PLOS ONE

RESEARCH ARTICLE

Methodology and validation of a new tandem mass spectrometer method for the quantification of inorganic and organic ^{18}O-phosphate species

Aimée Schryer[1], **Kris Bradshaw**[2], **Steven D. Siciliano**[1]*

1 Department of Soil Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada,
2 Federated Co-operatives Limited, Saskatoon, Saskatchewan, Canada

* steven.siciliano@usask.ca

Citation: Schryer A, Bradshaw K, Siciliano SD (2020) Methodology and validation of a new tandem mass spectrometer method for the quantification of inorganic and organic ^{18}O-phosphate species. PLoS ONE 15(2): e0229172. https://doi.org/10.1371/journal.pone.0229172

Editor: Fuzhong Wu, Sichuan Agricultural University, CHINA

Received: October 15, 2019

Accepted: February 1, 2020

Published: February 24, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Funding came from a Natural Sciences and Research Council (NSERC) Industrial Research Chair grant (IRCSA 478012-13) to SDS and the Sustainable In-Situ Remediation Co-operative Alliance (SIRCA). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The

Abstract

Phosphorus (P) fertilizers are crucial to achieve peak productivity in agricultural systems. However, the fate of P fertilizers via microorganism incorporation and the exchange processes between soil pools is not well understood. ^{18}Oxygen-labelled phosphate (^{18}O- P_i) can be tracked as it cycles through soil systems. Our study describes biological and geochemical P dynamics using a tandem mass spectrometry (MS/MS) method for the absolute quantification of ^{18}O- P_i. Soil microcosms underwent three treatments: (i) ^{18}O- P_i, (ii) unlabelled phosphate (^{16}O- P_i) or (iii) Milli-Q control, dissolved in a bio-stimulatory solution. During a 6-week series the microcosms were sampled to measure P by Hedley sequential fractionation and DNA extraction samples digested to 3′-deoxynucleoside 5′-monophosphates (dNMP). A MS/MS attached to a HPLC analyzed each P-species through collision-induced dissociation. The resin-extractable and bicarbonate ^{18}O- P_i and ^{16}O- P_i fractions displayed similar precipitation and adsorption-desorption trends. Biotic activity measured in the NaOH and dNMP fractions rapidly delabelled ^{18}O- P_i; however, the MS/MS measured some ^{18}O that remained between the P backbone and deoxyribose sugars. After 6 weeks, the ^{18}O- P_i had not reached the HCl soil pool, highlighting the long-term nature of P movement. Our methodology improves on previous isotopic tracking methods as endogenous P does not dilute the system, unlike ^{32}P techniques, and measured total P is not a ratio, dissimilar from natural abundance techniques. Measuring ^{18}O- P_i using MS/MS provides information to enhance land sustainability and stewardship practices regardless of soil type by understanding both the inorganic movement of P fertilizers and the dynamic P pool in microbial DNA.

Introduction

Phosphorus (P) is an essential macronutrient, yet it is frequently the limiting factor for biological activity in soils worldwide [1]. Applying P-phosphate fertilizers increases soil sustainability,

funders provided support in the form of stipend for the author AS, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The commercial affiliation of KB, Federated Co-operatives Limited Saskatoon, played a role in study design and preparation of the manuscript, but did not have any additional roles in the data collection and analysis, or the decision to publish. The specific roles of the authors are articulated in the 'author contributions' section. There are no financial, non-financial, professional or personal competing interests caused by the commercial affiliation of KB, including employment, consultancy, patents, products in development, marketed products, ect. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

crop yields and promotes other biological processes, such as contaminant bioremediation [2,3]. The primary source available for plants and microbial communities is inorganic phosphate; consequently, extraction methods emphasize quantification of the inorganic phases to estimate P availability [4–7]. Despite the importance of inorganic P, organic P represents a significant portion of both total and bioavailable P within soils [8]. A large part of the organic P fraction is bound in microorganisms, primarily in nucleic acids, phospholipids, inositol phosphate, sugar phosphates, and as condensed P [5,6]. Nevertheless, the precise composition of organic P within soils is poorly understood [5]. Microorganisms mediate key processes within the biogeochemical P cycle, such as immobilization and mineralization, strongly influence P bioavailability for other biotic species [9]. While many techniques estimate the concentration of organic P, including fumigation for microbial biomass and sequential fractionation for total organic P, they cannot identify the chemical nature or the cycling dynamics of organic P [10].

To investigate organic P dynamics, investigators typically resort to isotopic methods such as the isotope dilution protocol, which uses ^{32}P and ^{33}P to determine soil organic P permutation and concentration dynamics [8,11]. This technique monitors the exchange between a known concentration of dosed $^{32/33}$P-phosphate fertilizers and endogenous ^{31}P-phosphates in treated soils [12]. Work with $^{32/33}$P increased understanding of the P-cycle by assessing the sizes and rates of exchange of $^{32/33}$P between P pools and/or tracking fertilizer P fate from soil to biota, (e.g., plant and microbial communities) [4,13–16]. However, due to the radioactive nature of tracer $^{32/33}$P, current isotope dilution techniques are difficult to integrate with genomic pipelines. This includes difficultly in identifying what type of organic P is moving within biotic systems. The transmutation of $^{32/33}$P to daughter species $^{32/33}$sulfur produces an unstable coordination number and high vibrational energy [17]. Consequently, the half-lives of a radionucleotide is 5–20 times shorter than the radioisotope as the radionucleotides self-destructs [18]. Interactions with released energy or with any radiation-produced reactants (i.e., radicals) from labelled molecules causes damage to nearby biomolecules and nucleic acids [19]. Additionally, the half-lives of ^{32}P and ^{33}P (14.3 days and 24.4 days) restrict the length of studies due to self-radiolysis [4,18]. Equilibration times for the P species further complicates experimental design as a portion of mineral inorganic P is rapidly exchangeable with solution inorganic P [20]. For example, the isotopic equilibration rate between endogenous ^{31}P and experimental $^{32/33}$P fertilizers requires three months or between six (^{32}P) and three (^{33}P) half-lives [21]. While $^{32/33}$P studies provide the basis for understanding both soil fertility and P cycling, a non-radioactive tracer is needed to complement current work into organic P dynamics.

Oxygen is an ideal stable isotope to discern the biogeochemical cycle of P. Oxygen has three stable isotopes while P only has one (^{31}P) [1,22]. The natural abundance of ^{18}O is 0.204% and the two additional neutrons allow the separation between labelled and unlabelled fractions during downstream genomic applications [23,24]. Only enzyme mediated biological activity breaks the O-P bond under environmentally relevant conditions as it is stable under ambient temperatures and in abiotic environments [25–27]. The ubiquitous intracellular enzyme pyrophosphatase exchanges ^{18}O and ^{16}O present in cellular fluids and water until it reaches equilibrium [28]. Moreover, the enzyme is stable following cell lysis and will exchange atoms outside the cell [28]. Melby et al. [29] described that the half-life of ^{18}O-Orthophosphate (P_i) as 15 to 22 days in non-sterile soils and greater than 50 days in sterile soils. One option to track P trends is to measure the stable isotope ratio of oxygen($\delta^{18}O_p$) by isotope ratio mass spectrometry [IRMS, e.g. 22–24]. Samples are not directly analyzed. Alternatively, P_i undergoes processing to silver phosphate (Ag_3PO_4) with subsequent purification steps to minimize contamination from other O-isotope sources, such as oxyanions [30]. The samples are pyrolyzed in a thermochemolysis/elemental analyzer at 1460˚C, converted to C^{18}O and C^{16}O gas,

measured by IRMS and described using the following equation [30] [31];

$$\delta^{18}O_p = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1,000$$

However, δO vary by soils, sites and environments; this variation coupled with instrument sensitivity precludes the use of δO as a proxy for cycling of organic P [32–34]. In contrast, the use of enriched ^{18}O-P is well suited for stable isotope probing (SIP) [32,35]. Stable isotope probing tracks isotopically labelled substrates to determine nutrient movement within abiotic systems and organisms while concurrently identifying active microbial populations and biological processes [24,36,37]. The methodology can also follow both inorganic P pools within the environment using ^{18}O enriched fertilizers [38]. Thus, SIP experiments in soils need to balance the time required for refractory P-pools to be labelled with the decay in signature of the original source of ^{18}O-P by microbial communities[32,33]. Mass spectrometry is capable of following the unpredictable biotic and inorganic ^{18}O-SIP signature within soil systems due to its' sensitivity, accuracy, and its capability to concurrently measure analytes from a wide range of masses [39]. A MS instrument comprises of three elements: an ion source, mass analyzer and a detector [40,41]. The ion source produces charged gas phase ions from either liquid or solid phase samples [39]. Analyzers sort ions by mass using electromagnetic fields, thereby determining the isotopic composition of compounds [42]. To increase the selectivity of the analysis, the multiple step selection method known as tandem mass spectrometry (MS/MS) isolates precursor ions and produces known product ions [43]. Once through the mass analyzer, the detector performs both qualitative and quantitative analysis of the gas phase species through measuring the mass-to-charge (m/z) ratios and abundances [41,44]. Both the m/z accuracy and sensitivity for trace samples signifies that mass spectrometry is ideal to examine ^{18}O- P_i fertilizer movement and biotic exchange effects overtime; however, MS investigations into P cycling in soils are limited.

In this study, we conducted experiments to validate both MS and the use of ^{18}O labelled P_i to track the movement and dynamics of P in inorganic and organic pools. We hypothesize that the combination of labelled ^{18}O- P_i SIP with high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) provides new opportunities to follow the fate of P fertilizers to better comprehend the P cycle in soils. This was completed in four steps. First, two mass spectrometer (MS) methods were created to quantify the concentration of unlabelled (^{16}O)- and ^{18}O- P_i and ^{16}O- and ^{18}O-3′-deoxynucleoside 5′-monophosphates (dNMP). Secondly, we compared concentration of resin-extractable ^{16}O- and ^{18}O- P_i using the SEAL segmented flow analyzer (AA3) and the HPLC-MS/MS method to determine whether the extraction matrices or instrumentation hindered the analysis of P_i. Third, we doped soil with ^{16}O- and ^{18}O- P_i fertilizer in an ecologically relevant context to study P dynamics, ie. calcareous soil under anaerobic conditions, to both affirm the validity of the MS methods to track P and to view the differences in P movement in soils over a 6 week time series by sequential fractionation extraction. We used anaerobic conditions as our work focusses on P dynamics in polluted soils in which P is added to bio-stimulate remediation [3]. Fourth, we extracted DNA to measure the concentration of ^{16}O- and ^{18}O-dNMP between weeks to view changes in this significant portion of microbial organic P concentration and isotopic exchange over time. Finally, the sequential fractionation results were combined to create a mass balance of total P by isotopic composition to compare the recovery of each P_i species.

Materials and methods

Microcosm design

Soil samples were collected from Davidson (51˚15'46.7"N, 105˚59'36.9"W), Outlook (51˚28'27.3"N, 107˚06'04.6"W), and Allan (51˚53'42.38"N, 106˚03'22.02"W) in Saskatchewan, Canada. A total of 72 microcosms (3 treatments x 4 replicates x 6 time points) were created by homogenizing different quantities of soils from the three sites. Soil (10 g) was added to an acid bathed and autoclaved 30 mL serum bottle (Wheaton, Chicago, IL, USA). Each microcosm was filled with one of three treatments: (i) bio-stimulatory solution with either ^{18}O- P_i or (ii) ^{16}O- P_i as the P source or (iii) Milli-Q water only as a control. The ultra-purified Milli-Q water was obtained from an in-house purification system Milli-Q Direct 8/16 System (Millipore, Billerica, MA, USA). The bio-stimulatory solution comprised of 0.24 mM HNO_3 [3.4 mg/L N], 0.24 mM Fe(III)NH_4-citrate [13 mg/L Fe(III)], 22 mM SO_4 [700 mg/L S]), and 0.1 mM P-species (3.1 mg/L P) in Milli-Q water at a circumneutral pH. Both ^{16}O- and ^{18}O- P_i were synthesized based on the procedure published by Melby et al. [45] using Milli-Q $H_2{}^{16}O$ and $H_2{}^{18}O$ (97% ^{18}O; Millipore Sigma, Burlington, MA, USA) and Phosphorus Pentachloride (Millipore Sigma). The amount of labelling within each P source was checked by MS for isotopic purity. Each microcosm received 32 mL of the applicable solution to ensure complete saturation and was crimp sealed within an anaerobic chamber for minimal O_2 conditions. The closed microcosms were mixed for 1 hour following assembly on a horizontal rotary shaker (150 rpm) and incubated at room temperature.

Microcosms were randomly assigned incubation time points: 1 week, 2 weeks, 3 weeks 4 weeks, 5 weeks, and 6 weeks following construction. Following incubation, microcosms were destructively sampled using vacuum filter units fitted with autoclaved 0.45 μm filter paper into acid bathed and autoclaved Büchner flasks to separate the soil and water solution. Soil samples were collected for sequential P extraction and microbial DNA. Aliquots of soil samples were ground to 0.85 mm [46]. Soil P fractions were extracted following the Hedley method developed by Tiessen and Moir [46] using resin anion exchange strips, followed by 0.5 M bicarbonate (pH = 8.5), 0.1 M NaOH, and 1.0 M HCl. For 0.5 M bicarbonate (7.0 g/30 mL, end pH = 3.4-3.7) and 0.1 M NaOH (1.5 g/30 mL, end pH = 5.2-5.4) extractions. AG 50W-X8 cation exchange resin beads (Bio-Rad Laboratories, Hercules, USA) were added to exchange sodium ions with protons to clean and acidify the sample. Microbial DNA was collected using a PowerSoil® DNA isolation kit (MoBio Technologies, Vancouver, BC, Canada) and eluted at 40 μL, followed by quantification using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA).

Mass spectrometric optimization of ^{16}O- and ^{18}O-P_i and ^{16}O-dNMP standards

The collision-induced dissociation (CID) tandem mass spectrometric (MS/MS) optimization and analysis of ^{16}O- P_i, ^{18}O- P_i and ^{16}O-dNMP were conducted using a AB Sciex 4000 QTRAP® mass spectrometer (AB Sciex, Concord, ON, Canada) attached to an Agilent 1260 Infinity II HPLC System (Agilent Technologies, Santa Clara, CA, USA). The MS, a hybrid triple quadrupole–linear ion trap mass spectrometer (QqQ-LIT), is equipped with a Turbo V™ Ion Spray electrospray ionization (ESI) source with nitrogen utilized as the collision gas. The HPLC is composed of a binary pump with an autosampler that has temperature control. Both P_i and dNMP optimization were conducted in negative ion mode, where the collisional energy varied between -20.0 and -5.0 V, whereas the declustering potential remained fixed at -40 V. An integrated syringe pump (Harvard Apparatus, MA, USA) infused sample aliquots into the

mass spectrometer at a rate of 10 μL /min through a Turbo Ionspray Source, where the needle voltage was -4500 V. Nitrogen was used both as the drying gas and ESI nebulizing gas. The fractionation pattern, product ions and MS conditions for ^{16}O- P_i and ^{18}O- P_i (Table 1) were identified. Similarly, the fractionation pattern, product ions and MS conditions for each ^{16}O-dNMP (Table 2) were deduced.

Quantification of ^{16}O- and ^{18}O-P_i

The concentration of ^{16}O- and ^{18}O- P_i following sequential P extraction was performed by direct infusion analysis on the 4000 QTRAP. The HPLC-MS/MS calibrations curves were produced in their respective sequential fractionation matrices from synthesized ^{16}O- and ^{18}O- P_i stocks following quantification on the SEAL segmented flow analyzer (AA3; Seal Analytical, Mequon, WI, USA). The optimized chromatographic and instrumental parameters for ^{16}O- and ^{18}O- P_i quantification on the HPLC-MS/MS are in S1 Table. The quality assurance (QA)/quality control (QC) for the method included: duplicates; spikes; and low, medium and high QC concentrations from the calibration curve in order to determine accuracy and any variation occurring intra- and inter-day. The concentration of the P_i in mg/L was determined by reporting the chromatographic peak areas of the samples versus standard solution concentrations using AB Sciex Analyst® Software version 1.6.2 (SCIEX. 2013. Analyst 1.6.2 Software Installation Guide. Framingham, MA, USA). The concentration of P_i was converted to mg/g dry soil by multiplying by the extraction volume and dividing by the mass of dry soil.

Comparing instruments for the quantification of available ^{16}O- and ^{18}O-P_i

The resin anion exchange strips extracted P_i was measured on both the AA3 and direct infusion analysis on the 4000 QTRAP HPLC-MS/MS. The AA3 calibration curve was produced from 1000 mg/L stock P solution (Cole-Parmer, Vernon Hills, IL, USA). The QA/QC for the AA3 included: duplicates, blanks, and method spikes.

Digestion of DNA to dNMPs

Two enzymes were used to isolate dNMP from double stranded DNA following the method published by Bochkov et al. [47]. The double stranded DNA was combined with 2 μL DNAse I (1 unit (U)/μL, ThermoFisher, Waltham, MA, USA) and buffer and heated at 37˚C for 15 minutes (min). Then 1 μL Nuclease S1 (100 U/μL, Promega, Madison WI, USA) and buffer was added and the solution was heated at 37˚C for 15 min to release the dNMP (3′-deoxyadenosine 5′-monophosphate [dAMP], 3′-deoxythymidine 5′-monophosphate [dTMP], 3′-deoxycytidine 5′-monophosphate [dCMP] and 3′-deoxyguanosine 5′-monophosphate [dGMP]).

Table 1. The mass spectrometry parameters for quantification of ^{16}O- and ^{18}O-P_i.

ID	Q1	Q3	DP	EP	CE	CXP
	————amu———		——————————volts———————————			
^{16}O- P_i	96.9	78.8	-55	-10	-22	-5
		63.0	-55	-10	-62	-3
^{18}O- P_i	104.9	84.9	-55	-10	-20	-5
		67.0	-55	-10	-64	-1

Q1, quantifier precursor ion; Q3, quantifier product ions; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, Collision cell exit potential; amu, atomic mass unit (Daltons); ^{16}O- P_i; ^{16}O-orthophosphate; ^{18}O- P_i, ^{18}O-orthophosphate.

https://doi.org/10.1371/journal.pone.0229172.t001

Table 2. The mass spectrometry parameters for quantification of ^{16}O- and ^{18}O-dNMP, and internal standard dIMP.

ID	Retention Time	Q1	Q3	DP	EP	CE	CXP
	min	———amu———		——————————volts——————————			
^{16}O-dAMP	12.94	329.9	78.9	-105	-10	-58	-5
			134.1	-105	-10	-36	-9
^{18}O-dAMP		332.2	80.9	-105	-10	-58	-5
			134.1	-105	-10	-36	-9
		334.1	82.9	-105	-10	-58	-5
			134.1	-105	-10	-36	-9
		336.1	84.9	-105	-10	-58	-5
			134.1	-105	-10	-36	-9
		338.1	84.9	-105	-10	-58	-5
			134.1	-105	-10	-36	-9
^{16}O-dCMP	12.58	306.0	78.9	-85	-10	-58	-3
			110.1	-85	-10	-32	-7
^{18}O-dCMP		308.1	80.9	-85	-10	-58	-3
			110.1	-85	-10	-32	-7
		310.1	82.9	-85	-10	-58	-3
			110.1	-85	-10	-32	-7
		312.1	84.9	-85	-10	-58	-3
			110.1	-85	-10	-32	-7
		314.1	84.9	-85	-10	-58	-3
			110.1	-85	-10	-32	-7
^{16}O-dGMP	12.86	346.0	78.8	-90	-10	-66	-3
			150.1	-90	-10	-36	-11
^{18}O-dGMP		348.1	80.9	-90	-10	-66	-3
			150.1	-90	-10	-36	-11
		350.1	82.9	-90	-10	-66	-3
			150.1	-90	-10	-36	-11
		352.1	84.9	-90	-10	-66	-3
			150.1	-90	-10	-36	-11
		354.1	84.9	-90	-10	-66	-3
			150.1	-90	-10	-36	-11
^{16}O-dTMP	12.92	321.0	78.8	-70	-10	-78	-13
			124.8	-70	-10	-34	-13
^{18}O-dTMP		323.1	80.9	-90	-10	-66	-3
			124.8	-70	-10	-34	-13
		325.1	82.9	-90	-10	-66	-3
			124.8	-70	-10	-34	-13
		327.1	84.9	-90	-10	-66	-3
			124.8	-70	-10	-34	-13
		329.1	84.9	-90	-10	-66	-3
			124.8	-70	-10	-34	-13
^{16}O-dIMP	12.76	331.0	134.8	-85	-10	-34	-11
			194.9	-85	-10	-24	-5

Q1, quantifier precursor ion; Q3, quantifier product ions; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, Collision cell exit potential; amu, atomic mass unit (Daltons); dAMP, Deoxyadenosine monophosphate; dCMP, Deoxycystidine monophosphate; dGMP, Deoxyguanosine monophosphate; dTMP, Deoxythymidine monophosphate; dIMP, Deoxyidenosine monophosphate.

https://doi.org/10.1371/journal.pone.0229172.t002

Mass spectrometric analysis of ^{16}O- and ^{18}O-dNMP

Quantification of ^{16}O- and ^{18}O-dNMP species was completed using a calibration curve of ^{16}O-dCMP (≥95.0%), ^{16}O-dAMP (98-100%), ^{16}O-dGMP (≥99%), and ^{16}O-dTMP (≥99%) standards, all purchased from Millipore Sigma. The internal standard was deoxyinosine monophosphate (dIMP, Millipore Sigma), a structural analogue of the dNMP species. The chromatographic conditions and instrument parameters for dNMP quantification are in S2 Table. The QA/QC included: duplicates; spikes; and low, medium and high QC concentrations of the calibration curve. The concentration of dNMPs in mg/g soil was determined by reporting the chromatographic peak areas of the samples versus standard solution concentrations using AB Sciex Analyst® Software version 1.6.2 and correcting by the mass of soil used for DNA extraction and the final volume of the extraction (60 μL). The concentration of DNA- P_i from dNMPs was measured by adding the total concentration of each dNMP in each sample, where unlabelled dNMPs possessed 0 atoms of ^{18}O atoms in the dNMP and labelled dNMP represented dNMPs with 1, 2, 3, or 4 ^{18}O atoms.

Statistical analyses

Statistical analyses were completed using R v.3.5.1 (R Core Team, 2018). The lowest detectable concentration with a signal-to-noise ratio of 3 was designated as the limit of detection (LOD) for each species [48]. The lowest concentration in the calibration curve yielding precision and accuracy within ± 20% was defined as the lowest limit of quantification (LLOQ). These parameters were measured using AB Sciex Analyst® Software version 1.6.2.

Results

Comparing AA3 and 4000 QTRAP P_i concentrations

During the time series, the AA3 and the 4000 QTRAP measured comparable concentrations of exchangeable ^{16}O- P_i and ^{18}O- P_i (Fig 1). Both instruments revealed a decrease in exchangeable ^{16}O- P_i over the time series from ^{16}O- P_i doped microcosms (Fig 1A). In comparison, there were no trends in the quantity of endogenous ^{16}O- P_i in the control microcosms during the time series. Similar to ^{16}O- P_i, the concentration of exchangeable ^{18}O- P_i decreased overtime on both instruments with the exception of weeks 5 and 6 (Fig 1B). There were no differences in the 4000 QTRAP measured P_i from weeks 4 to 6; however, the AA3 revealed a decrease in the quantity of P_i from week 4 to weeks 5 and 6. The poor similarity between instrumental analysis of weeks 5 and 6 is likely due to human error rather than differences between instruments. The ^{18}O- P_i doped microcosms had a small invarying concentration of endogenous ^{16}O- P_i and control microcosms had no ^{18}O- P_i during the time series on the 4000 QTRAP. Generally, the 4000 QTRAP produced larger standard errors (SE) for each treatment in contrast to AA3 results. This may be because the AA3 is not as affected by the sample matrix in comparison to the MS. The LLOQ for both isotopic species on the AA3 was 0.1 mg/L. In contrast, the 4000 QTRAP LLOQ of ^{16}O- P_i was 0.2 mg/L but the LOD was 0.1 mg/L. The LLOQ for^{18}O- P_i was 0.1 mg/L and the LOD was 0.075 mg/L.

Temporal ^{16}O-P_i and ^{18}O-P_i trends by treatment

The average P_i concentration and temporal trends varied by sequential fractionation extraction method (Fig 2). There were shared characteristics between the trends of bicarbonate extracted ^{16}O- P_i and ^{18}O- P_i doped microcosms with no P_i concentration differences during the time series (Fig 2A). The quantity of ^{16}O- P_i was greater than ^{18}O- P_i as the instrument measures both doped and endogenous P_i. The bicarbonate fraction represented the most

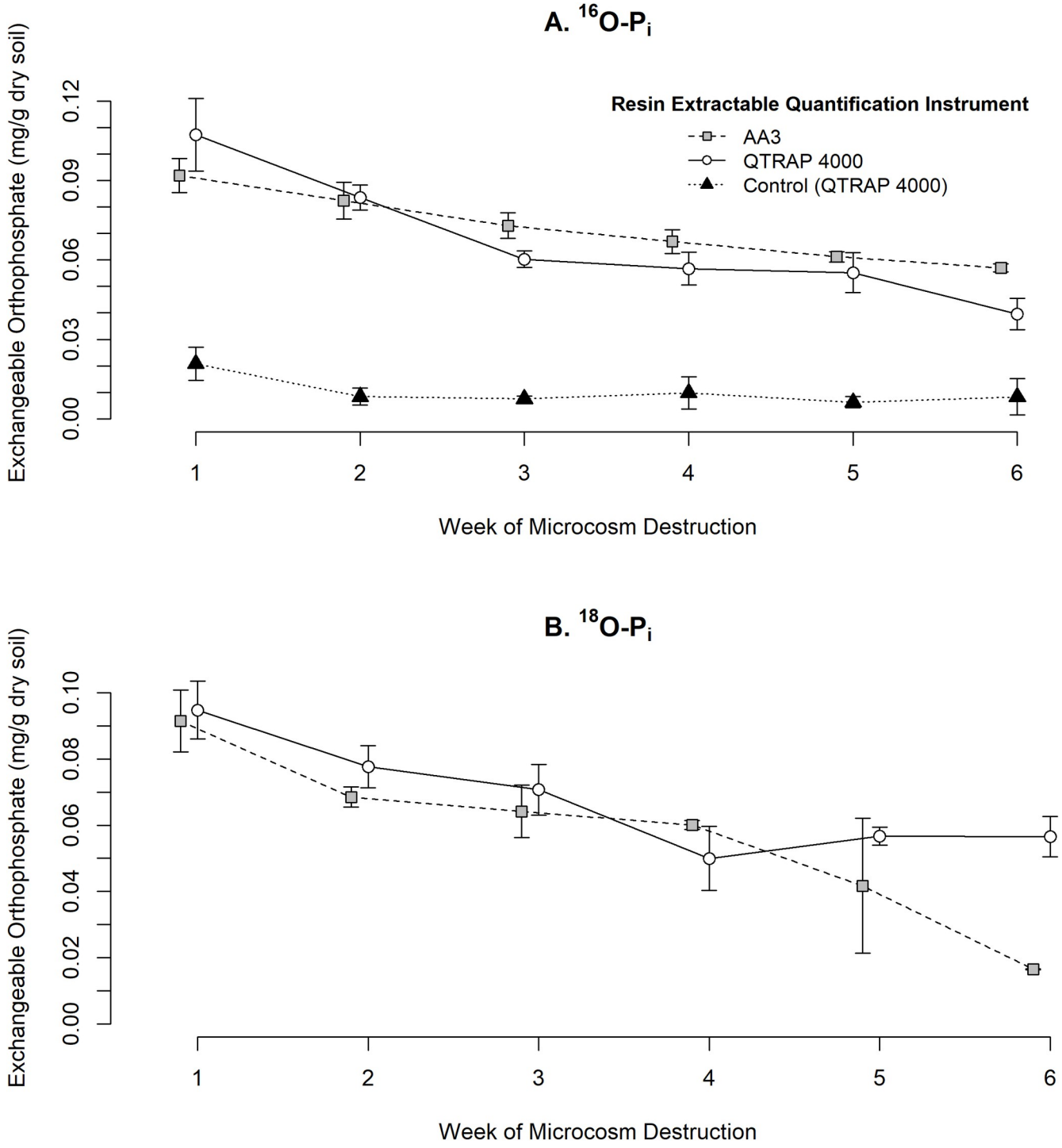

Fig 1. Concentration of resin-extractable P_i measured by AA3 and QTRAP 4000 versus week of microcosm destruction. At each time point, 0.5 g of soils were dried, sieved, and analyzed using strong anion resin strips. Each symbol represents the average of 4 microcosms ± standard errors of the estimates. Some symbols were offset on the x-axis to visualize the differences between treatment means. (A) The concentration of exchangeable ^{16}O- P_i from ^{16}O- P_i doped microcosms. (B) ^{18}O- P_i concentrations from ^{18}O- P_i doped microcosms.

https://doi.org/10.1371/journal.pone.0229172.g001

dominant source of P_i within all experimental microcosms. However, this fraction also had the greatest variation, embodied by larger SE values. Endogenous ^{16}O- P_i within control

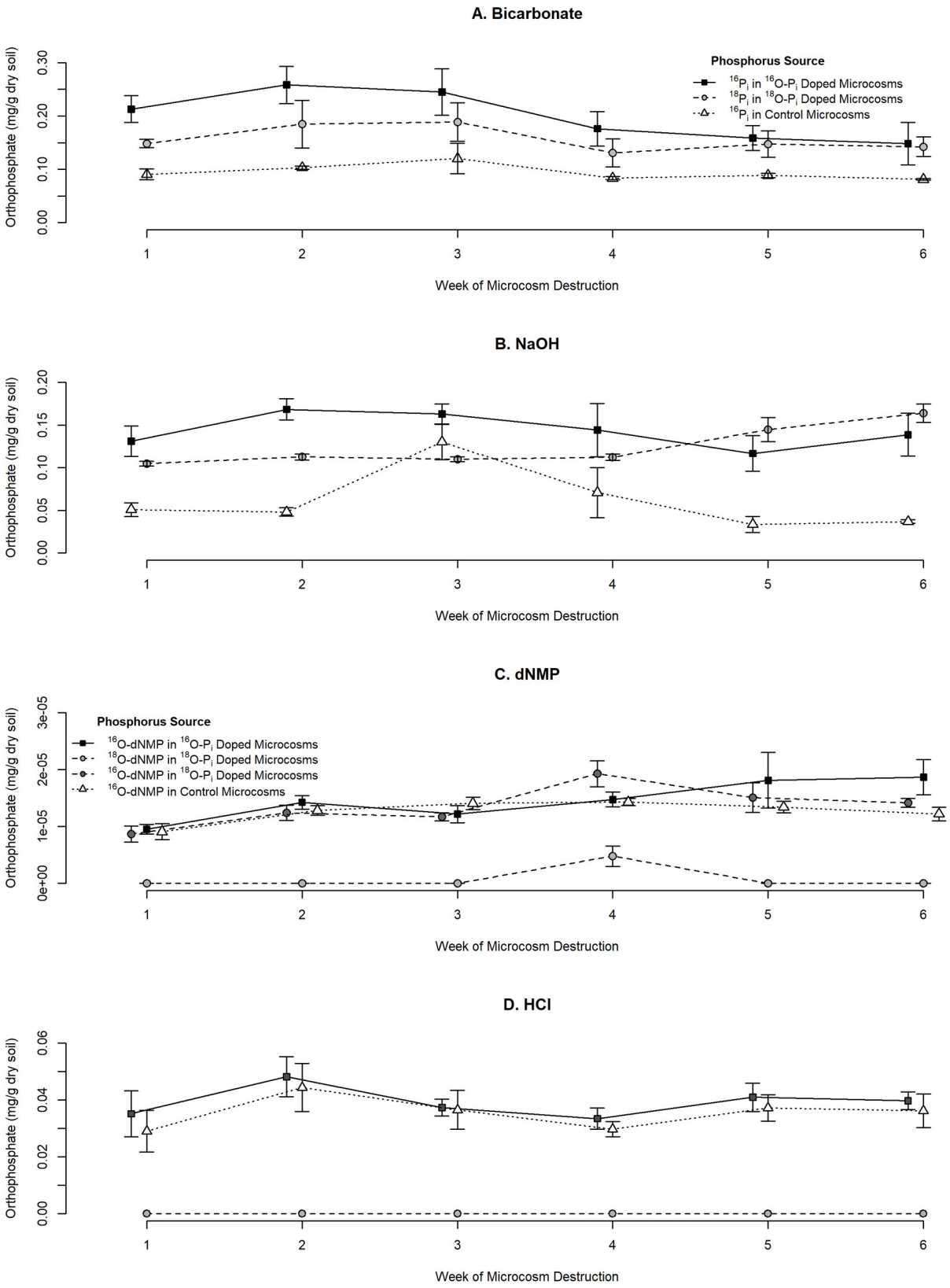

Fig 2. Temporal change in ^{16}O-P_i and ^{18}O-P_i in doped and control microcosms extracted by sequential fractionation. Each symbol represents the average concentration of 4 microcosms ± standard errors of the estimates. Sample means were off-set on the x-axes to see the

treatment differences. (A) Concentration of P_i extracted by 0.5 M bicarbonate solution. (B) The concentrtation of P_i extracted by 0.1 M NaOH solution. (C) The concentration of dNMP. (D) The concentration of P_i extracted by 1.0 M HCl solution.

https://doi.org/10.1371/journal.pone.0229172.g002

microcosms showed no trends during the time series. The LLOQ for both isotopic species was 0.3 mg/L and LOD was 0.2 mg/L.

The concentration of NaOH extracted ^{16}O- P_i and ^{18}O- P_i from doped microcosms was dissimilar during the time series (Fig 2B). The ^{16}O- P_i doped microcosms showed little variation between weeks during the time series. In comparison, weeks 5 and 6 showed a noticeable increase in NaOH extracted ^{18}O- P_i. The decrease of resin-extractable and bicarbonate fraction of ^{18}O- P_i suggests that labelled fertilizer shifted towards the NaOH pool. Once more, the concentration of ^{16}O- P_i was greater than ^{18}O- P_i as it characterized both doped and endogenous P_i. The quantity of endogenous ^{16}O- P_i within control microcosms increased until week 3 before rapidly decreasing at the end of week 6. Though the control microcosms did not receive a biostimulatory solution, the soil may have contained a small amount of endogenous nutrients that stimulated microbial communities. The LLOQ for both isotopic species was 0.2 mg/L and the LOD was 0.1 mg/L.

The first three weeks of the time series showed no differences between the treatments. Subsequently, treatments varied during week 4 (Fig 2C). The dNMP in ^{16}O- P_i doped microcosms increased until week 5, signifying a potential stall in the microbial growth. Within DNA ^{18}O concentrations were low. Specifically, the labelled portion of the dNMP molecules originated from the phosphodiester backbone, where a single ^{18}O atom was present on the product ion. The peak of ^{18}O-labelled DNA quantified on week 4 corresponded to the highest ^{16}O-labelled DNA concentration in the same microcosm. The control microcosms did not show variation between weeks. The LLOQ for both isotopic species was 0.01 mg/L and the LOD was 0.0055 mg/L.

No apparent trends were present from the 1.0 M HCl extracted P_i from all microcosms during the time series (Fig 2D). Specifically, the concentration of ^{16}O- P_i from ^{16}O- P_i doped microcosms and from the control microcosms strongly correlate, demonstrating no variances during the time series. The HCl-extracted fraction from ^{18}O- P_i microcosms measured no labelled species. This signifies that measuring isotopically labelled species within recalcitrant P fractions of soil requires a longer time series than provided. The LLOQ for both isotopic species was 0.2 mg/L and the LOD was 0.1 mg/L.

P mass balance

The total concentration of P_i during the time series varied by week, treatment, and extraction method (Fig 3). However, the amount of endogeous P_i strongly influenced the quantity of total ^{16}O- P_i during the time series (Fig 3A). The weekly mean of ^{16}O- P_i fluctuated from 0.37 to 0.56 mg/g dry soil and percent recovery varied from 94 to 144% (Table 3). As the experimental soil was inconsistently homogenized using the Japanese slabcake method before addition into the experimental units, the spatial variation in endogenous P within the experimental soils caused large disparity in mean and percent recovery of ^{16}O- P_i by week. In comparison, the average total concentration of ^{18}O- P_i by week shared similarity during the time series(Fig 3B). The mean of ^{18}O- P_i varies from 0.30 to 0.38 mg/g dry soil with a percent recovery ranging from 67 to 85%. These percent recoveries demonstrated that a substantial quantity of the doped ^{18}O- P_i was recovered during sequential fractionation. Any ^{18}O- P_i loss may be attributed to: not homogenizing the soil properly following microcosm destruction, incomplete extraction during sequential fractionation and isotope exchange between labelled biomarkers and unlabelled water by microorganisms. Overall, there was less dissimilarity in mean and

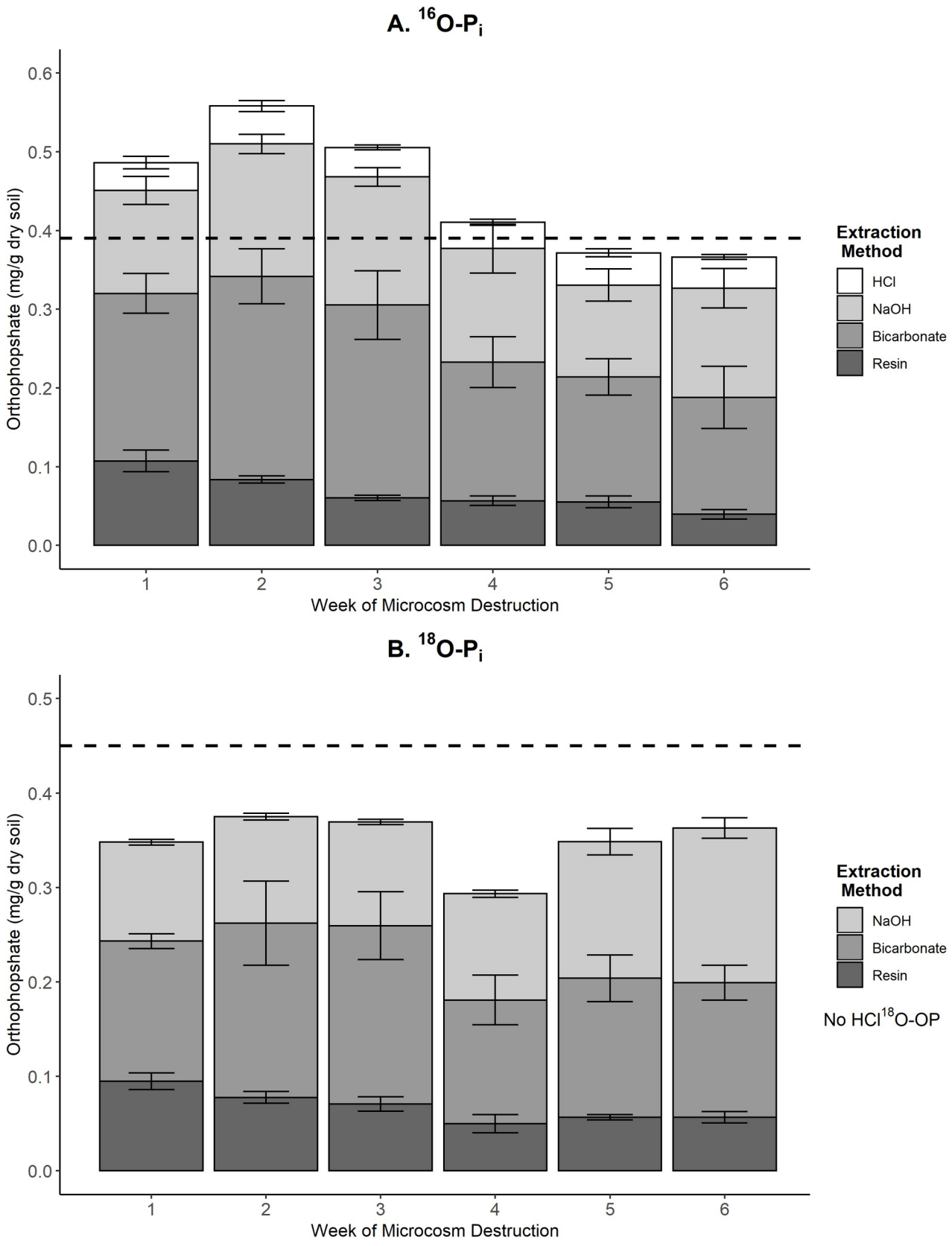
A. ^{16}O-P$_i$
Orthophopshate (mg/g dry soil)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
1
2
3
4
5
6
Week of Microcosm Destruction
Extraction Method
HCl
NaOH
Bicarbonate
Resin
B. ^{18}O-P$_i$
Orthophopshate (mg/g dry soil)
0.0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
6
Week of Microcosm Destruction
Extraction Method
NaOH
Bicarbonate
Resin
No HCl ^{18}O-OP

Fig 3. Mass balance of total P concentration from ^{16}O-P_i and ^{18}O-P_i doped microcosms by fractionation extraction. Each bar represents the average of 4 microcosms for the extraction methods (resin strips, bicarbonate, NaOH, HCl), with error bars indicating the standard error of the estimate. (A) Total ^{16}O-PI from ^{16}O- P_i doped microcosms. The dashed line represents ^{16}O- P_i doped into the microcosms (0.39 mg/g dry soil = 0.066 mmol PI). (B) Total ^{18}O- P_i from ^{18}O- P_i doped microcosms measured on the 4000 QTRAP. The dashed line represents ^{18}O- P_i doped into the microcosms (0.45 mg/g dry soil = 0.07 mmol P_i). No ^{18}O-HCl was measured in the ^{18}O- P_i doped microcosms.

https://doi.org/10.1371/journal.pone.0229172.g003

percent recovery in ^{18}O- P_i doped microcosms in comparison to ^{16}O- P_i doped microcosms. Additionally, the SE of the mean of ^{18}O- P_i doped microcosms were smaller than those of ^{16}O- P_i doped microcosms. As experimental addition was the sole source of ^{18}O- P_i into experimental units, a stronger ^{18}O- P_i percent recovery was expected. Therefore, tracking the movement of ^{18}O- P_i fertilizer produced robust information into experimental P movement over time relative to ^{16}O- P_i analyses.

Discussion

Benefits of methodology

In this study, we successfully tracked the movement of experimental ^{18}O- P_i using a novel mass spectrometry (MS) method. This methodology improves prior efforts to analyze ^{18}O- P_i by achieving absolute quantification of P from multiple soil pools using tandem mass spectrometry (MS/MS). Previous manuscripts focused on relative quantification of either pure samples or on a single P_i soil fraction, losing important insight into the movement of P in soils [29,38,45,49]. Absolute quantification allowed the creation of an ^{18}O- P_i mass balance to examine P pool movement and development over the time series, a unique feature to this study. While the MS and AA3 measured similar resin-extractable P_i results, the MS is a more robust instrument as it differentiates between ^{16}O and ^{18}O atoms. Additionally, the use of MS/MS provides significant benefits over ^{18}O- P_i studies that used single quadrupole instruments [29,38,45,49]. In comparison to MS/MS, single quadrupoles have lower selectivity due to interference from co-eluting compounds and matrices [50]. This is essential as the Hedley sequential extraction matrices have a negative effect on the LLOQs due to high salt concentrations, where measured limits varied from 0.075 mg/L for 0.5 M HCl to 0.3 mg/L for 0.5 M bicarbonate. Newer triple quadrupole instruments have the capacity to achieve greater sensitivity and selectivity into picogram/mL range [51], which will aid to decrease the LLOQ. Furthermore, the use of MS/MS allows for improved accuracy and reproducibility at the lower end of the calibration curve [50], permitting examination of P-deficient soils. Focusing on each dNMP of

Table 3. Mean, standard error, and percent recovery of P_i by treatment.

Week of Extraction	^{16}O-P_i			^{18}O-P_i		
	Mean	SE	PR	Mean	SE	PR
	——mg/g dry soil——		%	——mg/g dry soil—		%
1	0.49	0.034	125.3	0.34	0.005	77.0
2	0.56	0.044	143.8	0.38	0.051	84.7
3	0.51	0.032	130.3	0.37	0.021	82.6
4	0.41	0.067	105.8	0.30	0.025	66.6
5	0.37	0.030	95.8	0.35	0.018	77.7
6	0.37	0.059	94.4	0.37	0.021	81.9

The treatment of ^{16}O- P_i added 0.39 mg/g dry soil whereas the ^{18}O- P_i treatment added 0.45 mg/g dry soil to each applicable microcosm. Means represent the average of 4 microcosms by week of destruction. The percent recovery represents the P_i mean divided by P_i concentration added by treatment.
SE,standard error; PR, percent recovery ^{16}O- P_i; ^{16}O-orthophosphate; ^{18}O- P_i, ^{18}O-orthophosphate.

https://doi.org/10.1371/journal.pone.0229172.t003

DNA in an ^{18}O- P_i study is a distinct characteristic of our procedure to measure the organic P pool. Previous studies concentrated on a single dNMP (dTMP), and were unable to monitor the 3 other dNMPs present in DNA [52].

Geochemical and biological Phosphorus trends

The precipitation, adsorption-desorption, and biological effects of the PI fertilizers are like previous Hedley fractionation studies (Figs 2 and S1). Similar to our results, as P_i declined in the resin extractable pool, the concentration of bicarbonate P_i increased [53]. In agreement with both Qian and Schoenau [54] and Wagar et al. [55], we report that bicarbonate P_i represents the largest proportion extracted following fertilizer application. Short term studies often demonstrate a slight increase in P within recalcitrant fractions, where solubility decreases as P geochemically fixes to Ca-phosphates [10,56]. The ^{18}O- P_i NaOH fraction concentration increased overtime from more labile pools; however, this was not apparent in ^{16}O- P_i NaOH pool. As ^{18}O- P_i is not naturally occurring, the short term experiment provided greater sensitivity into the movement of labelled fertilizer to more recalcitrant fractions. Finally, the absence of fertilizer P movement to the HCl pools agrees with Helfenstein et al. [57] where the development of HCl-extractable P takes years to centuries to form.

Our study reveals that isotopic composition does not influence P movement; however, previous studies are divided on whether labelled PI influences geochemical and biological processes. The labile fractions results are in agreement with previous studies that reported the sorption of ^{16}O- and ^{18}O- P_i to synthetic ferrihydrite reached equilibrium after 20 hours under abiotic conditions [58]. Although, our findings are in disagreement with Melby et al. [29], which reported that multiple ^{18}O atoms within P_i causes greater sorption to soils. Moreover, the shared trends from resin-extractable and bicarbonate P extractions suggests that nutrient uptake by microbial communities is likely not influenced by isotopic composition of P_i. This is in contrast to results stating that microbial communities prefer lighter isotopologues [59]. Our study outcomes are consistent with Mamet et al. [24] who reported that microorganisms do not have a preference for P_i by isotopic composition. While the MS measured resin-extractable ^{18}O- P_i after 6 weeks, others found that the concentration of the labelled species becomes negligible after 50 days in aerobic non-sterilized soils [60]. Conflicting results may be attributed to anaerobic versus aerobic conditions as biological activity is much greater in the presence of O, producing a higher microbial P_i uptake [61]. Alternatively, Melby et al. [29] did not consider the movement of ^{18}O-P_i to other pools of P within the soil system.

Trends in NaOH and DNA P_i

The small concentration of P_i from dNMP, one of the largest pools of organic P [62], signifies that the majority of the NaOH pool is in inorganic forms of P, specifically Fe and Al species [46]. Nevertheless, NaOH and dNMP results displayed the greatest fluctuations over time and rapid ^{18}O- P_i delabelling compared to the other fractions; however, a small amount of labelled DNA remained within the macromolecule. Microorganisms negatively impacted DNA labelling as the greatest period of activity in ^{18}O- P_i microcosms synthesized a small concentration of ^{18}O-dNMP. The very small concentration ^{18}O-labelled DNA is in agreement with previous studies that found that biotic systems rapidly exchange isotopes between P_i and water [27,63]. Previous studies established that dNMP labels quickly following incubation in $H_2{}^{18}O$ doped soil [52,64]. However, as our study focuses on ^{18}O- P_i uptake by microorganisms, the amount of time required to incorporate the isotopically labelled substrates will differ from $H_2{}^{18}O$ studies. Future work into organic P movement requires consideration into the species not

measured by the NaOH fraction, such as organic matter isolated by the labile-resin and bicarbonate fraction [5].

Comparison of 32/33P to 18O-P_i mass spectrometry

The absence of ^{18}O- P_i in the HCl extraction fractions after 6 weeks confirmed the radioisotopes ^{32}P and ^{33}P are incapable of offering an appropriate experimental length to follow P fertilizers. Measuring the suitable kinetic equilibrium time to produce recalcitrant ^{18}O- P_i minerals may not be conducted using $^{32/33}$P as natural decay limits analysis to a few months [65]. The abiotic stability ^{18}O- P_i provides the availability of longer experimental times to follow the fate of fertilizer to inaccessible forms of P minerals. Furthermore, we were able to decipher temporal movement of biotic activity from the concentration of dNMP from DNA; a task not possible with radioisotopes.

Comparison of δ18O to 18O-P_i mass spectrometry

The limited sample preparation and the capability for absolute quantification favours HLPC-MS/MS measurement of ^{18}O- P_i over δ^{18}O to facilitate examination of P dynamics. In comparison to IRMS, ESI is a soft ionization MS technique that generates minimal fragmentation to the gas phase molecule, allowing for structural information [66]. Soils require substantial δ^{18}O characterization as isotopic values vary both temporally and spatially; therefore, individual sources of P_i within each soil will possess unique signatures [67]. Co-eluting anions, such as nitrates and sulfates, and ions, like Na^+ and Cl^-, interfere with δ^{18}O analysis in P_i by IRMS [49,68]. While the use of Ag_3PO_4 is considered the most suitable standard for ^{18}O measurement, there are current no certified standards [69]. Alternatively, P_i retains its shape during MS/MS quantification, as the instrument examines the mass to charge ratio of gas phase ions prior to and after the collision cell. Soil samples for MS analysis do not require background characterization as ^{18}O- P_i was absent from both the ^{16}O- P_i doped and control microcosms here and in previous studies [60]. The MS directly measures the concentration of P_i by using calibration curves for both ^{16}O- P_i and ^{18}O- P_i. While the isotopic forms of P_i co-elute, both may be used for MS/MS quantification as the species will not suppress the response of the analytes [70]. Mass spectrometry instruments do not affect P_i labelling as Alvarez et al. [49] reported that O exchange within phosphate species did not occur during MS quantification. Moreover, a quantifiable amount of naturally occurring ^{18}O- P_i is unlikely to occur due to low environmental abundance [59]. Therefore, replacing current δ^{18}O techniques with measuring P_i using MS will circumvent inconsistencies with quantification of the isotopically labelled substrates movement within soil ecosystems.

Sample clean-up

While the methodology for measuring experimental ^{18}O-P_i is applicable to all soil types, samples require cleanup prior to quantification on the MS/MS to remove excess salts from extraction solutions. Excessive salts interfere with detection and ionization by causing ion suppression [71]. Isolation of the respective P pools uses bicarbonate and NaOH solutions resulting in high sodium content and high pH. Our study sample preparations used resin beads to replace Na^+ with H^+, effectively lowering the concentration of salts and pH simultaneously. MS/MS requires lower pH to allow for protonation of gas phase ions [72]. For soils higher in Al and Fe, the Bray-1 and Mehlich-3 P extraction methods also generate a high volume of salts [73,74]. Resin beads can replace major cations and anions with H^+ and OH^- ions. Another option to overcome ion suppression is chromatographic separation; however, this will require longer chromatographic runs for sample and column clean-up [71]. Overall,

proper sample preparation for MS/MS measurement of ^{18}O- P_i allows the methodology to become available for all soil types to better understand the P cycle.

Conclusions

This document presents a MS method that improves current ^{18}O-isotope analysis to define inorganic and organic P cycling within soils. This protocol is accessible for all soil types; however, MS requires specific sample preparation to remove excess ions that inhibit ionization. Biological techniques such as SIP can use this method to verify isotopic incorporation into isopycnically separated DNA. While the purpose of this manuscript was to present the methodology, we found potential to provide new information in long-term P soil dynamics from the absence of ^{18}O- P_i in the HCl fraction. Future prospects of interpreting P dynamics using the ^{18}O- P_i MS method include the combination of spectroscopic and isotopic techniques as well as the combined use of radioisotopes $^{32/33}$P with ^{18}O to understand P fertilizer in soils [57]. The method we have outlined here provides new opportunities to resolve broken links in the P cycle.

Supporting information

S1 Fig. Total temporal change in ^{16}Oxygen-orthophosphate and ^{18}Oxygen-orthophosphate in microcosms extracted by sequential fractionation. Each symbol represents the average of 4 microcosms, with error bars indicating the standard error of the estimate. (A) Quantification of **^{16}Oxygen-orthophosphate** from **^{16}Oxygen-orthophosphate** doped microcosms. (B) Quantification of **^{16}Oxygen-orthophosphate** from **^{18}Oxygen-orthophosphate** doped microcosms. (C) Quantification of **^{18}Oxygen-orthophosphate** from **^{18}Oxygen-orthophosphate** doped microcosms.
(DOCX)

S1 Table. QTRAP 4000 parameters for the optimization of ^{16}Oxygen-orthophosphate and ^{18}Oxygen-orthophosphate and the deoxynucleoside monophosphate isotopologues.
(DOCX)

S2 Table. Chromatographic and QTRAP 4000 parameters for the quantification of ^{16}Oxygen-orthophosphate and ^{18}Oxygen-orthophosphate.
(DOCX)

S3 Table. Chromatographic and QTRAP 4000 parameters for the quantification of the deoxynucleoside monophosphate isotopologues.
(DOCX)

S1 Data.
(XLSX)

Acknowledgments

The authors thank Deborah Michel for all assistance with the MS instrument.

Author Contributions

Conceptualization: Aimée Schryer, Kris Bradshaw, Steven D. Siciliano.

Data curation: Aimée Schryer.

Formal analysis: Aimée Schryer, Steven D. Siciliano.

Funding acquisition: Kris Bradshaw, Steven D. Siciliano.

Investigation: Aimée Schryer.

Methodology: Aimée Schryer, Steven D. Siciliano.

Project administration: Steven D. Siciliano.

Resources: Kris Bradshaw, Steven D. Siciliano.

Supervision: Steven D. Siciliano.

Validation: Aimée Schryer, Steven D. Siciliano.

Visualization: Aimée Schryer, Steven D. Siciliano.

Writing – original draft: Aimée Schryer.

Writing – review & editing: Aimée Schryer, Kris Bradshaw, Steven D. Siciliano.

References

1. Zohar I, Shaviv A, Klass T, Roberts K, Paytan A. Method for the Analysis of Oxygen Isotopic Composition of Soil Phosphate Fractions. Environ Sci Technol. 2010; 44(19):7583–8. https://doi.org/10.1021/es100707f PMID: 20831152
2. Malik MA, Marschner P, Khan KS. Addition of organic and inorganic P sources to soil—Effects on P pools and microorganisms. Soil Biol Biochem. 2012; 49:106–13.
3. Siciliano SD, Chen T, Phillips C, Hamilton J, Hilger D, Chartrand B, et al. Total Phosphate Influences the Rate of Hydrocarbon Degradation but Phosphate Mineralogy Shapes Microbial Community Composition in Cold-Region Calcareous Soils. Environ Sci Technol. 2016; 50(10):5197–206. https://doi.org/10.1021/acs.est.5b05911 PMID: 27082646
4. Di HJ, Condron LM, Frossard E. Isotope techniques to study phosphorus cycling in agricultural and forest soils: A review. Biol Fertil Soils. 1997; 24(1):1–12.
5. Zhu Y, Wu F, He Z, Guo J, Qu X, Xie F, et al. Characterization of organic phosphorus in lake sediments by sequential fractionation and enzymatic hydrolysis. Environ Sci Technol. 2013; 47(14):7679–87. https://doi.org/10.1021/es305277g PMID: 23731033
6. Persson P, Andersson T, Nelson H, Sjöberg S, Giesler R, Lövgren L. Surface complexes of monomethyl phosphate stabilized by hydrogen bonding on goethite (α-FeOOH) nanoparticles. J Colloid Interface Sci. 2012; 386(1):350–8. https://doi.org/10.1016/j.jcis.2012.07.042 PMID: 22901376
7. Peak D, Kar G, Hundal L, Schoenau J. Kinetics and mechanisms of phosphorus release in a soil amended with biosolids or inorganic fertilizer. Soil Sci. 2012; 177(3):183–7.
8. Wyngaard N, Cabrera ML, Jarosch KA, Bünemann EK. Phosphorus in the coarse soil fraction is related to soil organic phosphorus mineralization measured by isotopic dilution. Soil Biol Biochem. 2016; 96:107–18.
9. Oberson A, Joner EJ. Microbial Turnover of Phosphorus in Soil. In: Turner BL, Frossard E, Baldwin DS, editors. Organic Phosphorus in the Environment. Oxfordshire: CABI Publishing; 2005. p. 133–64.
10. Kruse J, Abraham M, Amelung W, Baum C, Bol R, Kühn O, et al. Innovative methods in soil phosphorus research: A review. J Plant Nutr Soil Sci. 2015; 178(1):43–88.
11. Vu DT, Armstrong RD, Sale PWG, Tang C. Phosphorus availability for three crop species as a function of soil type and fertilizer history. Plant Soil. 2010; 337:497–510.
12. Bünemann EK, Marschner P, McNeill AM, McLaughlin MJ. Measuring rates of gross and net mineralization of organic phosphorus in soils. Soil Biol Biochem. 2007; 39:900–13.
13. Oehl F, Oberson A, Sinaj S, Frossard E. Organic Phosphorus Mineralization Studies Using Isotopic Dilution Techniques. Soil Sci Soc Am J. 2001; 65:780–7.
14. Hamon RE, McLaughlin MJ. Interferences in the determination of isotopically exchangeable P in soils and a method to minimise them. Aust J Soil Res. 2002; 40(8):1383–97.
15. McBeath TM, Lombi E, McLaughlin MJ, Bünemann EK. Polyphosphate-fertilizer solution stability with time, temperature, and pH. J Plant Nutr Soil Sci. 2007; 170:387–91.
16. Frossard E, Achat DL, Bernasconi SM, Bünemann EK, Fardeau JC, Jansa J, et al. The Use of Tracers to Investigate Phosphate Cycling in Soil–Plant Systems. In: Bünemann EK, Oberson A, Frossard E,

editors. Phosphorus in Action—Biological Processes in Soil Phosphorus Cycling. Berlin: Springer-Verlag; 2011. p. 59–91.

17. Lee BYWR, Sega GA, Alford CF. Mutations Produced by Transmutation of Phosphorus-32 to Sulfur-32 within Drosophila DNA. Proc Natl Acad Sci USA. 1967; 58(4):1472–9. https://doi.org/10.1073/pnas.58.4.1472 PMID: 5237881

18. Tijssen P. Labeling of probes and their detection. In: Tijssen P, editor. Hybridization with Nucleic Acid Probes Part II: Probe Labeling and Hybridization Techniques. 24th ed. Amsterdam: Elsevier Science Publishers; 1993. p. 269–374.

19. Tolbert BM, Adams PT, Bennett EL, Hughes AM, Kirk MR, Lemmon RM, et al. Observations on the Radiation Decomposition of Some C14 Labeled Compounds. J Am Chem Soc. 1953; 75(8):1867–8.

20. Fardeau JC. Le phosphore assimilable des sols: sa représentation par un Modèle Fonctionnel À plusieurs compartiments. Agronomie. 1993; 13(4):317–31.

21. Torrent J. Rapid and Slow Phosphate Sorption by Mediterranean Soils: Effect of Iron Oxdes. Soil Sci Soc Am J. 1987; 51(1):78.

22. Jaisi DP, Blake RE. Advances in Using Oxygen Isotope Ratios of Phosphate to Understand Phosphorus Cycling in the Environment. In: Advances in Agronomy. 1st ed. Elsevier Inc.; 2014. p. 1–53.

23. West JB, Bowen GJ, Cerling TE, Ehleringer JR. Stable isotopes as one of nature's ecological recorders. Trends Ecol Evol. 2006; 21(7):408–14. https://doi.org/10.1016/j.tree.2006.04.002 PMID: 16753238

24. Mamet SD, Ma B, Ulrich A, Schryer A, Siciliano SD. Who Is the Rock Miner and Who Is the Hunter? the Use of Heavy-Oxygen Labeled Phosphate (P18O4) to Differentiate between C and P Fluxes in a Benzene-Degrading Consortium. Environ Sci Technol. 2018; 52(4):1773–86. https://doi.org/10.1021/acs.est.7b05773 PMID: 29378402

25. Kolodny Y, Luz B, Navon O. Oxygen isotope variations in phosphate of biogenic apatites, I. Fish bone apatite—rechecking the rules of the game. Earth Planet Sci Lett. 1983; 64:398–404.

26. Blake RE O'Neil JR, Garcia GA. Oxygen isotope systematics of biologically mediated reactions of phosphate: I. Microbial degradation of organophosphorus compounds. Geochim Cosmochim Acta. 1997; 61(20):4411–22.

27. Lecuyer C, Grandjean P, Sheppard SMF. Oxygen isotope exchange between dissolved phosphate and water at temperatures $\leq$135˚C: inorganic versus biological fractionations. Geochim Cosmochim Acta. 1999; 63(6):855–62.

28. Granger SJ, Harris P, Peukert S, Guo R, Tamburini F, Blackwell MSA, et al. Phosphate stable oxygen isotope variability within a temperate agricultural soil. Geoderma. 2017; 285:64–75. https://doi.org/10.1016/j.geoderma.2016.09.020 PMID: 28050050

29. Melby ES, Soldat DJ, Barak P. Biological decay of 18O-labeled phosphate in soils. Soil Biol Biochem. 2013; 63:124–8.

30. Sun M, Jaisi DP. Distribution of inositol phosphates in animal feed grains and excreta: distinctions among isomers and phosphate oxygen isotope compositions. Plant Soil. 2018; 430(1–2):291–305.

31. Jaisi DP, Blake RE, Liang Y, Chang SJ. Investigation of Compound-Specific Organic-Inorganic Phosphorus Transformation Using Stable Isotope Ratios in Phosphate. In: He Z, Zhang H, editors. Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment. Springer; 2014. p. 267–92.

32. Stout LM, Joshi SR, Kana TM, Jaisi DP. Microbial activities and phosphorus cycling: An application of oxygen isotope ratios in phosphate. Geochim Cosmochim Acta. 2014; 138:101–16.

33. Young M, McLaughlin K, Kendall C, Stringfellow W, Rollog M, Elsbury K, et al. Characterizing the Oxygen Isotopic Composition of Phosphate Sources to Aquatic Ecosystems. Environ Sci Technol. 2009; 43(14):5190–6. https://doi.org/10.1021/es900337q PMID: 19708340

34. McLaughlin K, Cade-Menun BJ, Paytan A. The oxygen isotopic composition of phosphate in Elkhorn Slough, California: A tracer for phosphate sources. Estuar Coast Shelf Sci. 2006; 70(3):499–506.

35. Kreuzer-Martin HW. Stable Isotope Probing: Linking Functional Activity to Specific Members of Microbial Communities. Soil Sci Soc Am J. 2007; 71(2):611–9.

36. Radajewski S, Ineson P, Parekh NR, Murrell JC. Stable-isotope probing as a tool in microbial ecology. Nature. 2000; 403(6770):646–9. https://doi.org/10.1038/35001054 PMID: 10688198

37. Manefield M, Whiteley a. S, Bailey MJ. What can stable isotope probing do for bioremediation? Int Biodeterior Biodegradation. 2004; 54(2–3):163–6.

38. Melby ES, Soldat DJ, Barak P. Preferential Soil Sorption of Oxygen-18-Labeled Phosphate. Commun Soil Sci Plant Anal. 2013; 44(16):2371–7.

39. El-Aneed A, Cohen A, Banoub J. Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. Appl Spectrosc Rev. 2009; 44:210–30.

40. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003; 422(6928):198–207. https://doi.org/10.1038/nature01511 PMID: 12634793

41. Awad H, Khamis MM, El-Aneed A. Mass spectrometry, review of the basics: Ionization. Appl Spectrosc Rev. 2015; 50(2):158–75.

42. Patel R, Roy M, Dutta G. Mass spectrometry—a review. Vet World. 2012; 5(3):185–92.

43. Colinge J, Masselot A, Giron M, Dessingy T, Magnin J. OLAV: Towards high-throughput tandem mass spectrometry data identification. Proteomics. 2003; 3:1454–63. https://doi.org/10.1002/pmic.200300485 PMID: 12923771

44. Gross JH. Mass Spectrometry. 2nd ed. Heidelberg: Springer; 2011. 753 p.

45. Melby ES, Soldat DJ, Barak P. Synthesis and detection of oxygen-18 labeled phosphate. PLoS One. 2011; 6(4):e18420. https://doi.org/10.1371/journal.pone.0018420 PMID: 21483747

46. Tiessen H, Moir JO. Characterization of Available P by Sequential Extraction. In: Carter MR, Gregorich E., editors. Soil Samping and Methods of Analysis. 2nd Editio. Boca Raton, Florida: CRC Press; 2007. p. 75–86.

47. Bochkov D V., Khomov V V., Tolstikova TG. Hydrolytic Approach for Production of Deoxyribonucleoside- and Ribonucleoside-5'-Monophosphates and Enzymatic Synthesis of Their Polyphosphates. Biokhimiya. 2004; 71(1):97–102.

48. Buse J, Badea I, Verrall RE, El-Aneed A. A general liquid chromatography tandem mass spectrometry method for the quantitative determination of diquaternary ammonium gemini surfactant drug delivery agents in mouse keratinocytes' cellular lysate. J Chromatogr A. 2013; 1294:98–105. https://doi.org/10.1016/j.chroma.2013.04.031 PMID: 23659981

49. Alvarez R, Evans LA, Milham P, Wilson MA, Jaisi DP, Blake RE, et al. Analysis of oxygen-18 in orthophosphate by electrospray ionisation mass spectrometry. Int J Mass Spectrom. 2000; 203(1–3):177–86.

50. Josephs JL, Sanders M. Creation and comparison of MS/MS spectral libraries using quadrupole ion trap and triple-quadrupole mass spectrometers. Rapid Commun Mass Spectrom. 2004; 18(7):743–59. https://doi.org/10.1002/rcm.1402 PMID: 15052556

51. Luo G, Li Y, Bao JJ. Development and application of a high-throughput sample cleanup process based on 96-well plate for simultaneous determination of 16 steroids in biological matrices using liquid chromatography-triple quadrupole mass spectrometry. Anal Bioanal Chem. 2016; 408(4):1137–49. https://doi.org/10.1007/s00216-015-9213-1 PMID: 26738495

52. Aanderud ZT, Lennon JT. Validation of heavy-water stable isotope probing for the characterization of rapidly responding soil bacteria. Appl Environ Microbiol. 2011; 77(13):4589–96. https://doi.org/10.1128/AEM.02735-10 PMID: 21551285

53. Cross AF, Schlesinger WH. A literature review and evaluation of the Hedley fractionation: Applications to the biogeochemical cycle of soil phosphorus in natural ecosystems. Geoderma. 1995; 64:197–214.

54. Qian P, Schoenau JJ. Fractionation of P in soil as influenced by a single addition of liquid swine manure. Can J Soil Sci. 2000; 80:561–6.

55. Wagar BI, Stewart JWB, Moir JO. Changes with Time in the Form and Availability of Residul Fertilizer Phosphorus on Chernozemic Soils. Can J Soil Sci. 1986; 66:105–19.

56. Negassa W, Leinweber P. How does the Hedley sequential phosphorus fractionation reflect impacts of land use and management on soil phosphorus: A review. J Plant Nutr Soil Sci. 2009; 172:305–25.

57. Helfenstein J, Tamburini F, von Sperber C, Massey MS, Pistocchi C, Chadwick OA, et al. Combining spectroscopic and isotopic techniques gives a dynamic view of phosphorus cycling in soil. Nat Commun. 2018; 9(1):1–9. https://doi.org/10.1038/s41467-017-02088-w

58. Jaisi DP, Blake RE, Kukkadapu RK. Fractionation of oxygen isotopes in phosphate during its interactions with iron oxides. Geochim Cosmochim Acta. 2010; 74(4):1309–19.

59. Blake RE, O'Neil JR Surkov A V. Biogeochemical cycling of phosphorus: Insights from oxygen isotope effects of phosphoenzymes. Am J Sci. 2005; 305(6–8):596–620.

60. Melby ES, Soldat DJ, Barak P. Biological decay of 18O-labeled phosphate in soils. Soil Biol Biochem. 2013; 63:124–8.

61. Maloney LC, Nelson YM, Kitts CL. Characterization of Aerobic and Anaerobic Microbial Activity in Hydrocarbon-Contaminated Soil. In: Gavaskar AR, Chen ASC, editors. Remediation of Chlorinated and Recalcitrant Compounds—2004 Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds (Monterey, CA; May 2004). 2004.

62. McLaren TI, Smernik RJ, McLaughlin MJ, McBeath TM, Kirby JK, Simpson RJ, et al. Complex Forms of Soil Organic Phosphorus-A Major Component of Soil Phosphorus. Environ Sci Technol. 2015; 49(22):13238–45. https://doi.org/10.1021/acs.est.5b02948 PMID: 26492192

63. Larsen S, Middelboe V, Johansen HS. The fate of18O labelled phosphate in soil/plant systems. Plant Soil. 1989; 117(1):143–5.

64. Schwartz E. Characterization of growing microorganisms in soil by stable isotope probing with H218O. Appl Environ Microbiol. 2007; 73(8):2541–6. https://doi.org/10.1128/AEM.02021-06 PMID: 17322324

65. Bünemann EK. Assessment of gross and net mineralization rates of soil organic phosphorus—A review. Soil Biol Biochem. 2015; 89:82–98.

66. Wang Y, Sun J, Qiao J, Ouyang J, Na N. A "Soft" and "Hard" Ionization Method for Comprehensive Studies of Molecules. Anal Chem. 2018; 90:14095–9. https://doi.org/10.1021/acs.analchem.8b04437 PMID: 30422630

67. Granger SJ, Yang Y, Pfahler V, Hodgson C, Smith AC, Le Cocq K, et al. The stable oxygen isotope ratio of resin extractable phosphate derived from fresh cattle faeces. Rapid Commun Mass Spectrom. 2018; 32(9):703–10. https://doi.org/10.1002/rcm.8092 PMID: 29490108

68. Tamburini F, Pfahler V, von Sperber C, Frossard E, Bernasconi SM. Oxygen Isotopes for Unraveling Phosphorus Transformations in the Soil–Plant System: A Review. Soil Sci Soc Am J. 2014; 78(1):38.

69. Tamburini F, Bernasconi SM, Angert A, Weiner T, Frossard E. A method for the analysis of the δ18O of inorganic phosphate extracted from soils with HCl. Eur J Soil Sci. 2010; 61(6):1025–32.

70. Hewavitharana AK. Matrix matching in liquid chromatography–mass spectrometry with stable isotope labelled internal standards—Is it necessary? J Chromatogr A. 2011; 1218(2):359–61. https://doi.org/10.1016/j.chroma.2010.11.047 PMID: 21159347

71. Annesley TM. Ion suppression in mass spectrometry. Clin Chem. 2003; 49(7):1041–4. https://doi.org/10.1373/49.7.1041 PMID: 12816898

72. Liigand J, Laaniste A, Kruve A. pH Effects on Electrospray Ionization Efficiency. J Am Soc Mass Spectrom. 2017; 28(3):461–9. https://doi.org/10.1007/s13361-016-1563-1 PMID: 27966175

73. Bray RH, Kurtz LT. Determination of Total, Organic, and Available Forms of Phosphorus in Soils. Vol. 59, Soil Science. 1945. p. 39–46.

74. Mehlich A. Mehlich 3 soil test extractant: A modification of Mehlich 2 extractant. Commun Soil Sci Plant Anal. 1984; 15(12):1409–16.