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EDITED BY
Federica Tamburini,
ETH Zürich, Switzerland

REVIEWED BY
Sebastian Loeppmann,
University of Kiel, Germany
Steffen A. Schweizer,
Technical University of Munich, Germany

*CORRESPONDENCE
Daniel Wasner
daniel.wasner@usys.ethz.ch
Wolfgang Wanek
wolfgang.wanek@univie.ac.at

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Tracing ^{33}P-labelled organic phosphorus compounds in two soils: New insights into decomposition dynamics and direct use by microbes

Daniel Wasner[1,2]*, Judith Prommer[2], David Zezula[3], Maria Mooshammer[2], Yuntao Hu[4] and Wolfgang Wanek[2]*

[1]Soil Resources, Department of Environmental Systems Science, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland, [2]Division of Terrestrial Ecosystem Research, Department of Microbiology and Ecosystem Science, Center of Microbiology and Environmental Systems Science, Vienna, Austria, [3]Department of Environment and Biodiversity, Paris Lodron University of Salzburg, Salzburg, Austria, [4]Environmental Genomics and Systems Biology, Lawrence Berkeley National Laboratory, Berkeley, CA, United States

Introduction: Organic phosphorus (Po) compounds constitute an important pool in soil P cycling, but their decomposition dynamics are poorly understood. Further, it has never been directly tested whether low molecular weight Po compounds are taken up by soil microbes in an intact form, which reduces the dependence of their P acquisition on extracellular phosphatases.

Methods: We investigated the short-term fate (24 h) of five 33P-labelled Po compounds (teichoic acids, phospholipids, DNA, RNA and soluble organophosphates) and 33P-labelled inorganic P (Pi) in two soils.

Results: We found indications that soil microbial breakdown of phosphodiesters was limited by the depolymerization step, and that direct microbial uptake of Po occurred to a substantial extent.

Discussion: We postulate a trade-off between direct Po uptake and complete extracellular Po mineralization. These findings have profound consequences for our understanding of microbial P cycling in soils.

KEYWORDS

organic phosphorus, soil microbes, phosphorus uptake, decomposition, depolymerization, microbial activity, phosphatase, soil phosphorus cycle

1 Introduction

Phosphorus (P) is a key constituent of the cellular machinery across all life forms, and it is increasingly recognized that the P cycle has important implications for global carbon (C) and nitrogen (N) cycling, as the dynamics of these cycles are closely interlinked (1). In contrast to the high demand for P by organisms (2–4), the bioavailability of P in soils - the prime source of P in

terrestrial ecosystems – can be low, thereby often creating a situation of P limitation in ecosystems. The amount of bioavailable P in soils thus represents a crucial bottleneck in P cycling, as it sets a frame for how much P can enter the biotic component of the terrestrial P cycle.

Bioavailable P in soils is considered to be mainly supplied in inorganic form (P_i) by desorption/dissolution processes from the solid phase and by decomposition and mineralization of organic P (P_o) compounds, with their relative contribution strongly varying between ecosystems (5, 6). Organic P constitutes 20-80% of total soil P (7, 8), and is therefore an essential component in the terrestrial P cycle. The pool of P_o consists of a highly heterogeneous group of compounds, such as phosphate monoesters (phosphomonoesters), phosphate diesters (phosphodiesters) and organic phosphonates (9). Moreover, a range of organic phosphorus compounds in soil still remains uncharacterized (10, 11). A considerable fraction of extractable soil P_o has been shown to consist of large molecules (10), and to be closely associated with large structures of soil organic matter (11). Different P_o compounds have different chemical properties, pathways of decomposition and therefore degrees of bioavailability to soil microbes. Microbial turnover and root cell lysis release especially phosphodiesters into soils, as those are the major P components of all living microbial and plant cells, including nucleic acids (DNA and RNA), phospholipids and teichoic acids (12–14). Different from previous estimates of the predominance of phosphomonoesters (e.g. phytates – inositol polyphosphates) in soil P_o, 2D-NMR and back-calculation of the *in situ* soil P_o speciation showed that phosphodiesters like RNA (41%), phospholipids (28%) and DNA (22%) dominate the soil P_o pool (15).

Major strides have been recently made towards a better understanding of soil P speciation, but also in soil P_i dynamics (16–19) and in linking microbial community structure with soil P availability (20–22). In contrast to these advances, much less is known about (i) the pathways and controls of soil P_o decomposition and P_o mineralization and (ii) whether or not microbes can directly utilize low molecular weight P_o compounds (23, 24). The P_o decomposition process involves cleavage of the compounds by extracellular phosphatases, which are produced by plants and microbes (23). The cleavage of phosphodiesters is mediated by specific phosphodiesterases (25–27), and the final dephosphorylation of the formed phosphomonoesters to free P_i is performed by acid and alkaline phosphomonoesterases (28). If P_i is the main form acquired by all organisms to meet their P demand and given that phosphomonoesterases are abundant and ubiquitous in soils, soil P cycling might be rate-limited by the depolymerization and cleavage of diesters to smaller oligomers and to monomers (phosphomonoesters), while dephosphorylation of phosphomonoesters to P_i is rapid (Figure 1). In this case, the initial breakdown step may determine the rate of P_o mineralization. An analogous situation is known from soil N transformation processes, where depolymerization of high molecular weight organic N compounds such as proteins, chitin or peptidoglycan represents the bottleneck of soil N cycling (29–32).

Tracing the pathways and measuring the gross rates of P_o decomposition requires homogeneously radio-isotopically labelled (^{33}P or ^{32}P) and purified P_o compounds, which are rarely (nucleotides, glucose-6-phosphate) or not at all commercially available and very expensive to obtain by custom synthesis. To our knowledge, few studies have therefore directly traced the decomposition and fate of radiotracer-labelled organic P compounds in soils. To date, only radio-P labelled RNA (33), a cytosine dinucleotide (34), phytate (35), glucose-6-P (36, 37) and bacterial or fungal necromass (38, 39) have been studied. Further, except for Harrison (33), studies have been carried out with only one P_o compound per study, thus impeding comparative assessments of

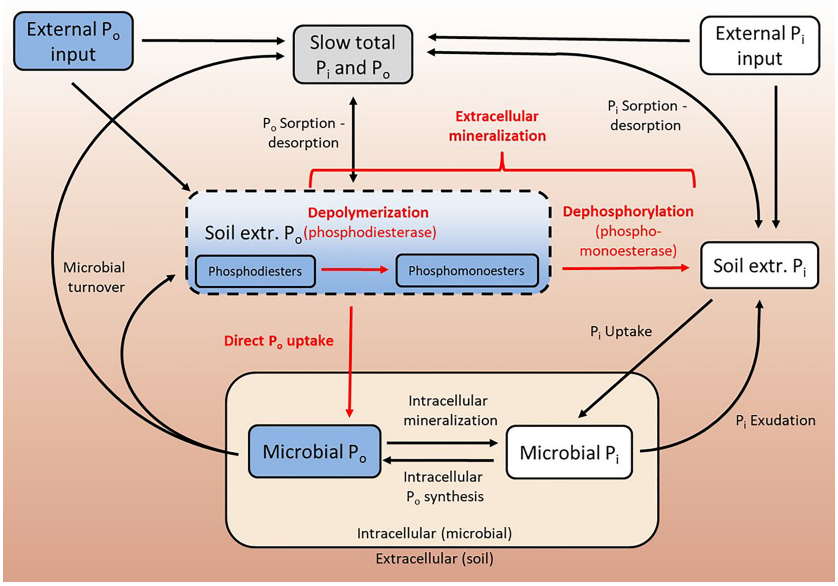

FIGURE 1
Schematic representation of soil P cycle processes. Bio-available P_o is mineralized extracellularly, where phosphodiesters are depolymerized by phosphodiesterases, yielding smaller phosphomonoesters. Phosphomonoesters can be dephosphorylated by phosphomonoesterases, and the resulting bio-available P_i can be taken up by the microbial community. In microbes, P_o compounds are synthesized and mineralized. P_i can be exuded by microbes, and P_o is mainly released into soil *via* microbial turnover. Soil P_i (and P_o) are subject to sorption and desorption processes. The current paradigm is that bio-available P_o cannot be directly taken up by soil microbes. Fluxes in red are in the focus of this study.

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decomposition dynamics of various P_o forms in different soils. Actual decomposition dynamics of most P_o compounds in soil and their controls have therefore remained largely elusive.

The current paradigm in soil microbial ecology is that microbes fully meet their P demand by the uptake of P_i. Utilization of P_o therefore only occurs after complete extracellular mineralization of P_o through phosphatases to P_i (Figure 1) (23, 24, 40). For this purpose, soil microbes produce a wide range of phosphomonoesterases (acid and alkaline phosphatases, phytases, phosphonatases) and phosphodiesterases (nucleases, phospholipases, glycerophosphodiesterases). However, some of the responsible genes are restricted to smaller phylogenetic groups while others are relatively ubiquitous (22, 41). Transporters for P_i-uptake are ubiquitous and well-studied in bacteria (39) and fungi (42). P_i is efficiently taken up against steep concentration gradients by high affinity phosphate: H^+ symporters (PHT protein family). In contrast, transporters for P_o compounds are less thoroughly studied. While some P_o uptake systems were only recently discovered (43), others are well understood in model organisms (44). For example, uptake systems for glycerol-3-phosphate (GlpT), hexose-6-phosphate (UhpT) and phosphoglycerate (PgpT) belonging to the organophosphate:phosphate antiporter (OPA) family are well known (41, 45), and have been characterized in several species of bacteria and eukaryotes (39, 46, 47). Despite awareness of the existence of microbial P_o transporters (39, 46, 48), we lack knowledge about the occurrence and potential ecological relevance of direct microbial P_o uptake in the soil environment (41). Direct uptake of P_o compounds could shortcut the extracellular mineralization step and potentially directly fuel salvage pathways or precede intracellular mineralization. Microbial use of P_o would therefore not be exclusively regulated by the production of extracellular enzymes, but rather be subject to metabolic controls that involve the balance between extracellular P_o mineralization and direct uptake.

In this study, we therefore assessed the decomposition kinetics of five ^{33}P-labelled P_o compounds and addressed the following research questions (1): Is the mineralization rate of phosphodiester compounds (teichoic acids, phospholipids, DNA, RNA) limited by the initial depolymerization and cleavage steps to monoesters, or by dephosphorylation of the intermediate phosphomonoester to free P_i? (2) Do soil microbes directly take up and utilize P_o in an intact form or only after complete mineralization to P_i? To tackle these questions, we developed a novel protocol to produce ^{33}P-labelled biochemical fractions that are relevant constituents of the soil P_o pool: cell walls (containing wall teichoic acids, TA), lipids including phospholipids (PL), DNA, RNA and soluble organophosphates (SOP). We added these five $^{33}P_o$-fractions in purified form as well as $^{33}P_i$ to two soils (temperate cropland and temperate pasture; Table 1) and traced the radioisotope with high temporal resolution over a time span of 24 h into the extractable P_o and P_i pools of soils and microbes, utilizing sequential P extraction and isobutanol fractionation of P_o and P_i.

2 Materials and methods

2.1 Soil characterization and experimental design

Two agricultural topsoils (0-15 cm, Luvisols on limestone) were collected from an arable field and a pasture site grazed by cattle at Moarhof [Trautenfels, Styria, Austria; 47°31'04" N, 14°04'31" E; 640 m a.s.l.; MAT: 7°C; MAP: 1230 mm; see (49)] in September 2016. We selected the arable and pasture soils under the assumption that contrasting management should lead to major differences in organic vs. inorganic P cycling. Phosphorus cycling in the pasture should be more dominated by organic P, through intense topsoil rooting and high microbial activity, while cycling in the cropland should be dominated by inorganic P through decadal mineral P fertilizer amendment. Soils were sieved to 2 mm and stored air dried, and two weeks prior to the experiments they were rewetted to 60% of their water holding capacity and kept at room temperature (25° C). Sand content was determined by wet sieving to 0.05 mm, after suspending the soil in 5% (w/v) sodium hexametaphosphate, clay content was calculated by using a pipette method (50) and silt content was calculated by difference. Soil pH was measured in 10 mM $CaCl_2$ in a 1:5 (w/v) soil slurry using an ISFET electrode (Sentron, Netherlands). Soil organic C and total soil N were measured by elemental analyser (CE1110, Thermo Fisher), after removal of carbonates by HCl treatment. Potential activities of extracellular phosphomonoesterases and -diesterases were measured one time after two weeks of pre-incubation, directly before the start of the experiment. The measurements were done photometrically in triplicates using p-nitrophenyl (pNP) coupled substrates (51), at native soil pH using a modified universal buffer (MUB, exact description in the reference) (52). Briefly, 1.5 g of soil were dissolved in 40 ml of MUB and dispersed by ultrasonication with an energy input of 8.5 J ml^{-1}. Subsequently, the soil slurry was mixed 1:1 (v/v) with 5 mM substrate solutions (pNP-phosphate for phosphomonoesterase, and bis-pNP-phosphate for phosphodiesterase, in MUB) in Eppendorf tubes, using wide pore pipette tips. After incubation for 1 h at 20°C under continuous shaking, the tubes were centrifuged at 10 000 g, and the supernatant was mixed with 1 M NaOH (10:1 (v/v)) to terminate enzyme activity and start

TABLE 1 Physicochemical properties and potential extracellular enzyme activities of the two soils used in this study.

Parameter	Soil	
	Arable	Pasture
Bedrock	Limestone	Limestone
Sand (%)	44.5	59.5
Silt (%)	46.6	34.7
Clay (%)	8.9	5.8
$pH_{(CaCl2)}$	5.3	5.0
Organic C (mg g^{-1})	47.0	47.9
Total N (mg g^{-1})	4.8	4.6
Total P (µg g^{-1})	1774.8	1720.8
Total extractable P (µg g^{-1})	41.6	13.7
Extractable P_i (µg g^{-1})	30.7	7.0
Extractable P_o (µg g^{-1})	10.9	6.7
Microbial P (µg g^{-1})	108.7	51.6
P diesterase (nmol pNP g^{-1} h^{-1})	391 (S.E. ± 11)	119 (S.E. ± 26)
P monoesterase (nmol pNP g^{-1} h^{-1})	630 (S.E. ± 48)	842 (S.E. ± 82)

All data presented in this table describes the soils in their unmanipulated state at the start of the incubation experiments. P_i, inorganic phosphorus; P_o, organic phosphorus; S.E., standard error.

the color reaction. Samples were diluted and transferred to a 96-well microtiter plate, and in addition a standard row was prepared by a 1:2 dilution series of 1 mM p-nitrophenol, as well as substrate controls without soil slurry. Absorbance of all wells was read at 410 nm with a microplate photometer (Tecan Infinite M200, Tecan Trading AG). Values were corrected for substrate controls, and potential extracellular enzyme activities were expressed as nmol pNP g^{-1} h^{-1}. Soil total P was determined colorimetrically with the malachite-green method (53) after ignition (450°C, 5 h) of 0.5 g dry soil and subsequent extraction with 20 mL 0.5 M H_2SO_4 for 16 h. Soil P_i was determined on unignited soils using the same protocol, and total P_o by difference. In brief, the malachite green method was applied to quantify P_i in soil extracts (after dilution with deionized water) as follows: 200 µL of the respective extracted solution were pipetted into a microtiter plate. First, 40 µL of malachite green reagent A (16.8 ml of 95 – 97% H2SO4 and 1.76 g $(NH_4)_6Mo_7O_{24}$ $4H_2O$, filled up to 100 ml with H_2O) were added, and after 10 min 40 µL of reagent B (0.875 g polyvinyl alcohol (MW = 72 000 g mol^{-1}) with 87 mg malachite green oxalate in 250 ml H_2O) were added. After 45 min of incubation, the absorbance was read at 610 nm with a microplate photometer. A 1:2 dilution series of 32 µM KH_2PO_4 was used as concentration standards. Total dissolved P (TDP) and P_i were measured in 0.5 M $NaHCO_3$ extracts after acidification with 2.75 M H_2SO_4 (10% (v/v)), with or without acid persulfate digestion with 5% (v/v) acidic persulfate (0.5 M H_2SO_4 + 0.5 M $Na_2S_2O_8$, autoclaved at 121°C for 60 min) (54), using the malachite green method. Microbial P was measured using chloroform ($CHCl_3$) fumigation (55). Soil was incubated in $CHCl_3$ saturated atmosphere for 48 h to lyse microbial cells. After fumigation, acid persulfate-digestion as described above was performed on 0.5 M $NaHCO_3$ extracts, yielding TDP of the soil plus the microbial biomass. P_i concentration was measured colorimetrically by the malachite green method. Microbial P was calculated as the difference in TDP between fumigated and unfumigated samples, applying an extraction factor k_{EP} of 0.4 based on an established literature value (55). Soil parameters are given in Table 1.

2.2 Production of ^{33}P-labelled organic compounds

To produce and purify ^{33}P-labelled organic compounds, the gram-positive bacterium *Bacillus subtilis* was grown in liquid medium amended with ^{33}P-labelled orthophosphoric acid. We then applied optimized biochemical fractionation protocols to fractionate the ^{33}P enriched culture into the five biochemical compound groups for use as substrates in the incubation experiment: cell walls containing wall teichoic acids (TA, glycerophosphate diester polymers), lipids including phospholipids (PL), DNA, RNA and soluble organophosphates (SOP). We did not investigate the fate of inositol phosphates, which are major P storage compounds in higher plants. Inositol phosphates, particularly myo-inositol hexakisphosphate (IP6), often accumulate in soils and constitute a large proportion of the soil phosphomonoester P_o pool, due to their resistance to mineralization and strong sorption (56, 57). Though microbes have been reported to produce IP6, the production of ^{33}P-labelled IP6 by plants or microbes remains very complicated, and we therefore did not include this compound class in this study. To keep losses of ^{33}P-substrate by radioactive decay as low as possible, the entire labelling and biochemical fractionation procedure was performed twice, directly before each of the two incubation experiments with either soil. A detailed description of the enrichment and fractionation protocol is provided in the supplementary material (Supplementary S1.1 Production of ^{33}P-labelled organic compounds).

2.3 Incubation experiment

We performed the incubation experiment twice, each time for one soil (i.e., arable and pasture). Each of the five ^{33}P-labelled organic substrates plus inorganic ^{33}P (P_i) were applied individually to the soils. The ^{33}P activity that was applied to each sample ranged between 1.5 and 44 kBq, depending on substrates and soils (Table 1). A schematic overview of the workflow of the incubation experiments is given in Figure S1.

Every soil x substrate combination was incubated in triplicates in 50 mL polypropylene tubes at room temperature, and incubations were stopped at seven time points (0, 0.5, 1, 2, 4, 10, 24 h). All substrates were applied in 200 µL deionized water on 2 g fresh soil equilibrated at 60% WHC, except for PL. Because of its amphiphilic character, the PL fraction was first dissolved in chloroform ($CHCl_3$) and added onto 180 mg acid washed glass beads (≤106 µm) in small aliquots. After the evaporation of $CHCl_3$, the beads were mixed with the soil aliquots and 200 µL deionized water was added. At the end of the incubations, we performed a sequential P extraction with 0.5 M $NaHCO_3$ to extract available soil P (P_{soil}) and subsequently microbial P (P_{mic}). The detailed protocol is given in the supplementary material (Supplementary S1.2 Sequential extraction). To separate P_i and P_o, isobutanol fractionation was conducted as described previously (58, 59), on aliquots of P_{soil} and P_{mic}, in which P_i binds to acidified molybdate which then is partitioned into the isobutanol phase, while P_o remains in the aqueous phase. The solution ratios applied were 1.5 mL extract plus 1.5 mL acidified molybdate, 3 mL isobutanol and 3 mL Milli-Q water. In all P fractions (P_o and P_i of each, P_{soil} and P_{mic}) as well as in unfractionated P_{soil} and P_{mic} extracts after acidification by addition of 10% (v/v) 2.75 M H_2SO_4, ^{33}P was quantified *via* liquid scintillation counting after addition of Ultima Gold Scintillation cocktail (10:1 and 16:1 liquid scintillation cocktail to sample ratio for isobutanol and aqueous samples, respectively) using a Tri-Carb1600-TR counter (Packard, PerkinElmer). To separate high molecular weight (HMW) and low molecular weight (LMW) fractions in the nucleic acid treatments, 1.5 mL of the P_{soil} extract was centrifuged through Amicon Ultra - 2 mL Centrifugal Filters (3 kDa molecular weight cut-off) for 90 min (6500 g, 15°C). The ultrafiltrate was then readjusted to 1.5 mL and rejoined the common workflow before isobutanol fractionation, whereas the concentrate was directly measured by liquid scintillation counting. Technical recoveries of added ^{33}P without soil were run in triplicates for all substrates (200 µL organic and inorganic ^{33}P substrates added to 20 mL 0.5 M $NaHCO_3$), in order to assess their behavior during isobutanol fractionation and to measure recoveries by 0.5 M $NaHCO_3$ extraction. To avoid potential contamination of P_i in the isobutanol

phase with extracted phospholipids, 2.5 mL of P_{soil} and P_{mic} of the PL treatment were briefly mixed with 1 mL of $CHCl_3$ and phases were separated before isobutanol fractionation of the aqueous phase.

Concentrations of P_i were measured colorimetrically in the isobutanol P_i fractions by applying the molybdenum-blue method (58), and in P_{soil} and P_{mic} after acidification with 2.75 M H_2SO_4 (10% (v/v)) by using the malachite-green method (53). Total dissolved P (TDP) was also quantified by the malachite-green method after digestion of P_o in P_{soil} and P_{mic} with 5% (v/v) acidic persulfate (0.5 M H_2SO_4 + 0.5 M $Na_2S_2O_8$, autoclaved at 121°C for 60 min). P_o concentrations were then calculated as difference between TDP and P_i.

2.4 Calculations and statistical analysis

All measured ^{33}P activities were corrected for radioactive decay. Technical recoveries showed that large organic molecules do not partition into the aqueous P_o fraction during isobutanol fractionation, but are precipitated at the interlayer between the two phases. The aqueous fraction of the isobutanol fractionation therefore does not represent the entire $^{33}P_o$ fraction. For this reason, in the treatments with TA, PL and SOP, we calculated the activity of $^{33}P_o$ in soil as $^{33}P_{soil}$ (*i.e.* total ^{33}P in P_{soil} - extract before isobutanol fractionation) minus $^{33}P_i$, and similarly the activity of $^{33}P_o$ in the microbial pool as $^{33}P_{mic}$ (*i.e.* total ^{33}P in P_{mic} extract before isobutanol fractionation) minus $^{33}P_i$. In the treatment with inorganic P, the ^{33}P activities in the P_i and P_o fraction of soil were proportionally fitted to P_{soil} (*i.e.* total ^{33}P in P_{soil} extract before isobutanol fractionation). In the nucleic acid treatments, the ^{33}P activities in the P_i and P_o fraction of soil were proportionally fitted to P_{soil} minus P in the HMW fraction (*i.e.* total ^{33}P in P_{soil} - extract before isobutanol fractionation minus ^{33}P in HMW-NA fraction). This is because ^{33}P in the concentrate of the ultrafiltration was quantified directly without undergoing isobutanol fractionation, and therefore was not affected by the bias arising through interlayer precipitation. ^{33}P recoveries in the different organic and inorganic fractions of P_{soil} and P_{mic} were calculated relative to the decay-adjusted technical recoveries, and specific activities (SA) of the P_o pools were calculated as Bq μg^{-1} P_o-P using the P_o concentrations obtained with acidic persulfate digestion and subsequent malachite-green quantification.

We calculated the degree of the mineralization of ^{33}P labelled P_o-substrates as the percentage of soil extractable ^{33}P present in inorganic form. This calculation was done for each time point and all five ^{33}P-labelled organic substrates and is subsequently referred to as the degree of substrate mineralization. This measure informs on the form (P_o vs. P_i) of available P (derived from the ^{33}P-labelled substrate) in the extractable soil pool.

We estimated the biosynthesis of $^{33}P_o$ from $^{33}P_i$ taken up for all organic treatments, based on our observations of P_o biosynthesis in the P_i treatments. For this, we assumed that the microbial communities of each soil allocate a fixed percentage of the available amendment-derived P_i into intracellular P_o biosynthesis. Based on the values from the P_i treatment, we calculated this percentage for each soil and time point as the microbial $^{33}P_o$ activity over the soil $^{33}P_i$ activity *100. To calculate the activities of ^{33}P invested into biosynthesis for each soil, time point and treatment, we multiplied the observed soil $^{33}P_i$ activities in the organic treatments with this factor. Finally, microbial $^{33}P_o$ was corrected for biosynthesis by subtraction of this activity, and to obtain $^{33}P_o$:$^{33}P_i$ ratios of cumulative ^{33}P uptake, the same activity was added to microbial $^{33}P_i$. By basing these calculations for each treatment and time point on the actual measured soil $^{33}P_i$ activities, the estimate of P_o biosynthesis from P_i with this method accounts for P_i sorption over time, extracellular substrate mineralization and P_i contaminations of the added substrates.

All statistical analyses were performed with R statistical software (R 3.5.2). Phosphatase activities and $^{33}P_i$ recoveries (P_i treatment, at 0 h and 10 h autoclaved) were compared between the soils with either Student`s t-Test or Welch`s t-Test after testing homogeneity of variance with the Bartlett test. After testing the assumption of variance homogeneity with Levene's tests, ANOVA was performed to test whether soil P_i or TDP pools changed after substrate addition. *Post-hoc*-tests (Fisher's least significant difference test) were applied to test for pair-wise differences between the treatments and native pool sizes. After testing the assumption of variance homogeneity with Levene's tests, two-way-ANOVA was applied to test for differences in extraction efficiencies between the two soils and the six substrates. Linear regression was applied to the $^{33}P_o$:$^{33}P_i$ ratios of microbes versus the degree of substrate mineralization of extractable soil P for all organic substrates in both soils. The assumption of heteroscedasticity of the models was tested with the Breusch-Pagan test, and in case of violation weighted least squares regression was applied, using the inverse of the fitted values as weights. We tested for differences in the dynamics of the labelled substrates between the soils, and for changes of ^{33}P recovery over time. For this, we applied factorial repeated measures ANOVA to the ^{33}P recoveries of all pools, using the R-package "ez". The assumption of sphericity was tested with the Mauchley's test, and in case of violation Greenhouse-Geisser (GG) adjustment was applied. Significant outliers from the time series were identified with Grubb`s test.

3 Results

3.1 General observations about the experimental design

The addition of organic ^{33}P substrates only caused negligible changes in extractable soil P_i (-8.8% to + 6.3%) and P_o (+ 0.8% to + 77.3%) concentrations relative to the P_i treatment (no 'spike', Table S2). This means that soil P concentrations of the treatments were similar to natural *in situ* conditions. More specifically, after $^{33}P_o$ substrate additions (at time point zero), only the extractable soil P_o pool of the pasture soil varied significantly between treatments (ANOVA, p-value < 0.05), increasing by 1%-77% relative to unamended controls, i.e. reaching 101% to 177% of the native P_o pool. Addition of $^{33}P_o$ substrate neither affected extractable soil P_i pools in pasture (ANOVA, p-value = 0.34) nor in arable (ANOVA, p-value = 0.58) soils.

Mineralization, uptake and immobilization processes of the applied ^{33}P compounds took place very rapidly, within the first 2 to 4 h of incubation (Figure 2), and markedly slowed down afterwards. This shows that the short observation period of 24 h was suitable to observe trends in the decomposition of the various P_o substrates, while minimizing the potential effect of intesa PC10 ndelay po ang response ng pc rfering longer-term processes such as remineralization and microbial turnover.

3.2 Recovery dynamics of ^{33}P in the extractable soil pool

3.2.1 General recovery dynamics of ^{33}P in the extractable soil pool

The ^{33}P recovery kinetics of the amended substrates (P_i, SOP, TA, PL, DNA, RNA) from soil showed differences and commonalities between the different $^{33}P_o$ compounds as well as between the two soils

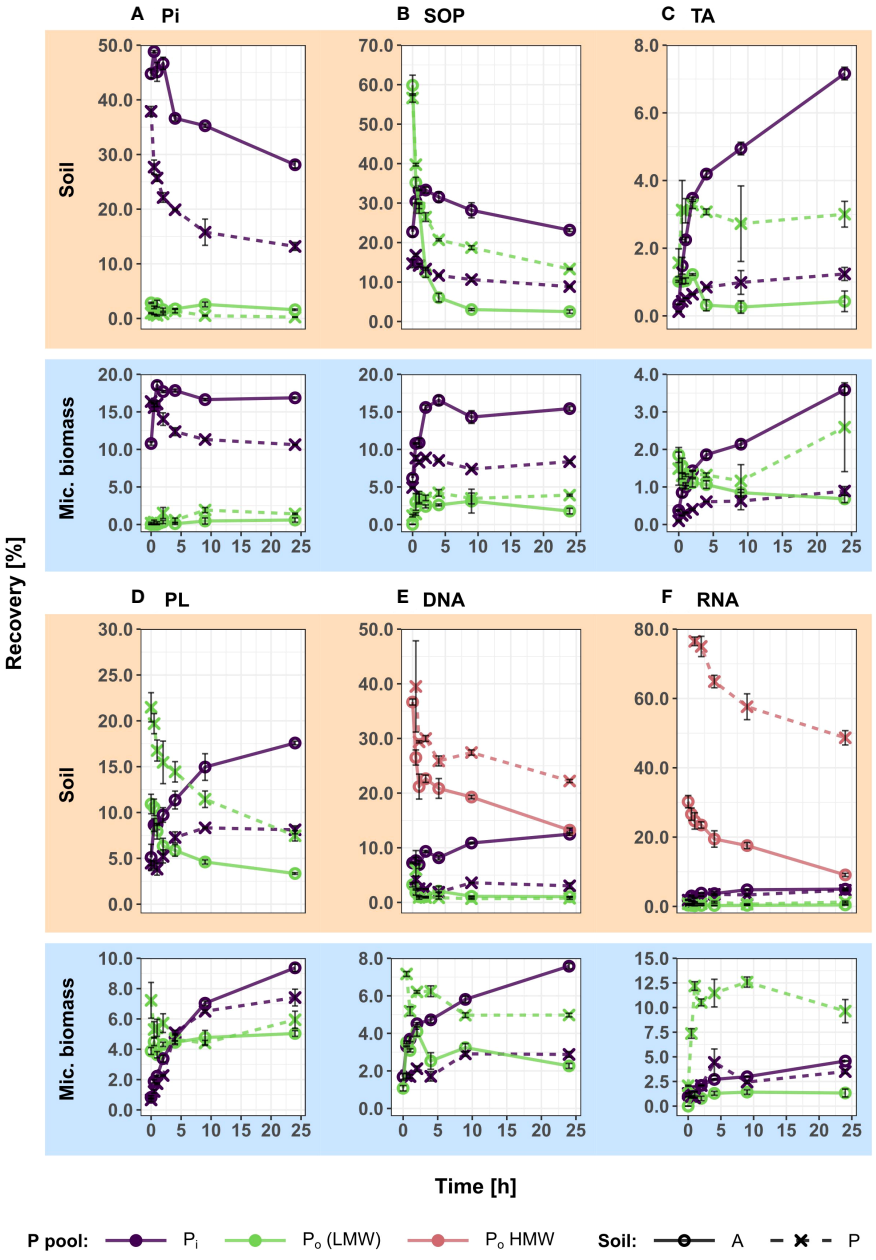

FIGURE 2
Recoveries of experimentally added ^{33}P in extractable soil and microbial organic (P_o) and inorganic (P_i) phosphorus pools over 24 h, showing the integrated temporal dynamics of decomposition processes, sorption and microbial P uptake for six different P compounds. ^{33}P recoveries (mean of triplicates ± S.E.) of all pools and time points are displayed as percentages of the initially added ^{33}P activity. In the nucleic acid treatments **(E, F)**, soil $^{33}P_o$ is fractionated into low molecular weight (LMW) P_o (<3kDa) and high molecular weight (HMW) P_o (>3kDa) size classes. In all other treatments **(A-D)**, P_o represents extracted P_o, without size fractionation. In the case of TA **(C)** and PL **(D)** additions, extractable P_o does not represent the intact added substrates (which are unextractable), but organic break down products. In the case of P_o additions **(B-F)**, microbial $^{33}P_o$ recovery was corrected for cell internal biosynthesis of P_o, as described in detail in the methods section. Pi, inorganic phosphate; TA, cell wall teichoic acids; PL, phospholipids; SOP, soluble organophosphates.

(Figure 2 and Table 2). Soil $^{33}P_o$ recoveries decreased significantly over time in all P_o treatments except for TA. Soil $^{33}P_o$ recoveries of the substrates and their breakdown products (except for LMW-DNA, i.e. the low molecular weight DNA fraction) differed significantly between the two soils. Further, the temporal dynamics of SOP and NA treatments were significantly different between the two soils (Table 2). We found higher extractable soil $^{33}P_i$ recoveries (from 3.4% for TA to 28.9% for SOP averaged across all time points) in the arable soil for all P_o treatments. This could be caused by lower $^{33}P_i$ sorption, and/or potentially higher extracellular mineralization activity in the arable soil as compared to the pasture soil. P_i sorption, assessed as the non-extractable fraction of added $^{33}P_i$, was significantly lower in arable soil (mean 51.0% ± 1.9% standard deviation (S.D.)) than in pasture soil (66.9% ± 5.9% S.D.) (Welch`s t-Test, p-value < 0.05). Moreover, phosphodiesterase activity was 3-fold higher in the arable soil (Student`s t-Test, p-value < 0.05) with 391 nmol pNP g^{-1} h^{-1}, whereas phosphomonoesterase activities did not differ significantly between the soils (Student`s t-Test, p-value = 0.07) with 630 nmol pNP g^{-1} h^{-1} for the arable soil and 842 nmol pNP g^{-1} h^{-1} for the pasture soil (Table 1).

TABLE 2 Differences of ^{33}P recovery from extractable soil and microbial organic (P_o) and inorganic (P_i) phosphorus pools, across time and between soils.

Treatment	Pool		Cor.	Soil			Time			Soil x time		
				d.f.	F	p-value	d.f.	F	p-value	d.f.	F	p-value
P_i	Soil	P_o	GG	1.00	142.15	**< 0.05**	1.60	0.55	0.25	1.60	0.90	0.14
	Soil	P_i	GG	1.00	967.20	**< 0.05**	2.20	34.16	**< 0.05**	2.20	2.13	**< 0.05**
	Mic.	P_o	GG	1.00	15.35	**< 0.05**	1.80	1.73	0.05	1.80	0.49	0.31
	Mic.	P_i		1.00	321.70	**< 0.05**	5.00	23.23	**< 0.05**	5.00	16.51	**< 0.05**
SOP	Soil	P_o	GG	1.00	232.48	**< 0.05**	2.05	177.55	**< 0.05**	2.05	14.08	**< 0.05**
	Soil	P_i	GG	1.00	807.81	**< 0.05**	1.50	11.86	**< 0.05**	1.50	2.45	**< 0.05**
	Mic.	P_o		1.00	0.69	0.45	5.00	1.50	0.23	5.00	2.96	**< 0.05**
	Mic.	P_i		1.00	352.68	**< 0.05**	5.00	23.72	**< 0.05**	5.00	24.89	**< 0.05**
TA	Soil	P_o		1.00	31.41	**< 0.05**	5.00	1.19	0.35	5.00	0.32	0.90
	Soil	P_i	GG	1.00	988.82	**< 0.05**	1.75	38.54	**< 0.05**	1.75	21.16	**< 0.05**
	Mic.	P_o		1.00	9.46	**< 0.05**	1.10	0.14	0.48	0.19	0.39	0.25
	Mic.	P_i		1.00	652.14	**< 0.05**	5.00	81.88	**< 0.05**	5.00	31.53	**< 0.05**
PL	Soil	P_o		1.00	102.30	**< 0.05**	5.00	24.15	**< 0.05**	5.00	2.08	0.11
	Soil	P_i		1.00	119.72	**< 0.05**	5.00	34.93	**< 0.05**	5.00	4.93	**< 0.05**
	Mic.	P_o	GG	1.00	10.36	**< 0.05**	1.75	0.67	0.22	1.75	0.72	0.20
	Mic.	P_i		1.00	30.85	**< 0.05**	5.00	342.12	**< 0.05**	5.00	6.34	**< 0.05**
DNA	Soil	P_o		1.00	61.55	**< 0.05**	5.00	25.53	**< 0.05**	5.00	3.56	**< 0.05**
	Soil	P_o HMW	GG	1.00	26.00	**< 0.05**	1.25	1.89	**< 0.05**	1.25	0.13	0.54
	Soil	P_o LMW		1.00	0.28	0.63	5.00	2.50	0.07	5.00	1.68	0.19
	Soil	P_i	GG	1.00	812.58	**< 0.05**	2.45	9.59	**< 0.05**	2.45	7.69	**< 0.05**
	Mic.	P_o		1.00	526.68	**< 0.05**	5.00	20.01	**< 0.05**	5.00	8.17	**< 0.05**
	Mic.	P_i		1.00	937.57	**< 0.05**	5.00	111.32	**< 0.05**	5.00	30.81	**< 0.05**
RNA	Soil	P_o	GG	1.00	5392.76	**< 0.05**	2.10	17.40	**< 0.05**	2.10	2.46	**< 0.05**
	Soil	P_o HMW	GG	1.00	5237.10	**< 0.05**	2.15	18.32	**< 0.05**	2.15	2.58	**< 0.05**
	Soil	P_o LMW	GG	1.00	45.85	**< 0.05**	1.20	0.82	0.13	1.20	0.45	0.24
	Soil	P_i		1.00	145.05	**< 0.05**	5.00	34.98	**< 0.05**	5.00	2.16	0.10
	Mic.	P_o		1.00	579.43	**< 0.05**	5.00	6.20	**< 0.05**	5.00	4.37	**< 0.05**
	Mic.	P_i		1.00	0.76	0.43	5.00	16.94	**< 0.05**	5.00	2.89	**< 0.05**

Recoveries were tested for differences between soils, changes over time and differences in temporal dynamics between the soils. Factorial repeated measures ANOVAs were performed with the ^{33}P recovery percentages of the time points 0.5 to 24 h, n = 3. If the assumption of sphericity was violated, Greenhouse-Geisser (GG) adjustment was applied, as indicated in the column "Cor.". The respective adjusted degrees of freedom (d.f.), F and p-values are reported. TA, cell wall teichoic acids; PL, phospholipids; SOP, soluble organophosphates. Soil, extractable soil P pool; Mic., extractable microbial P pool. P-values < 0.05 are bold.

3.2.2 Recovery dynamics of soluble organophosphates in the extractable soil pool

In the SOP treatments, the soil $^{33}P_o$ pool declined rapidly, leading to almost complete mineralization in the arable soil after 10 h. In the pasture soil, mineralization slowed down after 4 h as indicated by the time kinetics of $^{33}P_i$ formation from $^{33}P_o$, reaching P_i recovery values of 8.8% (± 0.3% S.D.) (Figure 2B).

3.2.3 Recovery dynamics of teichoic acids and phospholipids in the extractable soil pool

In the case of TA and PL additions, extractable soil $^{33}P_o$ continued to decline over the course of 24 h, with a slowdown after 2 to 4 h (Figures 2C, D). In both treatments, only a small fraction of the applied ^{33}P was recovered in soil $^{33}P_o$ (1.3% for TA and 16.2% for PL, averaged across both soils at 0 h), and this pool did not change significantly over time when TA was added, while it significantly declined in the PL treatments (Table 2). TA and PL are phosphodiesters which contain subunits of glycerophosphate. Both phosphodiester compounds are not directly extractable with $NaHCO_3$ as such, due to the following: in the case of TA their polymeric form and covalent binding within bacterial cell walls and in the case of PL their hydrophobicity render them unextractable by $NaHCO_3$. The labelled P_o in TA and PL therefore only becomes extractable after breakdown of the compounds into smaller and water-soluble units, such as glycerol-3-phosphate. The data therefore can be assumed to show the integral of two consecutive steps in the decomposition processes of ^{33}P labelled PL and TA: breakdown to small water-soluble molecules (extractable soil $^{33}P_o$), followed by complete mineralization (to soil $^{33}P_i$).

3.2.4 Recovery dynamics of nucleic acids in the extractable soil pool

In both nucleic acid (NA) treatments (DNA and RNA), ultrafiltration was performed to separate soil $^{33}P_o$ into low molecular weight-NA (<3 kDa; LMW) and high molecular weight-NA (>3 kDa; HMW) fractions. The LMW-fraction thus contained nucleotide monomers and small oligomers [max. 4 to 9 nucleotides, depending on the degree of hydration (60)], whereas the HMW-fraction contained NA oligomers and polymers larger than that. Again, the data showed two steps in the decomposition process of these ^{33}P labelled compounds: $^{33}P_o$ in the LMW-NA fraction is an indicator for breakdown of HMW compounds into oligonucleotides and mononucleotides, and soil $^{33}P_i$ is a measure for complete mineralization of the added ^{33}P-NA by phosphomonoesterases. In both soils, recovery of HMW-$^{33}P_o$ decreased significantly (from 51.6% at 0 h to 23.7% after 24 h, averaged across both NA treatments and both soils), while recovery of LMW-$^{33}P_o$ remained very low at all times without significant changes (between 1.5% at 0 h and 0.9% after 24 h, averaged across both NA treatments and both soils) and recovery of $^{33}P_i$ increased slowly in the arable soil and stagnated in the pasture soil (from 4.6% to 8.7% and between 2.9% and 3.9%, respectively, averaged across of both NA treatments) (Figures 2E, F; Table 2).

3.3 Microbial uptake of $^{33}P_o$ and $^{33}P_i$

3.3.1 Direct uptake of $^{33}P_o$

With the experimental approach in this study, ^{33}P derived from labelled P_o-substrates can be traced into the extractable organic fraction of the microbial biomass pool (P_{mic}). However, $^{33}P_o$ in P_{mic} can stem from direct uptake of intact P_o forms (pathway 1) and from extracellular $^{33}P_o$ mineralization to $^{33}P_i$, followed by cellular $^{33}P_i$ uptake and subsequent intracellular $^{33}P_o$ biosynthesis (pathway 2). To disentangle the source of measured intracellular $^{33}P_o$ between both pathways, we estimated intracellular $^{33}P_o$ biosynthesis using incubations with $^{33}P_i$ addition. Only a small amount of ^{33}P was recovered in the microbial P_o pools of these control treatments (0 to 2.5%, Figure 2A), indicating that cell internal biosynthesis of extractable P_o during the 24 h incubation period was limited. To correct microbial $^{33}P_o$ pools of the P_o treatments for intracellular P_o biosynthesis, we assumed that microbes allocated a fixed percentage (as observed in the ^{33}Pi additions) of the available substrate-derived P_i into biosynthesis. While strong increases of available P_i could theoretically change microbial P metabolism *via* induction of P_i assimilation or formation of polyphosphates, this should not have been triggered in our treatments. After substrate addition, soil P_i concentrations in the P_o treatments did not differ significantly from the P_i treatments (Table S2), supporting the assumption that microbial P metabolism was comparable between treatments. The observation of microbial $^{33}P_o$ that remains after accounting for P_o biosynthesis from P_i in all treatments thus indicates direct uptake of intact $^{33}P_o$ compounds into the microbial biomass.

Biosynthesis-corrected recoveries in the microbial $^{33}P_o$ pool after 24 h are therefore indicative for direct uptake of intact $^{33}P_o$ compounds into the soil microbial biomass. They ranged between 0.7% (± 0.2% S.D.) for TA in the arable soil and 9.6% (± 2.0% S.D.) for RNA in the pasture soil. Biosynthesis-corrected recoveries of ^{33}P in the microbial P_o-pool were significantly lower in arable (SOP: 2.4%, TA: 1.2%, PL: 4.4%, DNA: 2.8%, RNA: 1.0%, averaged across all time points) than in pasture soil (SOP: 3.0%, TA: 1.5%, PL: 5.5%, DNA: 5.8%, RNA: 9.4%, averaged across all time points), except for the SOP treatment which did not differ significantly between soils (Table 2).

3.3.2 Temporal dynamics of microbial $^{33}P_o$ and $^{33}P_i$ uptake

In the SOP and NA treatments, the temporal dynamics of microbial $^{33}P_o$ recoveries varied significantly between the soils. Across soils the temporal changes of microbial $^{33}P_o$ recoveries were not significant in the TA, PL and SOP treatments. In the SOP and NA treatments, extractable microbial $^{33}P_o$ recoveries increased rapidly

initially and then remained stable (Figures 2B-F).

The $^{33}P_o$:$^{33}P_i$ ratio of P_{mic} is an indicator for the amount of substrate-derived P_o that is directly taken up into microbial biomass relative to the amount of substrate-derived P_o that is taken up as P_i after external mineralization. The ratio is calculated for successive timesteps (0.5 to 24 h), based on timestep-integrated and biosynthesis-corrected $^{33}P_o$ and $^{33}P_i$ recoveries. In the arable soil, microbial $^{33}P_o$:$^{33}P_i$ ratios were lower than in the pasture soil (0.68 compared to 2.86, averaged across all P_o treatments and all considered time points). However, three potential methodological problems need to be considered: (i) Intracellular dephosphorylation of $^{33}P_o$ causes an increase in $^{33}P_i$ and a decrease in this ratio, underestimating intact P_o uptake. (ii) Continued extraction of soil P_i in the apparent P_{mic} pool could lead to an overestimation of $^{33}P_i$ in P_{mic} and a subsequent underestimation of the microbial $^{33}P_o$:$^{33}P_i$ ratio. (iii) On the other hand, sorption of microbial $^{33}P_i$ to soil during the extraction could lead to an opposite bias. As the pasture soil showed a higher P_i sorption capacity (66.9% compared to 51.0% in arable soil), these unquantified processes obstruct a direct comparison of the microbial $^{33}P_o$:$^{33}P_i$ ratio between the soils and may skew absolute values of the ratio. However, any such affect can be assumed to be independent of incubation time, thus allowing to consider the relative temporal patterns of treatment-specific microbial $^{33}P_o$:$^{33}P_i$ ratios. The $^{33}P_o$:$^{33}P_i$ ratios of P_{mic} decreased over time for most substrates, and this decrease was significantly related to the decline of available $^{33}P_o$ in the soil (Figure 3; Table S3). For all P_o amendments we found that the microbial $^{33}P_o$:$^{33}P_i$ ratios were well above 0 (in many cases above 1, ranging up to 8), indicating uptake of intact P_o compounds. Furthermore, the dynamics of the $^{33}P_o$:$^{33}P_i$ ratio of P_{mic} differed between soils (e.g. PL and TA versus NA dynamics) and between different P_o treatments. The microbial $^{33}P_o$:$^{33}P_i$ ratio for RNA after 1 h incubation time in pasture soil was a significant outlier (Grubb`s test, G = 1.661, p-value = 0.06) and was excluded from further analysis.

4 Discussion

4.1 Decomposition dynamics of $^{33}P_o$ compounds

4.1.1 Decomposition dynamics of teichoic acids and phospholipids

We observed comparable trends in the decomposition dynamics of TA and PL, which both contain P in the form of glycerophosphates, either polymeric in wall teichoic acids or linked to fatty acids in phospholipids. We found that the extractable intermediate breakdown products ($^{33}P_o$) of TA and PL were mineralized to a higher degree in the arable than in the pasture soil (72.7% compared to 26.1%, averaged across both treatments and all considered time points; see the degree of substrate mineralization, x-axis in Figure 3). This suggests that depolymerization by phosphodiesterases may be the rate limiting step of TA and PL decomposition in the arable soil (high degree of substrate mineralization). In the pasture soil, either mineralization through phosphomonoesterases seems to (co)limit complete TA and PL decomposition (lower degree of substrate

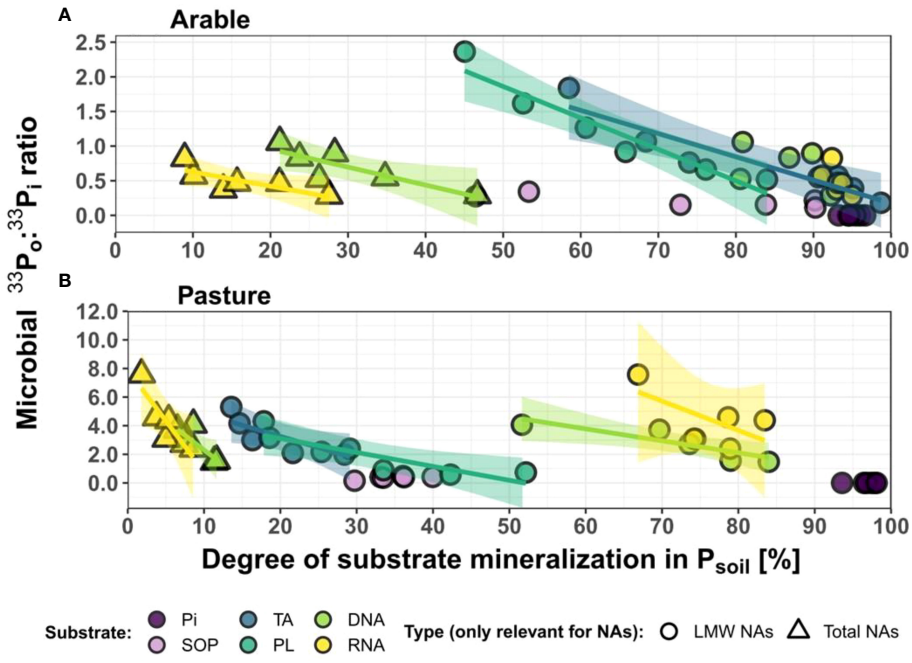

FIGURE 3
Ratios of cumulative P_o:P_i uptake from labelled substrates decline with progressing substrate mineralization in P_{soil} mineralization in Psoil. , shown for the arable **(A)** and pasture **(B)** soil. Data points are the means of triplicates for time points 0.5 to 24 h (the temporal pattern within each treatment proceeds from left to right). For both nucleic acid treatments (DNA, RNA), substrate mineralization is given for for total extractable NAs [high molecular weight (HMW) + low molecular weight (LMW)] and for the LMW fraction only. Fitted significant models are shown with 95% confidence intervals. For model descriptions, see Table S3. Microbial $^{33}P_o$:$^{33}P_i$ ratios are calculated based on ^{33}P activity [Bq] and were corrected for biosynthesis to obtain the $^{33}P_o$:$^{33}P_i$ ratio of cumulative ^{33}P uptake as explained in detail in the "methods" section. The degree of substrate mineralization is the percentage of extractable soil ^{33}P in inorganic form, *i.e.* P_i. Pi = inorganic phosphate; TA, cell wall teichoic acids; PL, phospholipids; SOP, soluble organophosphates.

mineralization), or a larger amount of mineralized P may be rendered bio-unavailable through higher P_i sorption as compared to the arable soil. The lack of accumulation of intermediate breakdown products in $^{33}P_{soil}$ over time demonstrates that glycerophosphate is promptly consumed, either by the direct use by microbes or by rapid dephosphorylation to $^{33}P_i$ by phosphomonoesterases, in line with earlier studies [e.g (61, 62)]. The observed decomposition and mineralization of TA is in contrast with the hypothesis that teichoic acids are resistant to decomposition (63). Mechanisms for the breakdown of wall teichoic acids under P limiting conditions are known (64, 65), and it has also been observed that soil bacteria can produce specialized phosphodiesterases (glycerophosphodiesterases) to utilize exogenous teichoic acid as the sole P source (66), thereby releasing glycerol-3-phosphate (27, 67, 68). Interestingly, enzymes from the same highly conserved family (glycerophosphodiesterases) are known to be involved in the breakdown of both TA and PL (69). We also observed rapid initial breakdown of PL, similar to Tollefson and McKercher (70). There are at least three alternative pathways for the first step of soil microbial breakdown of PL, all yielding different breakdown products (71–73). Since we observed direct uptake of $^{33}P_o$ (see discussion below), and transporters are only known for glycerol-3-phosphate, we hypothesize that a substantial part of the PL breakdown occurred *via* a pathway which yields glycerophosphate, choline and fatty acids as products (71). This pathway likely involves phospholipase D, which cleaves off the headgroup from the phospholipid (e.g. choline, ethanolamine) leaving phosphatidic acid behind, and phospholipase B (or A1 plus A2) that cleaves phosphatidic acid into two fatty acids and one glycerol-3-phosphate (26, 74, 75).

4.1.2 Decomposition dynamics of nucleic acids

The second group of compounds with commonalities in decomposition dynamics are the nucleic acids. While the ^{33}P activity recovered in HMW fractions in P_{soil} decreased (from 51.6% to 23.7% over 24 h, averaged across both NA treatments and both soils), the recovered activity of ^{33}P in the LMW fractions remained low (between 1.5% and 0.9% over 24 h, averaged across both NA treatments and both soils) and the recovery of $^{33}P_i$ generally increased over time (from 3.7% to 6.3% over 24 h, averaged across both NA treatments and both soils) (Figures 2E, F; Table 2). Intact nucleic acids (which are recovered in the HMW-NA pool) need to be broken down into nucleotides (which are recovered in the LMW-NA pool) before mineralization. Therefore, the LMW-NA pool reflects the net product of inputs *via* HMW-NA breakdown and outputs due to mineralization and microbial uptake (and sorption). There is a constant flux of ^{33}P through the LMW-NA pool, as nucleotides are the intermediate product between depolymerization and mineralization. The constant and low recoveries of LMW-$^{33}P_o$ (Figures 2E, F) therefore strongly indicate that substrate-derived nucleotides do not accumulate. This means that NA depolymerization to free nucleotides and oligonucleotides is the rate-limiting step in the decomposition of nucleic acids in soil, while dephosphorylation of phosphomonoesters (nucleotides) occurs rapidly and therefore is not rate limiting. Extracellular DNA in soil is thought to be cleaved into duplex oligonucleotides of 400 bp length by restriction endonucleases; studies in pure culture showed cleavage of HMW-DNA to oligonucleotides of ~7 bp likely by endonucleases and further hydrolysis *via* exonucleases into mononucleotides by DNases, although this has not yet been shown *in situ* in soils (76). Others observed that fragmentation dynamics during DNA decomposition varied between different soils (77). While it is still not clear which phosphodiesterases of the different nuclease types are involved in soil NA decomposition and who produces those in soils (77–79), our findings are in line with earlier observations. Phosphodiesterase activity was previously found to be rate-limiting in labile P_o turnover in pasture soils (80), and other studies reported rapid mineralization of free added nucleotides (61, 81). It has been implied that RNA can be used as a viability marker as it degrades more rapidly in soils in comparison with DNA, although comparable half-lives of 0.5 - 1.5 days of DNA and RNA in soils have also been reported previously (82–84). In this study, we found similar recovery dynamics of HMW-DNA and HMW-RNA within each soil, pointing to similar decomposition rates of environmental DNA and RNA.

4.1.3 Decomposition dynamics of soluble organophosphates

Recovery of intact organophosphates (SOP) from P_{soil} decreased rapidly (58.2% to 13.4% over the first 4 h, averaged across both soils), initially with concomitant but moderate increases in the soil $^{33}P_i$ pool (18.7% to 21.6% over the first 4 h, averaged across both soils). The SOP substrate presumably consisted mostly of phosphomonoesters (sugar phosphates, organic acid phosphates, nucleotides, nucleotide sugars, cofactors etc.), which are likely to be rapidly mineralized in soil by the ubiquitous acid and alkaline phosphomonoesterases. However, after one hour $^{33}P_i$ in P_{soil} also started to decrease, with continued decreases in $^{33}P_o$. This indicates that other processes aside from mineralization must have contributed to the ^{33}P removal from P_{soil}. Patterns of $^{33}P_i$ recovery resembled those observed in the P_i treatments and were thus likely caused by sorption of $^{33}P_i$ and by microbial uptake. However, it is unlikely that sorption played a large role in the observed $^{33}P_o$ decreases, since another study (81) found very rapid completion – within 15 to 45 minutes – of sorption processes of small phosphate monoesters in an Eutric Cambisol under meadow. It has been proposed that sorption dynamics of P_o compounds depend on the size and structure of their organic moieties, which have lower surface affinities than phosphate groups (85–87). Small molecules as in the SOP treatment may therefore exhibit comparatively rapid initial sorption upon addition to soil because the effect of the organic moieties over the phosphate groups may be less dominant due to their small size. In addition, phosphate monoesters also have a high ability to form insoluble complexes with polyvalent cations (86). The same study which observed rapid completion of initial sorption of phosphate monoesters (81) found rapid initial mineralization and respiratory utilization of ^{14}C-labelled glucose-6-phosphate and adenosine phosphates, gradually slowing down after 6 and 24 h, respectively. Two studies using ^{14}C and ^{33}P labelling (36, 37) also found rapid utilization of glucose-6-phosphate, with no more major changes in ^{33}P recovery after 2 to 3 days. Another possible explanation could be rapid uptake of ^{33}P, and assimilation into non-extractable P_{mic}. ^{33}P that is used to build up structures that are embedded in macromolecules (*e.g.* teichoic acids in cell walls) or that are hydrophobic (*e.g.* phospholipids) may not be extractable anymore with the methods applied in this study. Assimilation of

labelled substrate into non-extractable microbial pools has been observed previously elsewhere (88).

4.2 Direct microbial uptake of P_o versus uptake of P_i after extracellular mineralization

4.2.1 Direct uptake of $^{33}P_o$ in general

The low microbial P_o biosynthesis in soils amended with P_i clearly points to intermittent storage (e.g. polyphosphates) and slow metabolism of P_i in the soil microbial communities, showing a decoupling of uptake and metabolism of P_i. Direct P_o uptake contributed to ^{33}P uptake in all P_o treatments, and in both soils (Figure 2). The contribution of direct P_o uptake to cumulative ^{33}P uptake into P_{mic} seemed lower in the arable than in the pasture soil (with a $^{33}P_o$:$^{33}P_i$ ratio of P_{mic} of 0.68 compared to 2.86, averaged across all P_o treatments and all considered time, see also Figure 3). This could eventually be caused by a higher sorptive loss of microbial $^{33}P_i$ during extraction in the pasture soil. However, the arable soil also had a 4-fold higher native extractable P_i concentration than the pasture soil (30.7 µg g^{-1} compared to 7.0 µg g^{-1}, see Table 1), and a higher percentage of $^{33}P_{soil}$ was present as P_i (72.7% compared to 26.1%, averaged across both treatments and all considered time points, Figure 3). Both these aspects render a real difference between the soils plausible. The $^{33}P_o$:$^{33}P_i$ ratios of P_{mic} decreased in both soils, as the progression of extracellular substrate mineralization over 24 h decreased $^{33}P_o$ availability through substrate consumption and concomitantly increased $^{33}P_i$ (Figure 3). Direct P_o uptake therefore decreases with observational time due to the continuous action of the phosphomonoesterases converting $^{33}P_o$ to $^{33}P_i$. Hence, these patterns reveal that the P_o : P_i ratio of cumulative uptake of P_o-derived P is ultimately governed by the form in which it is available. This actually causes a situation in soils where microbial P_o uptake transporters compete with extracellular phosphomonoesterases for organic substrates. Direct uptake of organic P may be of greater relative (and absolute) importance under conditions of low P_i availability, regardless of whether low P_i availability is caused by lower extracellular mineralization or higher P_i sorption.

The observed stagnation of $^{33}P_o$ accumulation in P_{mic} of the TA, PL and SOP treatments (Table 2) is likely to be the net result of several (possible) processes: (i) *In situ* production and decomposition of unlabelled P_o compounds in P_{soil}, contributing to a decrease in the specific activities of soil P_o pools (Figure S2), thus leading to a decline in net $^{33}P_o$ accumulation at constant P_o uptake rates, (ii) intracellular P_o mineralization decreasing the microbial $^{33}P_o$ recoveries, and (iii) the intracellular production of insoluble (non-extractable) $^{33}P_o$ diesters. For instance, another study found that labelled DNA, which was cleaved and then assimilated by bacterioplankton, became non-extractable with time (88). Overall, the above mentioned processes would contribute to an underestimation of direct P_o uptake in this study.

4.2.2 Direct uptake of nucleic acids

It is commonly thought that NA-derived P cannot be taken up in an intact organic-bound form (*i.e.* nucleotide). Rather it is thought that nucleotides are first dephosphorylated, and that P-free nucleosides and P_i are taken up independently by nucleoside transporters and PHT transporters, respectively (89, 90). Empirical evidence for direct uptake of intact LMW nucleotides in non-parasitic organisms is scarce (91), but recently transporters of the nucleotide transport protein (NTT) family, previously known from intracellular parasites, have been found in free-living prokaryotes and eukaryotes (43). NTTs either exchange nucleotides across membranes (*e.g.* ADP for ATP, ADP for NAD^+) or are H^+:nucleotide symporters allowing efficient net uptake of nucleotides. NTTs could thus play an important role in the environment, contributing to the direct uptake of NA-derived P. Alternatively to nucleotide uptake, polymeric NA, such as DNA molecules, might also be actively transported across the cell envelope and into the cytoplasm, by a process called transformation in bacterial cells. Transformation aims at DNA molecules with ranges reported from 300 bp to more than 50 kb length (92, 93), and therefore would clearly relate to DNA-P_o from the HMW fraction in this study. This ability is taxonomically widespread but it is unknown whether it is common in soil microbes (76, 93, 94). Furthermore, RNA presumably cannot be taken up in intact form by any process analogous to transformation, although other studies found indications for intact uptake of HMW-RNA in yeast (95) and for intact, energy-dependent uptake of oligonucleotides including RNA by *Candida albicans* (96). Our data suggest that direct uptake of $^{33}P_o$ derived from DNA and RNA may have taken place *via* transporters for nucleotides (NTT), after decomposition of LMW-NA to oligo- and mononucleotides by exo- and endonucleases.

4.2.3 Direct uptake of teichoic acid and phospholipid decomposition products

Glycerolphosphodiesterases (GlpQ) hydrolyze wall teichoic acids and phospholipids, yielding glycerol-3-phosphate (G3P) amongst other byproducts. Uptake transporters for G3P (GlpT) are well known and belong to the organophosphate:phosphate antiporter (OPA) family. Often GlpT and GlpQ are co-localized (41). Recently, a novel potential P_o transporter was found in bacteria, which is a component of the phosphorus utilization system (PUS) clusters. These contain the genes of the two-component PusCD system alongside with phosphodiesterases, where PusC and D likely represent a G3P transporter and a lipoprotein cap (45). The known diversity of G3P transporters therefore renders direct uptake of G3P likely.

4.2.4 Direct uptake of soluble organophosphates

We also observed direct uptake of P_o compounds from the group of soluble organophosphates. However, in this treatment, the mineralization proceeded so rapidly that $^{33}P_i$ uptake outpaced that of $^{33}P_o$ right from the beginning, without major changes in the $^{33}P_o$:$^{33}P_i$ ratio of P_{mic} (Figure 3). The fraction of soluble organophosphates derived from bacterial cells is a complex mixture of compounds (sugar phosphates, nucleotide sugars, nucleotides, phosphoamino acids, organic polyphosphates, phosphonates *etc.*) and it is possible that only some of them can be taken up directly in an intact form, depending on the presence of suitable uptake transporters. For sugar phosphates, several transporters belonging to the OPA family are known, and these have been found in a variety of

bacteria (44, 46, 97). There is also evidence for direct uptake of hexose phosphates in fungi (98). Another uptake system from the same family is known to transport phosphoenolpyruvate and phosphoglyceric acid into bacteria, and further transporters for several phosphonates have been characterized (46, 48).

4.2.5 Cell internal fate of directly taken up $^{33}P_o$

In our study, the $^{33}P_o$ taken up directly remained at least partially in organic form, showing that complete intracellular mineralization did not take place within 24 h. The P derived from P_o compounds could undergo phosphoryl transfer to ATP and enter central metabolic pathways (48). Shortcutting cell internal metabolic pathways would be beneficial by saving costs for associated enzymes and skipping energy requiring steps. Salvage pathways using intact nucleotides have been suggested to be an important use of extracellular DNA (43, 76, 93). All antiporters of the OPA family transport intermediates of the glycolytic pathway (99), *e.g.* glycerol-3-phosphate, an intermediate of phospholipid synthesis (47, 73), which could directly contribute to the host metabolism. P_o uptake *via* OPA antiporters, however, has been suggested to be inefficient for bacterial P nutrition, because it requires efflux of P_i or P_o anions in order to take up P_o (46, 48). Similarly, many of the nucleotide transporter proteins (NTT) exchange ADP for ATP, or NAD^+ for ADP, serving purposes of energy gain with little net gain in P. Only a few NTTs for unidirectional proton-driven nucleotide import are known (100). However, efficient re-uptake of secreted P_i by phosphate:H^+ symporters (PHT family) might allow for continuous P_o uptake with net P gain. While P_i uptake clearly serves P nutrition, P containing organic molecules could also be used as sources of energy and C (36, 37) and in some cases N.

4.3 Ecological implications of direct P_o uptake by microbes

As direct microbial uptake of P_o is not perceived as an integrated component of the soil P cycle, we here consider some aspects that possibly have important ecological implications compared to complete extracellular mineralization. (i) The ability of direct uptake of P_o compounds reduces the dependence of P acquisition on extracellular phosphatases, especially phosphomonoesterases, since phosphate diesters still require a certain degree of extracellular enzymatic breakdown. Firstly, the efficacy of extracellular phosphatases in soils can be reduced by suboptimal pH, or by the presence of metal ions, polyvalent anions or chelating agents (56, 101), condensed organic molecules (102), and by sorption to clay minerals (103). Secondly, extracellular enzyme production requires an investment of N (and C), and thus, under N-limiting conditions, excretion of phosphatases may not be an adequate P acquisition strategy. N additions have been shown to enhance extracellular phosphatase activity under a wide range of conditions (104), suggesting that microbes can allocate excess N to phosphatase enzyme production. Under N-limiting conditions direct P_o uptake may be beneficial, because of reduced N investment into phosphatase production. (ii) Complete mineralization by extracellular enzymes might promote the risk of losing cleaved P_i to soil P_i sorption/precipitation processes. Sorption of P_i can remove a considerable fraction of free P_i or of mineralized P in soils, particularly in highly weathered soils with high content of Fe/Al oxyhydroxides and aluminosilicates (105). Since P_o sorption is weaker and slower than that of P_i (106, 107), P_i becomes more rapidly inaccessible compared to P_o. (iii) The general advantage of direct access to organic P substrates could also affect plant-microbe interactions, as plants and microbes compete for available P over short timescales (23, 108). Since there is limited evidence for direct P_o uptake by plants (109), P taken up in organic form by microbes might not be directly available to plants at any time, and microbes possessing this capability could therefore have an advantage over plants.

4.4 Methodological limitations

The applied methodology captures net changes in pool sizes (^{33}P recoveries relative to the activity added) over time, and therefore does not allow conclusions on underlying gross process rates. However, extractable soil ^{33}P pools ($^{33}P_o$ and $^{33}P_i$ in P_{soil}) provide a realistic picture of biologically available P and of the involved ecologically relevant net P processes. Aside from this, it must be considered that $^{33}P_i$ and $^{33}P_o$ are subject to abiotic immobilization processes such as sorption to soil particles and precipitation with cations (as indicated by soil P_o specific activities, Figure S2). Estimates of extracellular mineralization after accounting for P_i sorption indicate that mineralization of ^{33}P labelled organic substrates is likely underestimated by the quantification of $^{33}P_i$ in the extractable soil P pool (Figure S3). This is caused by continuous sorption of parts of the $^{33}P_i$ that is produced by mineralization of $^{33}P_o$ compounds. However, we argue that this ongoing sorption of $^{33}P_i$ does not confound the interpretation of the data when viewing P_{soil} as the available P pool; it just prohibits conclusions about gross mineralization rates. As for sorption of $^{33}P_o$, several studies that investigated the time kinetics of sorption of organic P compounds found a rapid slowdown of sorption after the first 60 min (81, 85, 87). According to this, losses of $^{33}P_o$ from the extractable soil pool by sorption should strongly decline after the fast initial sorption of added $^{33}P_o$ substrate is complete. Further, phosphomonoesters are known to have a higher affinity for complexation with cations and get more strongly stabilized by sorption than phosphodiesters (86). To directly quantify sorption of P_o compounds in soil remains an open challenge. There is yet no method for isotope pool dilution approaches or isotope exchange kinetic approaches (which are often used to assess P_i sorption and the size of the bioavailable P_i pool) which reliably inhibits microbial activity as well as the activity of extracellular phosphatases without affecting soil structure (6, 18, 110). However, as long as biotic mineralization of added $^{33}P_o$ compounds cannot be reliably inhibited without affecting sorption dynamics, P_o sorption dynamics in real soils remain hard to determine, and therefore empirical data on P_o sorption will remain constrained to artificial abiotic systems. Because of the above mentioned processes, the observed decreases in soil $^{33}P_o$ and the dynamics of soil $^{33}P_i$ do not necessarily reflect biological processes alone, and gaps in the balance between apparent P_o mineralization, the amount of soil $^{33}P_i$ recovery and microbial uptake of ^{33}P can arise. Similar restrictions apply to the quantification of P_o uptake; several

possibilities for hidden gross fluxes of ^{33}P – for instance incorporation into unextractable microbial P_o pools or intracellular P_o mineralization – might lead to an underestimation of actual P_o uptake.

Further uncertainty of the applied methodology comes from the unknown fate of polyphosphates during P extraction and fractionation of P_i and P_o from the soil. In the case of high soil P_i supply, bacteria and fungi can take up P_i in excess of their demand and store it in the form of inorganic polyphosphates, serving as intermittent stores for P_i and energy (111). The behavior of such polyphosphates during isobutanol fractionation is unknown, and polyphosphates might theoretically be partitioned into the microbial P_o fraction as an artefact. However, we can safely exclude that all observed microbial $^{33}P_o$ is polyphosphate synthesized from $^{33}P_i$, as this would require that polyphosphate synthesis had not occurred in the $^{33}P_i$ treatment, but consistently in all $^{33}P_o$ treatments. Moreover, significant *in situ* synthesis of polyphosphates by soil microbes is only expected under high P_i availability, while in this study substrate additions did not lead to significant changes in concentrations of available soil P_i relative to the P_i control treatments (Table 1).

4.5 Conclusions

We presented evidence that decomposition of all tested phosphodiester compounds (cell wall teichoic acids, phospholipids, DNA and RNA) was rate-limited by the initial breakdown step carried out by phosphodiesterases, while the resulting simple phosphomonoesters (such as nucleotides and glycerophosphates) were rapidly dephosphorylated by phosphomonoesterases in most cases. This indicates that depolymerization sets the pace for the decomposition of high molecular weight P_o compounds, with phosphodiesterase-activities representing the bottleneck in P_o decomposition. This study did not allow for direct quantification of the abiotic sorption of intact P_o compounds. However, we could show that abiotic sorption processes strongly reduced $^{33}P_i$ recoveries, and likely also competed with microbial uptake of P_i after extracellular P_o mineralization.

Up to date, the possibility of direct microbial uptake of P_o in soils has been widely overlooked. Here we presented indications that this process occurred to a substantial extent in two agricultural soils. Thus, we showed that direct microbial uptake of P_o constitutes a P-acquisition pathway in the soil P cycle that does not require complete extracellular mineralization and subsequent P_i uptake. We further found that cumulative net microbial P_o uptake seemed to be driven by the availability of intact phosphomonoesters. Direct P_o uptake could be competing or complementary to extracellular P_o mineralization, depending on whether it is subject to different or to interconnected controls. Further research will be necessary to investigate the interplay of these two pathways, to assess the quantitative importance of direct P_o uptake in soil systems, and to elucidate the ecological consequences of this process. Systematic studies of the distribution of phosphatases and P_o uptake transporters across the soil microbial diversity will be necessary to answer these questions. Organic P plays a central role in the P cycle of most soils, and accounting for direct microbial P_o uptake and the potential of phosphodiesterase activities to control overall P_o mineralization rates will substantially enhance our understanding of soil P cycling (Figure 1).

Data availability statement

The raw data supporting the conclusions of this article is available in the Supplementary Material as Data Sheet 2.

Author contributions

WW designed the experiment, DW and WW developed the methods with contributions from YH. DW, JP and DZ conducted the laboratory work, DW analyzed the data, and DW and WW interpreted the data and wrote the manuscript with contributions from MM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2023.1097965/full#supplementary-material

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