid soil. *Soil Science Society of America Journal*, **43**, 292–297.

Hsieh, J.C.C., Chadwick, O.A., Kelly, E.F. & Savin, S.M. 1998. Oxygen isotopic composition of soil water: quantifying evaporation and transpiration. *Geoderma*, **82**, 269–293.

IUSS Working Group WRB. 2006. *World Reference Base for Soil Resources 2006*. World Soil Resources Report No 103, FAO, Rome.

Jaisi, D.P. & Blake, R.E. 2010. Tracing sources and cycling of phosphorus in Peru Margin sediments using oxygen isotopes in authigenic and detrital phosphates. *Geochimica et Cosmochimica Acta*, **74**, 3199–3212.

Karl, D.M. & Tien, G. 1992. MAGIC: a sensitive and precise method for measuring dissolved phosphorus in aquatic environments. *Limnology and Oceanography*, **37**, 105–116.

Liang, Y. & Blake, R.E. 2006. Oxygen isotope signature of P_i regeneration from organic compounds by phosphomonoesterases and photooxidation. *Geochimica et Cosmochimica Acta*, **70**, 3957–3969.

Liang, Y. & Blake, R.E. 2009. Compound- and enzyme-specific phosphodiester hydrolysis mechanisms revealed by δ^{18}O of dissolved inorganic phosphate: implications for the marine P cycling. *Geochimica et Cosmochimica Acta*, **73**, 3782–3794.

Longinelli, A. & Nuti, S. 1973. Revised phosphate-water isotopic temperature scale. *Earth and Planetary Science Letters*, **19**, 373–376.

Matsuhisa, Y., Tainosho, Y. & Matsubaya, O. 1973. Oxygen isotope study of the Ibaragi granitic complex, Osaka, southwest Japan. *Geochemical Journal*, **7**, 201–213.

Melby, E.S., Soldat, D.J. & Barak, P. 2013a. Biological decay of ^{18}O-labelled phosphate in soils. *Soil Biology & Biochemistry*, **63**, 124–128.

Melby, E.S., Soldat, D.J. & Barak, P. 2013b. Preferential soil sorption of oxygen-18-labeled phosphate. *Communications in Soil Science and Plant Analysis*, **44**, 2371–2377.

Middleboe, V. & Saaby, H. 1998. Quantification of cumulative bioactivity in soil via replacement of oxygen in labelled phosphate. *Applied Radiation and Isotopes*, **49**, 855–857.

Murphy, J. & Riley, J.P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, **27**, 31–36.

Negassa, W. & Leinweber, P. 2009. How does the Hedley sequential phosphorus fractionation reflect impacts of land use and management on soil phosphorus: a review. *Journal of Plant Nutrition & Soil Science*, **172**, 305–325.

Paytan, A., Kolodny, Y., Neori, A. & Luz, B. 2002. Rapid biologically mediated oxygen isotope exchange between water and phosphate. *Global Biogeochemical Cycles*, **16**, 13-1–13-8.

Pfahler, V., Durr-Auster, T., Tamburini, F., Bernasconi, S.M. & Frossard, E. 2013. ^{18}O enrichment in phosphorus pools extracted from soybean leaves. *New Phytologist*, **197**, 186–193.

Rothfuss, Y., Biron, P., Braud, I., Canale, L., Durand, J.L., Gaudet, J.P. *et al.* 2010. Partitioning evapotranspiration fluxes into soil evaporation and plant transpiration using water stable isotopes under controlled conditions. *Hydrological Processes*, **24**, 3177–3194.

Rothfuss, Y., Braud, I., Le Moine, N., Biron, P., Durand, J.L., Vauclin, M. *et al.* 2012. Factors controlling the isotopic partitioning between soil evaporation and plant transpiration: assessment using a multi-objective calibration of SiSPAT-isotope under controlled conditions. *Journal of Hydrology*, **442–443**, 75–88.

Tamburini, F., Bernasconi, S.M., Angert, A., Weiner, T. & Frossard, E. 2010. A method for the analysis of the δ^{18}O of inorganic phosphate extracted from soils with HCl. *European Journal of Soil Science*, **61**, 1025–1032.

Tamburini, F., Pfahler, V., Buenemann, E.K., Guelland, K., Bernasconi, S.M. & Frossard, E. 2012. Oxygen isotopes unravel the role of microorganisms in phosphate cycling in soils. *Environmental Science & Technology*, **46**, 5956–5962.

Tamburini, F., Pfahler, V., von Sperber, C., Frossard, E. & Bernasconi, S.M. 2014. Oxygen isotopes for unraveling phosphorus transformations in the soil-plant system: a review. *Soil Science Society of America Journal*, **78**, 38–46.

Tiessen, H. & Moir, J. 2008. Characterization of available P by sequential extraction. In: *Soil Sampling* and Methods of Analysis (eds M.R. Carter & E.G. Gregorich), pp. 293–306. CRC Press, Taylor & Francis Group, Boca Raton, FL.

Tudge, A.P. 1960. A method of analysis of oxygen isotopes in orthophosphate – Its use in the measurement of paleotemperatures. *Geochimica et Cosmochimica Acta*, **18**, 81–93.

Wang, P., Song, X.F., Han, D.M., Zhang, Y.H. & Zhang, B. 2012. Determination of evaporation, transpiration and deep percolation of summer corn and winter wheat after irrigation. *Agricultural Water Management*, **105**, 32–37.

Webster, R. 2007. Analysis of variance, inference, multiple comparisons and sampling effects in soil research. *European Journal of Soil Science*, **58**, 74–82.

Young, M.B., McLaughlin, K., Kendall, C., Stringfellow, W., Rollog, M., Elsbury, K. *et al.* 2009. Characterizing the oxygen isotopic composition of phosphate sources to aquatic ecosystems. *Environmental Science & Technology*, **43**, 5190–5196.

Zohar, I., Shaviv, A., Klass, T., Roberts, K. & Paytan, A. 2010. Method for the analysis of oxygen isotopic composition of soil phosphate fractions. *Environmental Science & Technology*, **44**, 7583–7588.