Method for the Analysis of Oxygen Isotopic Composition of Soil Phosphate Fractions

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The isotopic signature of oxygen in phosphate ($\delta^{18}O_P$) of various soil fractions may shed light on P transformations, including phosphorus (P) recycling by soil microorganisms, uptake by plants and P adsorption, precipitation and release by oxides and minerals, thus increasing our understanding on P cycling and lability in soils. We developed and tested a protocol to extract and purify inorganic phosphate (Pi) from different soil fractions distinguished by binding strength and precipitate it as silver phosphate (Ag_3PO_4) for $\delta^{18}O_P$ analysis. Soil P is extracted sequentially using water, $NaHCO_3$, NaOH and HCl and Pi in each solution is purified and precipitated as Ag_3PO_4. The unique characteristics and possible interferences of the soil solution extracts are addressed. Two agricultural soil samples receiving reclaimed wastewater or fresh water were analyzed, and results indicate that all soil fractions analyzed have been impacted to some degree by biologically enzyme mediated cycling of P in the soil.

Introduction

Phosphorus (P) is an important nutrient for all living organisms. P availability can limit productivity in many environments therefore the study of P cycling in both natural and impacted ecosystems is of interest (*1, 2*). Determining P availability for natural vegetation and agriculture crops and gaining a better understanding of processes within the soil P cycle which enhance P lability in top soils is important (*3*). Specifically, high P availability in agricultural systems may be transported from soil to water bodies, contributing to eutrophication (*4, 5*). Accordingly, understanding P cycling in soils has implications to water quality concerns.

The distribution of stable isotopes in natural systems has been used extensively for deciphering the biogeochemical cycling of various elements (e.g., C, N, S). P has only one stable isotope therefore stable isotope tracing cannot be applied to this element. However, much of the P in nature is bound to oxygen which has three stable isotopes. Indeed the oxygen isotopic signature of phosphate (Pi) has been used for tracking dissolved Pi sources and cycling in water bodies including oceans (*6*–*9*), estuaries (*10*), and lakes (*11, 12*). The P—O bond in phosphate is resistant to inorganic hydrolysis at the temperature and pH of most natural systems and inorganic processes such as mineral precipitation/dissolution or adsorption/desorption are associated with small (~1‰) fractionation effects (*13*). In contrast, biological processes are associated with large equilibrium or kinetic effects (*14*). Therefore, where rates of biological uptake and recycling through the biomass are relatively low, $\delta^{18}O_P$ (the ratio $^{18}O/^{16}O$ in phosphate) values predominantly reflect the isotopic signature of sources whereas biologically mediated transformation will alter these source signatures. Specifically, intracellular P processing (e.g., pyrophosphatase hydrolysis), imprints a temperature-dependent equilibration signature with oxygen in ambient water, and extracellular enzymatic hydrolysis involves kinetic effects with preferential uptake of the light oxygen isotopes. The fractionation effects depend on the enzyme used (e.g., alkaline phosphatase, 5′-nucleotidase) and the hydrolysis substrate (e.g., monoesterases, diesterases) (*15, 16*).

Analysis of $\delta^{18}O_p$ in minerals has long been used for elucidating the temperature or water isotopic signatures of the solutions from which the minerals precipitated (*17*–*19*). Mizota et al. (*20*) have analyzed $\delta^{18}O_p$ of apatite minerals from volcanic ash soils and found disequilibrium with soil solution. Larsen and co-workers (*21*) have tracked ^{18}O labeled Pi (enriched spike) in resin extracted P from soil and concluded that loss of labeled ^{18}O is caused by biochemical processes, and may be used as a measure of biological activity in soil. Tamburini et al. (*22*) investigated $\delta^{18}O_p$ variability of the HCl leachable fraction of soils and noted that differences in P status and availability are reflected in the isotope ratio. However, the natural $\delta^{18}O_p$ signature of different soil Pi pools, distinguished by different bonding types, to our knowledge, has not yet been used for elucidating P cycling in soils. Different soil pools have different reactivity and thus vary in their availability to plants and soil microorganisms and in their susceptibility to leaching and transport into surface and groundwater. Accordingly, such information is likely to contribute to the understanding of P availability and transformations in soils and processes that affect P lability and loss from soils. Specifically, it might be possible to elucidate which of the P pools is bioavailable for plants and how this changes under different environmental, climatic, agriculture practices or soil development conditions. Although, radioactive P isotopes (^{32}P, ^{33}P) have been used to investigate P transformations in soils (e.g., refs *23, 24*), the use of natural stable isotope signatures has advantages as it does not perturb the system (e.g., by adding Pi) and integrates over longer time scales. Moreover, the $\delta^{18}O_p$ signature in soil fractions that are not readily reactive, like Pi in mineral phases could be determined, which is not possible in radioactive labeling experiments.

P concentrations associated with various soil fractions determined using sequential extraction procedures have been used to elucidate P associations and transformations in soils (e.g., refs *25, 26*). The soil sequential extraction protocol developed by Hedley and co-workers (*27*) is aimed at quantifying soil Pi and organic phosphate (Po) pools. Specifically labile P, Fe, and Al associated P, Ca associated P, and the residual (nonreactive) P are targeted. Although the fractions are operationally defined, each extract corresponds to a certain P pool in the soil and is assigned some role in P transformations, thus soil P status can potentially be elucidated (*28*).

To determine $\delta^{18}O_p$ in various soil fractions, we have coupled and modified two well established procedures: the

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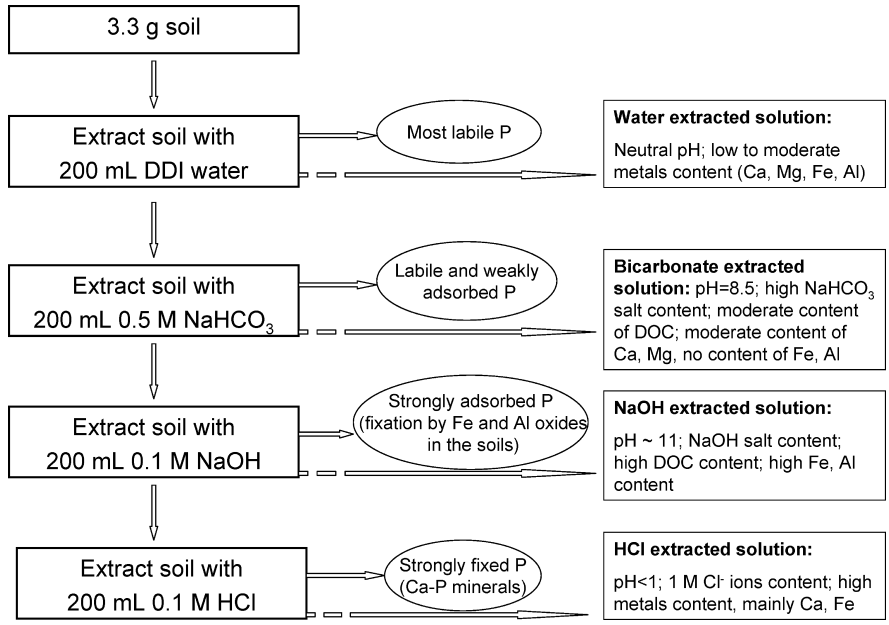

FIGURE 1. Soil sequential extraction (Modification of Hedley et al. (*28*), soil P fractions and typical resulting solutions characteristics. It can be assumed that the extracting solutions possess similar characteristics even when employed to different types of soils. It is, however, recommended to know ahead the characteristics of the soil of interest, specifically, Pi concentration, DOC and the metals content for each extracting solution. Knowing these should allow making adjustments to the procedure as needed when preparing samples for isotopic analysis.

Hedley soil P sequential extraction (*27*) and the oxygen isotope analyses of Pi in water samples (*29*). Modifications were made to account for the chemical nature of each of the extraction solutions and the presence of organic matter (OM) and metal ions in solution, while ensuring the integrity of the isotopic signature of the sample. We present here a detailed protocol and assessment of the modified procedure and demonstrate its utility using two agricultural soil samples, which were irrigated with either reclaimed wastewater (RWW) or with fresh water and fertilizer (FWF) and thus have distinct P content and have likely undergone different transformations over time.

Materials and Methods

Lists of required reagents and equipment, as well as basic instructions can be found in the original published procedures: the Hedley et al. (*27*) sequential extraction of soil ("the Hedley procedure") and the McLaughlin et al. (*29*) procedure for analysis of $\delta^{18}O_p$ of Pi in seawater ("the McLaughlin procedure"). In this manuscript, we only describe the modifications and adjustments made to the original protocols, such that they are useful for analyses of $\delta^{18}O_p$ of soil extracts.

1. Soil Sequential Extraction. Soil is sequentially extracted using a modified Hedley procedure (see also Tiessen and Moir (*28*)) yielding different soil P fractions. The extraction solutions are (by order of use) distilled water (modified from resin in distilled water); 0.5 mol L^{-1} $NaHCO_3$, pH 8.5; 0.1 mol L^{-1} NaOH, and 1 mol L^{-1} HCl, (a digestion step of the residual soil with sulfuric acid and H_2O_2 was not included). Soluble reactive P (SRP) concentration in each solution is determined by spectrophotometry at 880 nm using the molybdate blue procedure after pH neutralization. Total P (Pt) concentrations are determined on the first three extraction solutions, after acid digestion in an autoclave according to Tiessen and Moir (*28*). Figure 1 presents the principle steps of the modified sequential extraction used in this study, along with description of the P fractions targeted and solution characteristics relevant for the isotopic procedure (pH, metal ions and OM content) for each step.

The following modifications have been made to the Hedley procedure:

Soil sample size was increased to ensure sufficient amount of Pi for a final yield of ~8 mg of Ag_3PO_4 from each soil fraction, while maintaining soil to solution ratio of 0.016 as in the Hedley procedure (e.g., subsample replicates of 3.3 g soil extracted with 200 mL solutions). The soil sample was split into subsamples for ease of handling and centrifugation (e.g., most centrifuges accept 250 mL bottles). SRP concentration in each fraction is determined and the total amount of soil to be processed is calculated ensuring sufficient Ag_3PO_4 for each step (this depends on the step containing the least amount of Pi, typically the DDI water extract). The solutions of the multiple sample splits for each extraction are combined before processing the samples for isotope analyses. For extraction steps containing high concentrations of Pi (e.g., NaOH, HCl) the number of subsamples combined should be limited to the needed Pi in order to reduce the impact of interfering constituents (e.g., OM and metals). In the first extraction step we use DDI water only, without resin, as this better represents the fraction of P that will be washed away from the soil to the environment following rain and runoff events (the resin extracted component is thought to represent plant available P not all of which is likely to be washed by water). Pt was measured in all three first extracts (including DDI) in order to determine Po concentrations. The last digestion step (H_2SO_4 and H_2O_2) was not included, since we focus on the more reactive fractions of soil P and because such harsh treatment is likely to compromise the isotopic integrity of the sample.

In addition dissolved organic matter (OM) content (measured as dissolved organic carbon (DOC)) and relevant metal ion concentrations (e.g., Ca^{2+}, Mg^{2+}, $Fe^{3+/2+}$, Al^{3+}) are determined for each extract. While not required for $\delta^{18}O_p$ analysis, this information sheds light on the soil components with which P is associated and facilitates data interpretation.

2. Preparation of Samples for Isotopic Analysis. McLaughlin et al. (*29*), describe a protocol for the concentration and purification of Pi from seawater for $\delta^{18}O_p$ analysis (Supporting Information (SI) Figure S1). However, soil

extraction solutions are substantially different than seawater, thus we modified the procedure to address potential interferences of OM, metal ions, salts and solution pH and verified that the whole process does not alter the extracted Pi $\delta^{18}O_p$ (see "assessment" section). Water oxygen isotopic composition ($\delta^{18}O_w$) is also determined and used for calculating deviation from isotopic equilibrium (see SI).

2.1. General Considerations. McLaughlin et al. (*29*) concentrate Pi from solution by coprecipitation with seawater Mg^{2+} as $Mg(OH)_2$, after increasing the pH of seawater (the MAGIC step). Soil extracts do not contain high Mg^{2+}, hence Mg^{2+} is added to each solution as $MgCl_26H_2O$ or $Mg(NO_3)_2$ to obtain "seawater-like" Mg^{2+} concentration (~50 mM) and enable $Mg(OH)_2$ precipitation (pH adjustment to ~10.5 precedes Mg^{2+} addition). The MAGIC step should be performed on the same day of extracting the soil to prevent bacterial processes that could alter $\delta^{18}O_p$. Some batches of $MgCl_26H_2O$ and $Mg(NO_3)_2$ have trace amounts of Pi, thus the contribution of this reagent to the sample (blank) should be determined. For our samples the blank was <0.1 μg Pi per gram $MgCl_26H_2O$ and the total contribution of this to any of our samples was always much less than 1% of the sample Pi. The color of the $Mg(OH)_2$ precipitate for soil extracts may be tan or even brown due to coprecipitation of soil OM and some metals along with Pi. Specifically, humic substances (including humic acid (HA) and fulvic acid (FA)), which constitute the major organic fraction in soils (*30*) are compatible with P (*31*−*33*), and may partially coprecipitate with $Mg(OH)_2$. Organic matter will interfere with the precipitation of Ce phosphate and could persist until the final Ag_3PO_4 precipitation step in some samples (*29*). Therefore it is necessary to remove the OM. This can be done by HA precipitation at pH < 1 (*34*; see Step II below) and/or using DAX-8 Amberlite resin after the Hedley extraction, following the protocols of (*22*). Any residual OM left after the sample is fully processed should be removed from the Ag_3PO_4 by reaction with H_2O_2. Isotopic fractionation is not expected to take place in any of the OM treatment steps mentioned above (see "assessment" section). We have applied the procedure described below successfully to two soil samples reported here and to two additional samples (Aquolls, Tahoe, CA and Mollisols, Santa Cruz, CA), however due to the distinct and complex nature of soils, application of this procedure to any new soil type may have to be adjusted to ensure complete OM removal and corrections/tests for any processing artifacts related to OM hydrolysis have to be applied (see below). Information regarding the isotopic analysis of Ag_3PO_4 is detailed in (*29*) and in the SI.

2.2. Modifications Specific for Each Extracting Solution. Modifications of the "McLaughlin procedure" for each extracting solution are described below (step numbers are according to SI Figure S1). Details relevant for handling and preparation of each specific solution are also described.

Water Extraction Solution. Step I: Immediately after collecting the water soil extraction solution, raise pH to ~10.5. Add 10 g of $MgCl_26H_2O$ for each 1 L of solution, shake to dissolve; Let $Mg(OH)_2$ precipitate (allow to settle for ~2 h). Centrifuge and collect the precipitate; discard supernatant.

Step II: Dissolve the precipitate using acetic and nitric acids, bring solution to pH <1. Let HA precipitate overnight. Centrifuge and keep the solution; discard precipitate (after removing the HA, the solution color might still be slightly tanned). Fix pH of the solution to 5.5 using KOH (pH should be measured precisely using a pH electrode). Note, organic matter can also be removed using DAX-8 either before or after the $Mg(OH)_2$ precipitation following the procedure described in ref *22*.

Continue without modifications from step III thru step IX, following the "McLaughlin procedure".

Step X: After the Ag_3PO_4 has precipitated overnight and before its collection on filter paper, rinse it several times with DDI water (add water, shake, centrifuge and decant water). Add 1 mL of 15% H_2O_2 to the Ag_3PO_4 and let react at room temperature for a few hours. Rinse with DDI, decant H_2O_2 and repeat (in most samples the color of the silver phosphate will change to yellow and the OM will be removed). Continue as instructed in step X of "McLaughlin procedure".

Bicarbonate Extraction Solution. Pretreatment step: Immediately after collecting the $NaHCO_3$ soil extraction solution lower the pH of the solution to ~1 while stirring constantly with a magnetic stirrer. This is to ensure all the carbonate is transformed into CO_2 and degassed out of the solution.

Step I: For 1 L of solution, raise pH to ~10.5 using NaOH (NaOH beads added directly to the solution) while stirring with a magnetic stirrer, add 10 g of $MgCl_26H_2O$, shake; Let $Mg(OH)_2$ precipitate; Centrifuge and collect the precipitate; discard supernatant.

Step II: Dissolve the precipitate using acetic and nitric acids; if the precipitate is colored (containing organic matter) bring solution to pH < 1. Let HA precipitate overnight. Centrifuge and keep the solution; discard precipitate. Raise the pH of the solution to 5, using KOH. Let FA precipitate overnight. Centrifuge and keep the solution; discard precipitate. Fix pH to 5.5 using KOH. Note that after removing HA and FA, some OM might still be present and the solution may be of orange-brown color; this OM can be removed with DAX-8 or H_2O_2 (see below).

Continue without modifications from step III thru step IX, following the "McLaughlin procedure".

Step X: After the Ag_3PO_4 has precipitated overnight and before its collection on filter paper, rinse it several times with DDI water (add water, shake, centrifuge and decant water). Add 1 mL of 15% H_2O_2 to the Ag_3PO_4 and let react at room temperature for a few hours. Rinse with DDI, decant H_2O_2 and repeat (in most samples the color of the silver phosphate will change to yellow and the OM will be removed). Continue as instructed in step X of "McLaughlin procedure".

NaOH Extraction Solution. Step I: This solution is already at high pH; only addition of $MgCl_2$ is required for the MAGIC step. For 1 L of solution, add 10 g of $MgCl_26H_2O$, shake to dissolve. Let $Mg(OH)_2$ precipitate; Centrifuge and collect the precipitate; discard supernatant.

Step II: Dissolve the precipitate using acetic and nitric acids; bring solution to pH < 1. Let HA precipitate overnight. Centrifuge and discard precipitate; keep the solution; discard precipitate. Fix pH of the solution to 5.5 using KOH. Organic matter can also be removed using DAX-8 following the procedure of (*22*). Fix pH of the solution to 5.5 using KOH.

Continue without modification from step III thru step IX, following the McLaughlin procedure to precipitate P with cerium. Expect a big amount of brownish precipitate to form at the end of this step. This is likely a result of metal ions precipitating along with the Ce^{3+} (in addition to Pi). In a later step (VII) the metal ions are removed by the resin and the color of the solution becomes lighter. For soils rich in OM and metal ions, the sample should be split into several subsamples after step V and recombined after step VII (similar to the treatment of the HCl extracting solution, see below).

Step X: After the Ag_3PO_4 has precipitated overnight and before its collection on filter paper, rinse it several times with DDI water (add water, shake, centrifuge, and decant water). Add 1 mL of 15% H_2O_2 to the Ag_3PO_4 and let react at room temperature for a few hours. Rinse with DDI, decant H_2O_2 and repeat (in most samples the color of the silver phosphate will change to yellow and the OM will be removed). Continue as instructed in step X of McLaughlin procedure.

HCl Extraction Solution. The HCl solution contains high concentrations of chloride ions (~1 M) as well as high concentration of $Fe^{3+/2+}$ and Ca^{2+} ions. Cl^-, Ca^{2+}, and $Fe^{3+/2+}$

TABLE 1. Isotopic Composition ($\delta^{18}O_p$) of the Irrigation Water (RWW and FWF) and of the Different Soil Fractions Extracted from the Soil Samples Irrigated with Either RWW or FWF[a]

irrigation water	extraction solution	RWW, $\delta^{18}O_p$, ‰ (sd)	FWF, $\delta^{18}O_p$, ‰ (sd)
		18.5 (0.6)	28.0 (0.5)
	H_2O	RWW soil, $\delta^{18}O_p$, ‰	FWF soil, $\delta^{18}O_p$, ‰
soil P sequential extractions	H_2O	18.5 (0.6)	10.7 (1)
	0.5 mol L^{-1} $NaHCO_3$	20.3 (0.2)	19.0 (0.9)
	0.1 mol L^{-1} NaOH	9.1 (0.2)	7.8 (0.5)
	1 mol L^{-1}HCl	6.3 (0.1)	7.7 (0.7)

[a] Standard deviations (SD) are based on at least three replicates and are sometimes higher than the 0.4‰ analytical error found for standards. This is expected since natural variability in real samples may result in larger standard deviation.

ions all interfere with the final precipitation of Ag_3PO_4. The "McLaughlin procedure" includes a step in which Cl^- ions (originating in seawater) are removed by thorough rinsing of the sample (as $CePO_4$ precipitate) with K-acetate buffer. Excess Cl^- ions from the HCl solution are removed at this step as well; however, due to the high Cl^- concentrations, it is necessary to divide each $CePO_4$ sample into several subsamples and to repeat the rinsing step more times (check with $AgNO_3$ for the complete removal of Cl^- ions). Sample division is also useful for the subsequent step, where $CePO_4$ is dissolved and cation exchange resin is used to remove Ce^{3+}, as well as excess Ca^{2+} and $Fe^{3+/2+}$ ions.

Step I: Pi, Cl^- and metal ions concentrations in this extract are expected to be high, thus limit the amount of soil replicates used to that required to produce only the needed 8 mg of Ag_3PO_4. Raise pH to ~10.5 using NaOH beads while stirring with a magnetic stirrer. For 1 L solution add 10 gr of $MgCl_26H_2O$, shake; let $Mg(OH)_2$ precipitate. Centrifuge and discard supernatant; expect the MAGIC precipitate to be orange in color (due to Fe ions).

Continue with steps II and III following the "McLaughlin procedure". Expect a big amount of brownish precipitate at the end of step III (Ce−P precipitation) since a lot of the metal ions, including $Fe^{3+/2+}$, will also precipitate at pH ~5.5.

Step IV: Add K-acetate buffer, shake to dissolve the precipitate and divide the original sample into 4−6 subsamples in order to wash thoroughly the Cl^- ions and for better operation of the resin in the next step (sample splitting is required even for small soil samples). For each of the sub samples, rinse the precipitate with K-acetate buffer several times to remove chloride ions (5−6 rinses); check with $AgNO_3$ for the complete removal of Cl^- ions (see "McLaughlin procedure").

Step V: Dissolve precipitate with as little as possible 1 M nitric acid and dilute solution with water to 0.2 M nitric acid (for each of the subsamples, separately).

Step VI: Add "BIORAD AG 50" resin to each of the subsamples separately; shake overnight to let the resin adsorb Ce^{3+} ions.

Step VII: Separate solution from resin (for example using columns as in the "McLaughlin procedure"); retain solution, collect solution of all subsamples and combine. Make sure the solution is clear, if it is not, repeat the resin step by repeating steps VI−VII using fresh resin. Rinse all the resin used in all subsamples with DDI water, then combine all treated solutions and rinsewater.

Continue without modification from step VIII on following the "McLaughlin procedure" (H_2O_2 treatment for the HCl extracting solution is typically not required, since typically no OM is left at this stage).

Assessment

To test the integrity and utility of the method we conducted an isotope spiking experiment (Table S1) and applied the procedure to two soil samples (Table 1 and SI Table S2). Hydrolysis of Po compounds (e.g., phosphate-diesters, phosphonates) or condensed P compounds (e.g., pyrophosphate and polyphosphates) may add Pi to the sample. These processes are associated with incorporation of oxygen from solution (*10*) and thus may compromise the integrity of the isotope data if they occur at any point in the procedure. McLaughlin et al (*29*) and Colman et al. (*7*), reported that their published protocols do no involve isotope exchange. The Hedley procedure involves extractions at similar pH conditions, thus, we do not expect isotopic alterations during soil extraction. Moreover, the Hedley procedure was developed to distinguish between Pi and Po of soil fractions and thus was designed to minimize Po hydrolysis (although condensed P may be hydrolyzed if present). Modifications we made to the Hedley and McLaughlin procedures do not involve any step that introduces different chemicals or conditions (e.g., temperature, pH) compared to the original procedures, thus $\delta^{18}O_p$ values obtained using the combined protocol presented above are expected to be reliable. Regardless, to demonstrate that no isotopic exchange or hydrolysis occur in the procedure, we processed a soil sample (the RWW soil described below) with ^{18}O enriched reagents. In brief, solutions and reagents used were prepared with ^{18}O enriched water and the soil was treated according to the protocol described above. Hydrolysis or other processes that would result in incorporation of oxygen from the reagent would be detected as elevated $\delta^{18}O_p$ values in the final Ag_3PO_4 compared to the same sample processes without spiked reagents. Results (SI Table S1) show that the isotopic composition of Ag_3PO_4 obtained for all extractions was not altered in the process (e.g., samples processed with and without the ^{18}O spiked reagents were identical within analytical error) suggesting that Po hydrolysis or isotopic fractionation have not compromised the integrity of the data. For detailed description of the spiking experiment and results, see online SI.

The utility of this method is demonstrated by an application to two soil samples (of calcareous alluvial clay soil; Acre, Israel) obtained from agricultural plots with distinct P concentrations and application history. One plot was irrigated with low grade, secondary, reclaimed wastewater (RWW), and the other with fresh water amended with a chemical fertilizer (FWF). Information about soil sampling, irrigation history, and chemical characteristics of the soils (Pi, Pt, DOC, pH, Fe, Al, Mg, and Ca) is given in SI Table S2. The soils were sequentially extracted, and Pi was purified and precipitated from each solution as Ag_3PO_4 using our combined protocol as detailed above. Samples were then analyzed for $\delta^{18}O_p$. Phosphate in irrigation water was also sampled and analyzed for $\delta^{18}O_p$. The irrigation solution used at the FWF plot was prepared according to McLaughlin et al. (*29*), after adding Mg^{2+} to the solution (without further modifications), whereas the irrigation solution used at the RWW plot was treated

according to the protocol for water extract of the soil described above. The isotope values of the phosphate in the irrigation water and of the various soil extracts are given in Table 1 for both soil samples (RWW and FWF soils).

The $\delta^{18}O_p$ of the water extracted fraction of the RWW soil (18.5 ‰, Table 1) is identical to that of the irrigation water but it is also quite close to the expected oxygen isotopic equilibrium value. Longinelli and Nuti (*35*) have described the relation between $\delta^{18}O_p$ and the isotopic composition of the water oxygen, $\delta^{18}O_w$, when in equilibrium at any given temperature as follows:

$$T(^0C) = 111.4 - 4.3(\delta^{18}O_p - \delta^{18}O_w) \quad (1)$$

Assuming soil temperature of approximately 24 °C in August (when the soil was sampled) and the measured $\delta^{18}O_w$ in RWW used for irrigation in Acre (−2.7‰), the $\delta^{18}O_p$ in equilibrium would be +17.6‰. This value is close to the measured value of +18.5‰ ± 0.6‰, considering possible variability in soil temperatures and soil solution $\delta^{18}O$. Thus, the measured $\delta^{18}O_p$ in this fraction may reflect either the Pi applied during irrigation or equilibrium with water at the soil's temperature.

If indeed the $\delta^{18}O_p$ of the DDI water extract of the RWW soil reflects equilibrium, this indicates that the soil biological activity is quite high, effectively bringing the labile water-soluble Pi to equilibrium values. However, as noted above, because the $\delta^{18}O_p$ of the RWW soils' DDI extracted fraction is identical to the $\delta^{18}O_p$ of the irrigation water (Table 1), it may suggest that the water labile Pi fraction originates predominantly from the irrigating water. It is hard to distinguish between these two scenarios based on these results alone, however, based on the results from DDI fraction of the FWF soil which are very different than the applied $\delta^{18}O_p$ of the irrigation water it is more likely that the DDI extract in this soil represents equilibrium values.

As mentioned above for the FWF soil, the $\delta^{18}O_p$ of the DDI extract (10.7‰) does not reflect the $\delta^{18}O_p$ of the fertilizer used (28‰) indicating that this value is modified by P processing in the soil. The $\delta^{18}O_p$ of the DDI extract is however lower than the expected equilibrium value determined based on water isotopes (−4.9‰) and temperature (24 °C) (equilibrium = 15.4‰). Lack of isotopic equilibrium suggests that intracellular P cycling, that appears to dominate the $\delta^{18}O$ signature of dissolved Pi in many systems (*14, 36, 37*) does not control the $\delta^{18}O_p$ of the water extract in the FWF soil. A possible explanation for the relatively low isotopic value recorded in the DDI fraction of this soil which has low Pi concentrations (see SI Table S2), is fractionation associated with extracellular enzymes. Specifically, when Po is remineralized to form Pi, in processes mediated by extracellular phosphohydrolase enzymes, the resulting Pi incorporates one oxygen if hydrolyzed by phosphomonoesterase or two oxygen atoms is hydrolyzed by phosphodiesterase from the water with an isotope fractionation of −10‰ to −30‰, depending on enzymes involved (*15, 16*), leading to lower values compared to those expected from equilibrium. The resemblance of $\delta^{18}O_p$ in the DDI extract (10.7 ‰) to that of the NaOH and HCl extracts (7.8 ‰ and 7.7 ‰, respectively) may indicate that all three fractions are controlled by similar processes (e.g., all of these fractions are/were controlled at some point by extracellular enzyme mediated cycling). Measurement of enzyme activities may shed more light on determining rates and specific enzymes involved in determining the $\delta^{18}O_p$ of this fraction. Alternatively, the above-noted similarity may imply that Pi in the soil solution or Pi weakly sorbed (e.g., DDI fraction) are being impacted by Pi released from mineral phases through processes of desorption and/or dissolution. Geochemical equilibrium with soil minerals may be prevalent in soils where biological activity is slow such as in the FWF soil. Application of this procedure in other locations characterized by low microbial activity along with the enzyme analyses mentioned above would help determine which of the above explanations is more likely. Characterization of the oxygen isotopic signature of soil P_o which was not assessed here will also enable further understanding of the bioavailability and cycling of specific compounds in the soil.

The $\delta^{18}O_p$ of the bicarbonate extracted solution for both soils is almost identical (∼20 ‰, Table 1) and higher than the water extract and the expected equilibrium values. This extraction targets easily exchangeable P adsorbed on mineral surfaces. It is expected that this fraction will be relatively available to plants growing on the soil and to the soil microbial community. Plants and microorganisms will preferentially utilize the light isotopes resulting in enrichment of the residual Pi in this bioavailable pool. The higher than equilibrium values indeed suggest preferential loss of Pi with low $\delta^{18}O_p$ via uptake.

The isotopic compositions of the NaOH and HCl extracts of both soils are much lower than the first two extractions (in the range of 6−9 ‰). The NaOH solution extracts P strongly fixed by Fe/Al-hydroxides and the HCl dissolves Ca−P minerals (*28*). It is possible that the $\delta^{18}O_p$ of Pi of these much more stable soil fractions retains the $\delta^{18}O_p$ of the original parent rock/mineral material. However, low $\delta^{18}O_p$ indicates Pi of igneous origin (*11*), which is unlikely to reflect the bedrock (carbonates) in this area. Thus, the $\delta^{18}O_p$ likely represents some newly precipitated minerals in the soil which reflect disequilibrium extracellular enzymatic effects (as was mentioned above for DDI extract of the FWF soil). We note that Pi produced in this process (at any point of the soil's history) can then be sequestered and immobilized by adsorption/precipitation within these phases without further transformations or interactions with the soil more labile Pi pool.

Our application of the procedure to two soil samples with different P concentrations, distribution and application history demonstrates that different soil fractions have different $\delta^{18}O_p$ signatures and that the $\delta^{18}O_p$ of the same soil fraction can differ among soil samples that differ in their P dynamics and agriculture use. Results obtained using this method expand the interpretation possible based on P concentration data for the various soil P fractions by linking the distribution of P in the soil's fractions to potential biogeochemical processes that affect them. These results illustrate the potential of this isotopic tool to understand P dynamics in soil systems.

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Supporting Information Available

Additional details are provided regarding the experimental and the assessment sections, including 2 figures and 3 tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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