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Microorganisms drive stabilization and accumulation of organic phosphorus: An incubation experiment

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ABSTRACT

At the earliest stage of pedogenesis, microorganisms are the main biological drivers of nutrient transformation and mobilization from rock minerals. However, this driving process is not yet well understood despite its importance for the formation and fertility of soil. The aim of this study was to determine the microbial accumulation of organic phosphorus (Po) and microbial release of bioavailable phosphorus (bio-P) from rock minerals and to analyse which factors control these processes. We hypothesized that microorganisms contribute to stabilization and accumulation of Po. For this purpose, we carried out a series of incubation experiments with model soils (with soil Po removed) and soil extracts of natural soil from the earliest stage of pedogenesis in the newest retreat area of the Hailuogou glacier. A modified Hedley fractionation method was used to characterize the P forms in soils. Microbial synthesis of Po was clearly observed in this study. In view of the fact that carbon (C) and nitrogen (N) are the main energy and material sources of microorganisms and that their stoichiometry plays an important role in microbial metabolism, we hypothesized that the C and N (ratio and level) play a regulatory role in microbial transformation of P. Therefore, the different C/N gradients were constructed in our incubation experiments. Results showed addition of C and N resulted in an accumulation of stable Po and decrease in pH in soils. These results suggest that not all microbial biomass P is easily degraded. Along two C/N ratio gradients (same ratio but keeping C vs N constant), opposite patterns of total Po (TPo) changes was observed. Opposite patterns were also observed for bio-P along the two C/N ratio gradients. Regardless of the C/N ratio, high CN additions always led to lower TPo accumulations, higher bio-P releases and higher phosphatase activity compared to low CN additions. We conclude that microbial synthesis of Po contributed to the P fractions in soil stable residual pools and that microbial release of bioavailable P was driven by two modes (i.e., N-driven mode and C-driven mode). Our study highlights that microorganisms drive the accumulation of soil Po during the earliest stage of pedogenesis in our study area.

1. Introduction

Phosphorus (P) is a crucial macronutrient for the soil biota community. In soil, all living organisms require considerable amounts of P for energy transport and storage (e.g., ATP and inorganic polyphosphate), transmission and storage of genetic information (e.g., RNA and DNA) and building cell structures (e.g., phospholipid bilayers). However, P easily becomes a limiting nutrient for soil biota communities (Marklein and Houlton, 2012) because (1) in terms of the ultimate source of P, provision of new P in the land that has not been disturbed by human fertilization activities (e.g., new land exposed after glacier retreat and virgin forest soil) is almost entirely derived from the slow weathering of soil minerals (Walker and Syers, 1976) and (2) available P for soil organisms is easily precipitated or adsorbed by metal cations (e.g., Fe and Al) and their oxides. Moreover, the available P is frequently removed from soil ecosystems by water flows. P limitation can lead to significant ecological consequences, such as a decline in soil productivity, changes in plant diversity (Laliberte et al., 2014) and nutrient

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acquisition strategies (Zemunik et al., 2015). These findings point to the importance of P mobilization for the nutrition of the soil biota community; after all, soil mineral P needs to be transformed before becoming available for soil organisms (Sun et al., 2020).

The transformation and mobilization of soil P are largely driven by soil microorganisms. On the one hand, microorganisms transform insoluble P forms (e.g., phospholipids and rock phosphates) into free orthophosphate by using two different processes: (i) organic P (Po) mineralization and (ii) inorganic P (Pi) solubilization. Most available P from organic sources is released by soil microorganisms because the majority of phosphatase enzymes are probably produced by microorganisms (Buenemann, 2015). The microbial mineralization abilities of organic P are attributed to their functional genes coding for a series of phosphatases, such as the microbial *app*A gene for phytase (Rodriguez et al., 2006) and *pho*C and *pho*D for nonspecific phosphatases (Fraser et al., 2017). In addition, P solubilization processes that release available P from inorganic P pools (e.g., apatite) can greatly enhance soil P availability. Microorganisms can solubilize P through several mechanisms. They can (1) acidify the soil environment by producing protons to enhance the dissolution of rock phosphates and their desorption reactions (which are pH dependent), and then to release free orthophosphate from the solid phase in soil (Brucker et al., 2020). Microorganisms may (2) secrete low-molecular-weight organic acids (e.g., gluconic and citric), which chelate the cations that are bound to phosphate through their hydroxyl and carboxyl groups and thus transform rock phosphates into soluble forms (Chen et al., 2006). At a genetic level, the microbial metabolic basis of P solubilization is associated with genes coding for quinoprotein glucose dehydrogenase (PQQGDH), which controls the acidification of periplasmic space and the direct oxidation pathway of glucose (Bergkemper et al., 2016). The mineralization of soil Po has been widely and intensively studied in previous research work (Buenemann, 2015), but only a few studies have addressed P solubilization in soil inorganic P (Pi) pools. Our understanding of microorganisms that drive the solubilization of soil Pi is mostly limited to the characterization of isolates or the effect of P-solubilizing microorganisms in pure microbial cultures (Song et al., 2007; Sun et al., 2020). Especially in the soil environment, the factors and mechanisms that drive microbial P solubilization from complex minerals are not well understood (Brucker et al., 2020).

On the other hand, soil microorganisms absorb available P as part of their own biomass, which involves microbial synthesis of Po and immobilization of free orthophosphate. The forms of microbial P include nucleic acids + phospholipids (54–62%), polyphosphate (16–25%), cytoplasmic Po (1.9–17%) and cytoplasmic Pi (5.9–12.5%) in P-limited bacteria (Vadstein, 2000). The total P concentrations in microbial biomass (MBP) range from 15.0 to 29.3 g/kg for bacteria and from 6.3 to 14.5 g/kg for fungi (Buenemann et al., 2008a), which are much higher than those of plants (i.e., 0.9–4.5 g/kg for leaves and 0.2–0.5 g/kg for wood, according to Netzer et al.'s data (2017)). In a temperate rainforest, the proportion of MBP to total biomass P is more than 68% (Turner and Condron, 2013). Although these findings were derived from pure microbial cultures or crude methods of assessing soil MBP (e.g., chloroform fumigation methods), they indicate the importance of microorganisms for soil Po formation. However, since the same broad chemical P forms occur in all organisms (Buenemann et al., 2008b), the origin of soil Po is difficult to determine, and consequently, the origin of soil Po is still a matter of some debate (Buenemann et al., 2008a). In addition, recent studies have increasingly indicated that microorganisms have the ability to produce recalcitrant compounds that supplement the stable carbon (C) pool in soils (Liang et al., 2011). According to this, soil recalcitrant P is likely to be directly synthesized by microorganisms to expand the stable Po pool in soils. Nevertheless, little is known about microbial contribution of recalcitrant Po accumulations in soil.

In terms of the above microbial processes involving P transformation (P solubilization, Po mineralization and synthesis), C and nitrogen (N) are key factors as the necessary material and energy for microbial processes. Based on solution incubation experiments, Sun et al. (2017) demonstrated C and N addition control microbial phosphate-solubilizing function by driving the changes of microbial community structure and metabolic activities. For Po mineralization, Spohn and Kuzyakov (2013) conducted isotope labeling experiments (^{14}C and ^{33}P) and showed Po mineralization were driven by microbial need for C, although the role of N was not evaluated in their experiments. In addition, C also drives the synthesis of Po. Based on soil incubation experiments with repeated addition of C source, Buenemann et al. (2008b) showed that compared to non-carbon-amended controls, C addition significantly increased microbial synthesis of Po. Although the above studies showed that C is an important factor driving microbial transformation of P, N is also an important factor affecting microbial community structure, microbial biomass and metabolic process (Sun et al., 2017). Therefore, in order to acquire a better understanding of microbial transformation of P, the N factor should be considered in the study of microbial transformation of P. However, these studies provide very little information about effects of N on the microbial transformation of P.

In pedogenesis, an accumulation of Po and decline in Pi have been confirmed by observations along soil chronosequences (Zhou et al., 2019). In particular, during pedogenesis, its beginning stage without vegetation provides ecological references and experimental materials (e.g., mineral mixtures and microbial consortia extracted from it) for our controlling experiments to understand the microbial mechanisms of P transformation in the earliest stage of pedogenesis.

To confirm the microbial-mediated processes causing soil P mobilization, as well as to elucidate the effects of nutritional factors on microbial P transformations during the initial stage of pedogenesis, we conducted a series of incubation experiments that used microbial consortia and soils from the beginning stage of the soil chronosequence that were located in the retreat area of the Hailuogou glacier in SW China. We tested the following hypotheses. First, soil microorganisms can contribute to the production of stable Po that accumulates in soil. Second, the same initial gradient of C/N ratios causes the same change pattern of bioavailable P (bio-P) along the gradient. Third, high availability of C and N leads to high accumulations of soil Po.

2. Materials and methods

2.1. Study site and soil

The study site is located in the new retreat area of the Hailuogou glacier (29°34′08″N, 101°59′41″E) at the southeastern edge of the Tibetan Plateau, southwest China (Fig. S1). The elevation of this study site is 2990 m above sea level (a.s.l). Soil temperature (at depth of 10 cm) ranges from minimum of −1.5 °C to maximum of 20.5 °C over the year. The annual precipitation is 1586–2175 mm. In the chronosequence of the Hailuogou glacier retreat area, the exposure age of the studied soil is less than 2 years. There are no plants in the soil. According to the soil property data (Table 1 and Fig. S1), strictly speaking, the studied soil is more like rock debris, although there is a small amount of organic matter in the soil. Please see more soil details in Table 1.

2.2. Soil sampling and creation of model soils

In May 2016, six sampling plots were set up in the newest retreat area of the Hailuogou glacier. The size of each plot was 1 × 1 m, and the interval between plots was greater than 5 m. In each plot, five sampling points were randomly selected along diagonal lines in the plot. At each sampling point, fresh soils were collected using a sterile blade from depths of 0–10 cm. Soil samples from the six sampling plots were homogenized using the same weight to obtain a representative soil sample of the study site. One subsample was stored at 4 °C for determining soil physical and chemical properties, and another subsample was stored for creating model soils.

Table 1
Properties comparison between model soil and natural soil from the newest retreat area of the Hailuogou glacier. Data are shown as the means ± SD. A same letter indicates no significant differences between natural soil and model soil at the $p > 0.05$ level by independent-samples T test.

Items	Natural soils	Model soils
SOC(g/kg)	3.61 ± 0.24	ND
TN(mg/kg)	83.80 ± 5.16	ND
TP(g/kg)	1.13 ± 0.02 a	1.14 ± 0.02 a
Fe(g/kg)	36.25 ± 2.87 a	37.65 ± 1.89 a
Al(g/kg)	70.76 ± 2.79 a	71.59 ± 2.44 a
pH	8.56 ± 0.11 a	8.52 ± 0.03 a
Coarse grains (2–0.2 mm, %)	41.10 ± 1.03 a	40.86 ± 1.43 a
Fine grains (0.2–0.02 mm, %)	48.37 ± 0.86 a	48.42 ± 0.95 a
Silt + clay (<0.02 mm, %)	10.53 ± 0.69 a	11.12 ± 0.69 a
Quartz (%)	26.1 ± 0.8 a	27.9 ± 0.9 a
Plagioclase (%)	20.5 ± 0.4 a	20.4 ± 0.3 a
K-feldspar (%)	7.7 ± 0.4 a	7.4 ± 0.3 a
Calcite (%)	4.9 ± 0.1 a	4.9 ± 0.2 a
Dolomite (%)	0.3 ± 0.1 a	0.3 ± 0.1 a
Augite (%)	2.4 ± 0.2 a	2.8 ± 0.2 a
Hornblende (%)	11.9 ± 0.3 a	11.4 ± 0.3 a
Apatite (%)	2.4 ± 0.2 a	2.1 ± 0.1 a
Biotite (%)	20.2 ± 0.4 a	19.4 ± 0.7 a
Chlorite (%)	3.3 ± 0.1 a	3.2 ± 0.1 a

The ND indicates values below the limit of detection.

Since the natural soil in the study area is mostly composed of rock particles and the degree of weathering is very low, we created model soils by crushing natural soil gravel and mixing the debris to mimic the natural soil particle size distribution. According to the natural soil texture (i.e., particle-size distribution, Table 1), the model soils were created by mixing coarse grains (2–0.2 mm, 41.1%), fine grains (0.2–0.02 mm, 48.4%) and silt + clay (<0.02 mm, 10.5%). The preparation methods for these rock grains were as follows: gravels (>2 mm) are collected from the representative soil sample, and the gravels were washed with ultrapure water until no Po was detected in the water. After drying, the gravels were crushed and ground. The broken gravels were sieved to obtain enough coarse grains and fine grains. To obtain the silt + clay portion of the model soil, a pipette method (Gee and Or, 2002) was used. Finally, the coarse grains, fine grains and silt + clay were sterilized for further use. In addition, the most important reason for using model soils is to ensure that the experimental soil does not contain Po at the beginning of the experiment.

2.3. Incubation experiments

We conducted incubation experiments using the model soils and soil extracts to inoculate microorganisms and avoid introduction of Po. Preparation of the soil extracts was performed in the following manner. Twenty grams of fresh soil (screened with a 2-mm sieve) were extracted in sterile ultrapure water for 2 h on a rotary shaker. After standing for 0.5 h, the supernatants containing microorganisms were collected. Each model soil (100 g) was placed in a glass culture jar, and the suspension (5 ml) was then sucked up with a pipette and injected into the jar. All experimental treatments and control treatments were performed in six replicates. Depending on the experimental purpose, all treatments were classified into three subexperiments, i.e., experiment 1, experiment 2 and experiment 3:

For different treatments of experiment 1, the levels of C addition were maintained but the C:N ratios were changed. In brief, in experiment 1, the C source was added to the culture jars at the same dose (3.6 g C/kg), and the N source was then added at three doses (i.e., 16.8 mg N/kg, 84 mg N/kg and 420 mg N/kg), respectively. See more details in Table 2.

For different treatments of experiment 2, the levels of N addition were maintained but the C:N ratios were changed. In brief, in experiment 2, the N source was added to the culture jars at the same dose (84 mg N/kg), and the C source was added at three doses (i.e., 18 g C/kg, 3.6 g C/kg and 0.72 g C/kg), respectively. See more details in Table 3.

Although experiment 1 and experiment 2 have the same C:N ratio gradient, the differences between them are as follows: in experiment 2, the gradient of C:N ratios was only caused by the change of C addition; in experiment 1, by contrast, the gradient of C:N ratios was only caused by the change of N addition.

For different treatments of experiment 3, the C:N ratios were maintained but levels of C and N additions were changed. Namely, a high CN addition vs a low CN addition here is referring the low vs high levels of C and N addition for the same C:N ratio. For example (Table 4), at a C:N ratio of 250, two levels of CN addition were designed, i.e., High CN addition (HighCN250: 18 g C/kg and 84 mg N/kg) and Low CN addition (LowCN250: 3.6 g C/kg and 16.8 mg N/kg). See Table 4 for other treatments of experiment 3.

Moreover, we set up three types of control treatments (Table 2, Table 3, Table 4 and Fig. 1). The differences between them and their corresponding experimental treatments were as follows: 1) -CK1: blank control treatments (i.e., only sterilized soil extracts), in which there were no living microorganisms and no CN additions; 2) -CK2: microbial control treatments (i.e., CN + sterilized soil extracts), in which no living microorganisms were present to confirm the microbial effects of the experimental treatments containing living microorganisms; 3) -CK3, CN control treatments (i.e., only soil extracts), in which no CN addition was present to confirm the CN effects of the experimental treatments containing CN additions. All culture jars were sealed with screw caps, and the soil moisture was adjusted to 80% of the soil water holding capacity. Under sterile conditions, the culture jars were inverted repeatedly by hand to mix the cultures every 3 days, and the culture jars were then opened to allow air exchange. The incubation experiment was conducted for 201 days at 20 °C. At the end of the experiment, the soil samples in the culture jars were collected and divided into two

Table 2
The treatments of experiment 1(the levels of C addition were maintained but the C:N ratios were changed in experimental treatments).

	Treatments	C/N	C	N	soil extracts	sterilized soil extracts
		Atomic ratio	g/kg	mg/kg	ml	ml
Experimental treatments	N1	250	3.6	16.8	5	0
	N2	50	3.6	84.0	5	0
	N3	10	3.6	420.0	5	0
Control treatments	N-CK1	0	0	0	0	5
	N1-CK2	250	3.6	16.8	0	5
	N2-CK2	50	3.6	84.0	0	5
	N3-CK2	10	3.6	420.0	0	5
	N-CK3	0	0	0	5	0

C and N were added by glucose, NH_4NO_3, respectively. The C source was added to the culture jars at the same doseratio (3.6 g C/kg), and the N source was then added at three doses (i.e., 16.8 mg N/kg, 84 mg N/kg and 420 mg N/kg), respectively. Therefore, for the CN addition treatments of experiment 1, the gradient of C:N ratios was only caused by the change of N addition. In control treatments, -CK1: blank control treatments (i.e., only sterilized soil extracts); -CK2: microbial control treatments (i.e., CN + sterilized soil extracts); -CK3: CN control treatments (i.e., only soil extracts). For each treatment, set up 6 replicates.

Table 3
The treatments of experiment 2 (the levels of N addition were maintained but the C:N ratios were changed in experimental treatments).

	Treatments	C/N	C	N	soil extracts	sterilized soil extracts
		Atomic ratio	g/kg	mg/kg	ml	ml
Experimental treatments	C1	250	18	84.0	5	0
	C2	50	3.6	84.0	5	0
	C3	10	0.72	84.0	5	0
Control treatments	C-CK1	0	0	0	0	5
	C1-CK2	250	18	84.0	0	5
	C2-CK2	50	3.6	84.0	0	5
	C3-CK2	10	0.72	84.0	0	5
	C-CK3	0	0	0	5	0

C and N were added by glucose, NH_4NO_3, respectively. The N source was added to the culture jars at the same dose (84 mg N/kg), and the C source was added at three doses (i.e., 18 g C/kg, 3.6 g C/kg and 0.72 g C/kg), respectively. Therefore, for the CN addition treatments of experiment 2, the gradient of C:N ratios was only caused by the change of C addition. In control treatments, -CK1: blank control treatments (i.e., only sterilized soil extracts); -CK2: microbial control treatments (i.e., CN + sterilized soil extracts); -CK3: CN control treatments (i.e., only soil extracts). For each treatment, set up 6 replicates.

Table 4
The treatments of experiment 3 (the C:N ratios were maintained but levels of C and N additions were changed in experimental treatments).

	Treatments	C/N	C	N	soil extracts	sterilized soil extracts
		Atomic ratio	g/kg	mg/kg	ml	ml
Experimental treatments	HighCN250	250	18	84.0	5	0
	LowCN250	250	3.6	16.8	5	0
	HighCN50	50	18	420	5	0
	LowCN50	50	3.6	84.0	5	0
	HighCN10	10	3.6	420	5	0
	LowCN10	10	0.72	84.0	5	0
Control treatments	CN-CK1	0	0	0	0	5
	HighCN250-CK2	250	18	84.0	0	5
	LowCN250-CK2	250	3.6	16.8	0	5
	HighCN50-CK2	50	18	420	0	5
	LowCN50-CK2	50	3.6	84.0	0	5
	HighCN10-CK2	10	3.6	420	0	5
	LowCN10-CK2	10	0.72	84.0	0	5
	CN-CK3	0	0	0	5	0

C and N were added by glucose, NH_4NO_3, respectively. HighCN250: high CN addition at a C:N ratio of 250; LowCN250: low CN addition at a C:N ratio of 250; HighCN50: high CN addition at a C:N ratio of 50; LowCN50: low CN addition at a C:N ratio of 50; HighCN10: high CN addition at a C:N ratio of 10; LowCN10: low CN addition at a C:N ratio of 10. Namely, a high CN addition vs a low CN addition here is referring the low vs high levels of C and N addition for the same C:N ratio. In control treatments, -CK1: blank control treatments (i.e., only sterilized soil extracts); -CK2: microbial control treatments (i.e., CN + sterilized soil extracts); -CK3: CN control treatments (i.e., only soil extracts). For each treatment, set up 6 replicates.

subsamples. One subsample was stored at 4 °C for analysing soil physicochemical properties, and the other subsample was freeze-dried and stored at −80 °C for determining soil P forms.

2.4. Sequential fractionation of soil P

A modified Hedley fractionation method (Tiessen and Moir, 1993) was used to extract the forms of P from freeze-dried soil (0.5 g). The extraction procedure was executed sequentially in the following five steps: 1) resin-extraction (resin: BDH 551642S, 9 × 62 mm, two strips per tube); 2) $NaHCO_3$-extraction (0.5 M); 3) NaOH-extraction (0.1 M); 4) HCl-extraction (1 M); and 5) concentrated HCl-extraction (cHCl). In each extraction, the soil-extractant solution was shaken (16 h, 280 rpm), centrifuged (10,000 g for 10 min at 0 °C) and filtered (pore size 0.45 μm) before being collected. The molybdenum blue colorimetric method was used to determine the Pi concentration in each extraction. For the extractions with low P concentrations, they were concentrated by a vacuum drying process before being measured. After digestion with ammonium persulfate at 121 °C, the total P (P_t) in each extraction was determined. The Po was determined as the difference between P_t and Pi. Finally, the soil P was presented in the following forms: resin-Pi, $NaHCO_3$-Pi, $NaHCO_3$-Po, NaOH-Pi, NaOH-Po, HCl-Pi, cHCl-Pi and cHCl-Po. Generally, resin-Pi and $NaHCO_3$-Pi are considered to be bio-P, and $NaHCO_3$-Po considered to be labile P, whereas NaOH-Pi and NaOH-Po are assigned to moderately labile P, and cHCl-Pi and cHCl-Po are assigned to a stable residual pool (Tiessen and Moir, 1993). Therefore, the P forms in this study were grouped as: bio-P = resin-Pi + $NaHCO_3$-Pi, stable Po = cHCl-Po, and total Po (TPo) = $NaHCO_3$-Po + NaOH-Po + cHCl-Po. Gu et al. (2020) showed the Hedley scheme have some degree of uncertainty in accurate estimates of P pools which are based on chemical property classification. However, Helfenstein et al. (2018) reported that the resin-P, NaOH-Pi and HCl–P pools turn over in minutes, weeks-months, and years-millennia, respectively. Therefore, the Hedley scheme can imply the stability (or availability) of P because of its physical and chemical treatment steps in the scheme. For example, before the cHCl-Po is extracted, the same soil has been shaken violently (280 rpm) for 4 times (4 extraction solutions) for a long time (16 h each time), and subjected to desorption effects of $NaHCO_3$ and NaOH. This process can strongly infer the physical stability (or physical accessibility) of cHCl-Po. Moreover, it is well known that the hot concentrated HCl is a strong driving force for Po hydrolysis. After sequential extraction with 4 extraction solutions (i.e, pure water, $NaHCO_3$, NaOH and

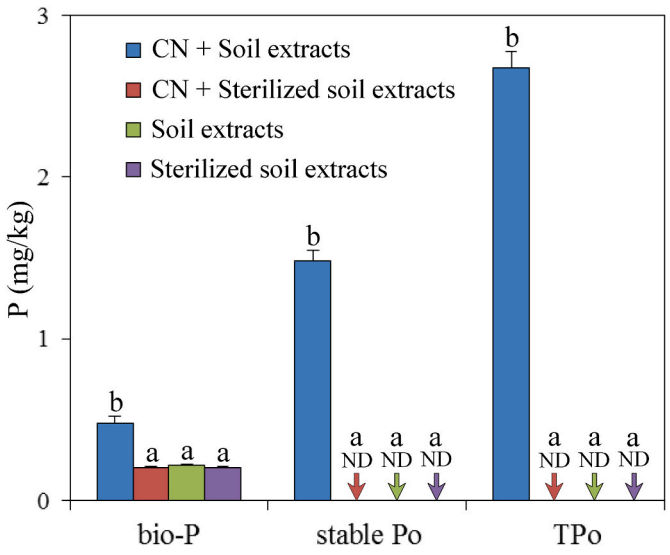

Fig. 1. Mean concentration (mg P/kg) of bioavailable phosphorus (bio-P), stable organic phosphorus (stable Po) and total organic phosphorus (TPo) in carbon- and nitrogen-depleted, sterile, model soils, built from gravel collected from the newest retreat area of the Hailuogou glacier. The 'CN + Soil extracts' treatment (n = 72) is the mean value for the average of all experimental additions of C and N. A full description the 3 types of controls ('CN + Sterilized soil extracts' (n = 72), 'Soil extracts' (n = 18), and 'Sterilized soil extracts' (n = 18)) can be found in the text, Table 2, Table 3 and Table 4; these were all below detection ('ND'), and the 'ND' was defined as a value of 0 in statistical analysis. Error bars represent one standard error. Significant differences ($p < 0.05$) by a one-way ANOVA followed by a post-hoc test (Tukey or Games-Howell) were indicated by different letters above bars.

dilute-HCl), the cHCl-Po is extracted by the hot concentrated HCl, and thus such process can strongly infer the chemical stability of cHCl-Po.

2.5. Microbial lipid analyses

Phospholipid fatty acids (PLFAs) were extracted to measure the total viable microbial biomass. According to the procedure of Sun et al. (2018), PLFAs were extracted from 8.0 g of freeze-dried soil by a single-phase mixture of chloroform/methanol/citrate buffer (40 ml at a 1:2:0.8 vol ratio). After the extracts were dried, fractionated and methylated, the fatty acid methyl esters were obtained and determined by gas chromatography (GC, Agilent 6890) with MIDI peak identification software (version 4.5, MIDI Inc., Newark, DE, USA). The GC was equipped with an Agilent capillary column (19091B-102E, 25.0 m long × 0.2 mm internal diameter × 0.33 μm film thickness) using H_2 as the carrier gas. Nonadecanoic acid (19:0) was added as an internal standard before extraction and methylation. Wang et al. (2003) indicated that phospholipids are lipids that contain phosphoric residues and are thus ampiphilic molecules possessing polar head groups and non-polar lipid chains. Moreover, Mamun et al.(2020) showed that major phospholipids in the living system have a phosphate group. Therefore, in order to simplify the estimation of P content in phospholipids (PLFA-P), we assume that 1 mol of the fatty acid molecule comes from 1 mol of phospholipid with one phosphate group. Therefore, the mole of each phospholipid can be calculated by the mole of fatty acid, and then the P in phospholipids (PLFA-P) can be calculated by the mole of each phospholipid (i.e. the mole of PLFA).

2.6. Soil physicochemical properties

Soil moisture (SM) levels were determined gravimetrically by oven-drying soil to a constant weight at 105 °C. Soil pH values were measured in CO_2-free water (soil: water = 1 : 2.5) using a pH meter (METTLER TOLEDO). Soil total organic carbon (SOC) was determined using an elemental analyser (Elementar Vario EL) after the soil was acidified for 48 h with HCl to clear inorganic carbon. Total nitrogen (TN) concentration was also determined using an elemental analyser. Because the soil is alkaline, soil phosphatase activities are expressed by the activities of alkaline phosphomonoesterase (ALP, EC 3.1.3.1), which were measured using the method recommended by Tabatabai (1994): The activities of alkaline phosphomonoesterase were assayed with the substrate *p*-nitrophenyl Phosphate: fresh soil (1.0 g) was put into a flask and incubated (37 °C for 1 h) with buffered substrate solution(pH = 11), and then NaOH–$CaCl_2$ solution was added in the flask to end the reactions. Then, the flask was swirled, and the soil suspension was filtered. The yellow color intensity of the filtrate was measured with a spectrophotometer (400 nm). Controls were performed with the substrate being added after the reactions were stopped. Ammonia (NH_4^+-N) was extracted from 2 g of soil with KCl (2 M, 20 ml) for 1 h, and its concentrations were determined using a continuous flow autoanalyser (Skalar San++ 8505, Netherlands). After the freeze-dried soil (0.5 g) was digested by an $HClO_4$–HNO_3–HF–HCl mixture, soil total P, Fe and Al were determined using an American Leeman Labs Profile inductively coupled plasma atomic emission spectrograph (ICP-AES).

2.7. Statistical analysis

The Shapiro-Wilk test was used to determine whether the data were normally distributed. If the data were not normally distributed, a logarithmic transformation was performed. For data with multiple treatments (≥3), one-way analysis of variance (ANOVA) combined with Tukey's post-hoc test were conducted to compare these data. If the variances of the data were not equal, a Games-Howell test was used. For data with two treatments, an independent-samples T test was used. At a statistical level of $p < 0.05$, significant differences were accepted in this study. The above analyses were executed with PASW software (SPSS 13.0 softwareIL).

3. Results

3.1. Soil P in the experimental and control treatments

In all experiments of the present study, the contrasting results between the experimental and control treatments showed that CN inputs promoted microbial synthesis of Po (Fig. 1). In the 3 control treatments (i.e., I. CN + sterilized soil extracts, II. soil extracts, and III. sterilized soil extracts), for stable Po and TPo, all of their concentrations were below the limit of detection (Fig. 1). As a result, bio-P (0.47 ± 0.05 mg/kg), stable Po (1.5 ± 0.1 mg/kg) and TPo (2.6 ± 0.1 mg/kg) in the experimental treatments were higher than in the control treatments ($p < 0.001$).

3.2. P fractions and other parameters along the C/N ratio gradient

In experiment 1, 3.6 g/kg of C source was added, and the changes in C/N ratios were then controlled by N addition to form a gradient of C/N ratios (i.e., C/N = 10, 50, and 250) (Table 2 and Fig. 2). Compared to the control treatments with no CN addition, the TPo (2.9 ± 0.2 mg/kg) and stable Po (1.6 ± 0.1 mg/kg) accumulations clearly occurred (Fig. 2a). Overall, the bio-P concentrations (0.31 ± 0.02 mg/kg) decreased with increasing C/N ratios, while the TPo and stable Po concentrations increased with increasing C/N ratios, although there was no significant difference between the two C/N ratios (50 and 250) (Fig. 2a). All soils were alkaline. The soil pH values (8.26 ± 0.04) in the experimental treatments were lower than those in the CK treatments (Fig. 2b). At a C/N ratio of 50, soil pH showed a significantly low value than those in the CK treatments, but there were no significant differences in soil pH values along the C/N ratio gradient (Fig. 2b). No ALP activity was detected in the control treatments (Fig. 2b). In the experimental treatments, the ALP

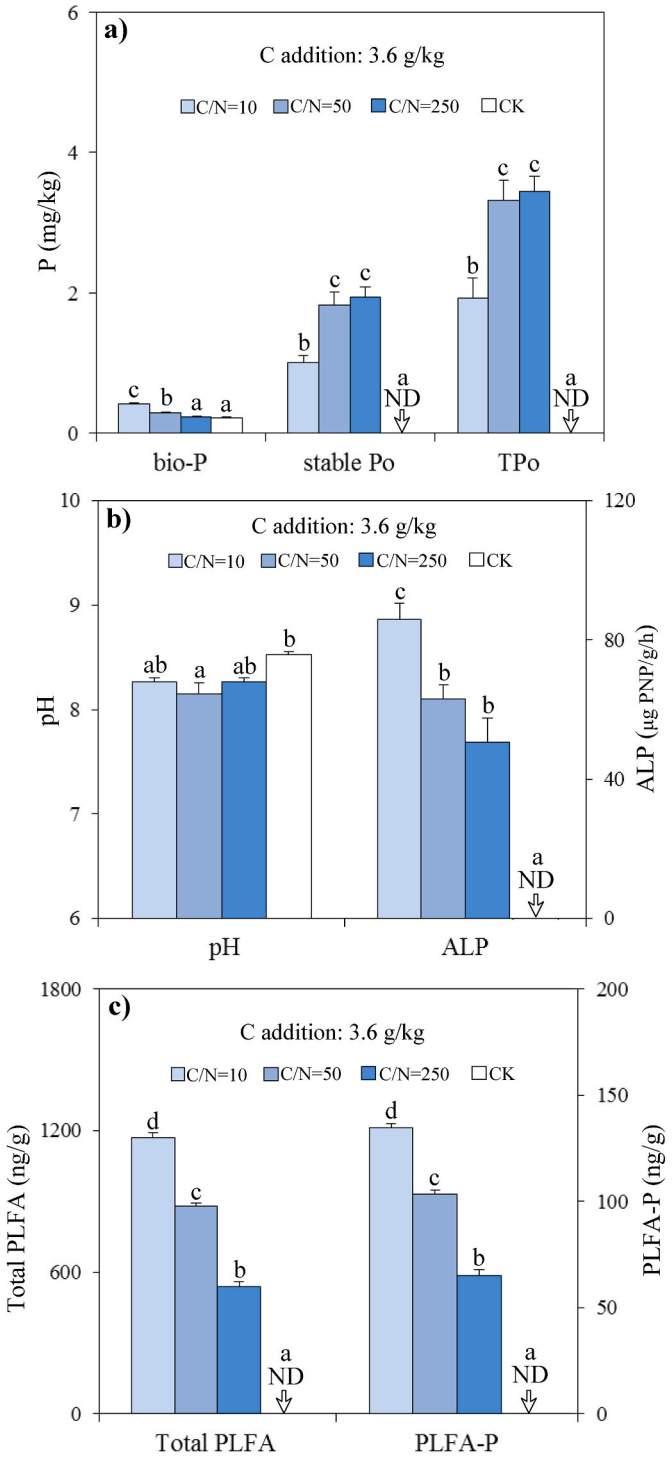

Fig. 2. Results from experiment 1 (the levels of C addition were maintained but the C:N ratios were changed in experimental treatments, see Table 2 for the treatments). Columns show means and the error bars indicate the standard errors (n = 6). 'ND' means that the measured value is below the detection limit, and the 'ND' is defined as a value of 0 in statistical analysis. Significant differences ($p < 0.05$) by a one-way ANOVA followed by a post-hoc test (Tukey or Games-Howell) were indicated by different letters above bars. The control treatments containing code 'N-CK3' (i.e., only soil extracts, see Table 2) are used as the CK in the figure. ALP: alkaline phosphomonoesterase activity. The PLFA-P means the P in phospholipids, and it is calculated by the mole of each phospholipid (i.e, the mole of each PLFA).

activities showed a decreasing trend ($p < 0.05$) with increased C/N ratios. In experiment 1, the total PLFA and PLFA-P decreased with increase in the C/N ratios (Fig. 2c).

Similarly to experiment 1, experiment 2 had the same C/N ratio gradient (i.e., also C/N = 10, 50, and 250). However, compared to experiment 1, the changes in C/N ratios were controlled by C addition, while N addition was a set value (i.e., 84 mg N/kg) in experiment 2 (Table 3 and Fig. 3). In the experimental treatments, significant TPo (2.6 ± 0.2 mg/kg) and stable Po (1.4 ± 0.1 mg/kg) accumulations were also observed (Fig. 3a). Along the C/N ratio gradient, experiment 2 showed opposite patterns of changes in bio-P (and/or TPo, stable Po) compared to experiment 1. Overall, the bio-P concentrations (0.6 ± 0.1) increased with increasing C/N ratios, while the TPo and stable Po concentrations decreased with increasing C/N ratios (Fig. 3a). Soil pH also significantly decreased in the experimental treatments (Fig. 3b). The lowest value of soil pH was observed at a C/N ratio of 250. The soil pH showed a decreasing trend ($p < 0.05$) with increased of C/N ratios. There were no significant differences in ALP activity among the three C/N ratios (Fig. 3b), but the ALP activity increased with increasing C/N ratios. The patterns of total PLFAs (and/or PLFA-P) along the same gradient of C/N ratios were clearly different between experiment 1 and experiment 2. In experiment 2, the total PLFAs and PLFA-P increased with increases in C/N ratios (Fig. 3c).

3.3. *P fractions and other parameters under different levels of CN addition*

In experiment 3, the effects of the CN addition levels on the P fractions were checked at C/N ratios of 10, 50 and 250 (Fig. 4a, b, and c). Regardless of the C/N ratio, when compared to the low CN addition, the high CN addition always led to lower accumulation of TPo (2.6 ± 0.1 mg/kg) and stable Po (1.5 ± 0.1 mg/kg), and higher release of bio-P (0.51 ± 0.07 mg/kg) (Fig. 4a, b, and c). For TPo (or stable Po and bio-P), the difference between the high CN addition and low CN addition was significant at a statistical level of $p < 0.05$. Significant differences in soil pH between the two levels of CN additions were detected only at C/N ratios of 250 (Fig. 4f), while no significant difference was observed for the other two C/N ratios (Fig. 4d and e). ALP activities with high CN additions were always high ($p < 0.05$) compared to those with low CN addition (Fig. 4d, e, f). Compared to the low CN additions, high CN additions always led to high values of total PLFA (or PLFA-P) (Fig. 4h, i, j).

4. Discussion

4.1. *Microbial synthesis of Po*

The microbial origin of soil Po was clearly demonstrated in this study. In the present experiment, the absence of plants and Po were strictly set as the initial conditions. Hence, the production of Po forms manifested the key role of soil microbial communities in synthesizing Po and controlling the dynamics of soil P forms.

In an incubation experiment that aimed at the net microbial uptake kinetics of ^{14}C and ^{33}P from glucose-6-phosphate, Spohn and Kuzyakov (2013) found that 16.4% of the ^{14}C from glucose-6-phosphate was recovered in the microbial biomass, while ^{33}P incorporation into the microbial biomass was one-third less. In their analysis, they concentrated on one aspect of the effects of C on P (i.e., C drives Po mineralization by microorganisms). However, their results also demonstrated another aspect of effects of C on P (i.e, C drives the synthesis of Po by microorganisms). In our experiments, we also observed that the net synthesis levels of Po ranged from 0.9 to 4.2 mg/kg soil by soil microorganisms under C addition conditions. In addition, when using a NaOH-EDTA solution as the extractant, Bünemann et al. (2008) found that repeated additions of carbon substrates caused soil microorganisms to synthesize more Po (0.06–0.37 mg/kg soil/day). Therefore, these

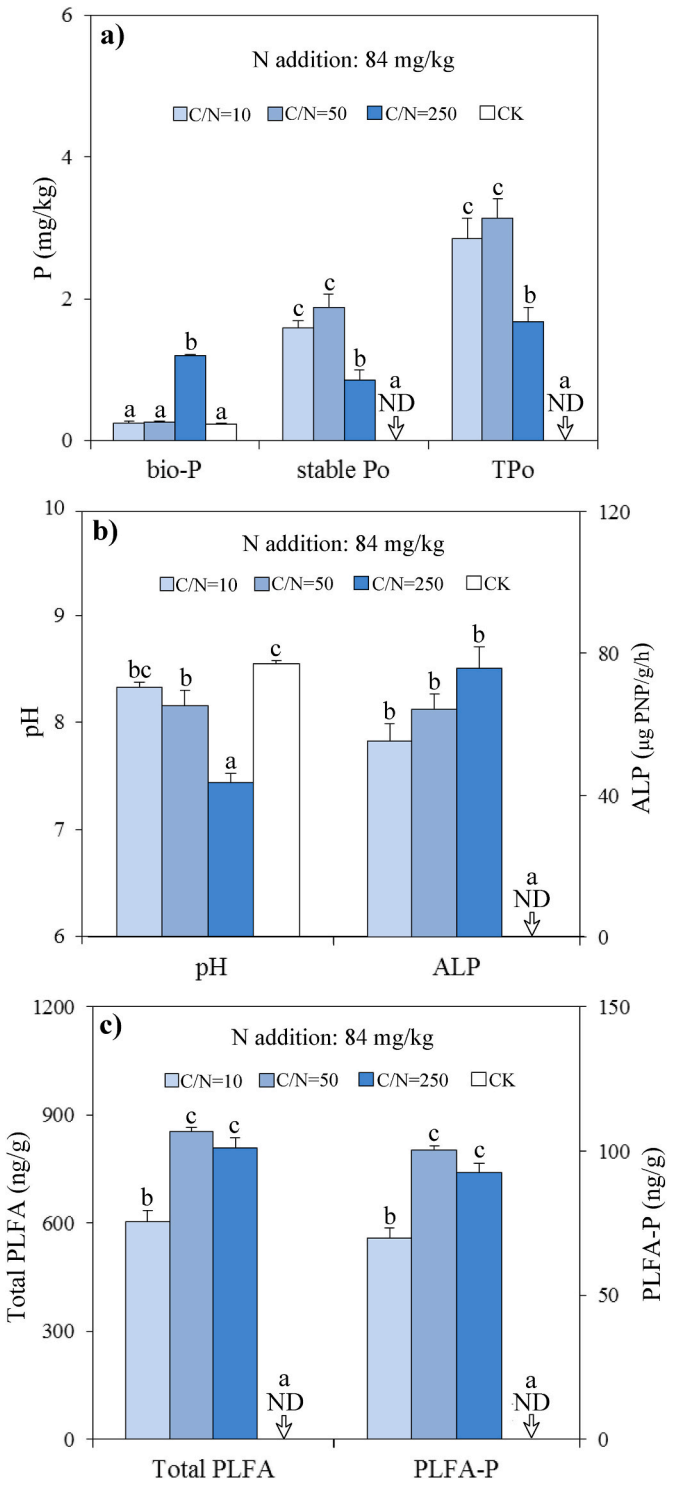

Fig. 3. Results from experiment 2 (the levels of N addition were maintained but the C:N ratios were changed in experimental treatments, see Table 3 for the treatments). Columns show means and the error bars indicate the standard errors (n = 6). 'ND' means that the measured value is below the detection limit, and the 'ND' is defined as a value of 0 in statistical analysis. Significant differences ($p < 0.05$) by a one-way ANOVA followed by a post-hoc test (Tukey or Games-Howell) were indicated by different letters above bars. The control treatments containing code 'C-CK3' (i.e., only soil extracts, see Table 3) are used as the CK in the figure. ALP: alkaline phosphomonoesterase activity. The PLFA-P means the P in phospholipids, and it is calculated by the mole of each phospholipid (i.e, the mole of each PLFA).

results show experimentally that organic C drives not only the microbial mineralization of Po but also drives the synthesis of Po by soil microbial communities. Thus, in a field soil ecosystem, estimations of the relative intensities between the two processes (microbial synthesis and mineralization of Po) should be one important point for studying the dynamics of soil P pools. Moreover, the synthesis of Po can exceed its mineralization under certain conditions. For example, our experiment showed positive Po accumulation. These suggested that, in addition to plants, microorganisms were also a driver of Po accumulation at the earliest stage of pedogenesis.

4.2. *Stability of microbially derived Po*

In the soil environment, the important, positive influence of microbial P on the stabilization of P is somewhat unexpected and has been rarely addressed in the past. Microbial biomass P (MBP) usually refers to P in living microorganisms (Dalal, 1998). Then, there is usually a prevailing viewpoint: after microbial biomass P (i.e., P in living microorganisms) is released into the soil (because of microbial cell ruptures with death) and become microbial necromass P, the necromass P is still easily degradable and then transforms into available P (Oberson and Joner, 2005; Achat et al., 2010). However, in agreement with our first hypothesis, our sequential extraction of soil P showed that cHCl-Po (stable Po), considered equivalent to stable residual pools (Tiessen and Moir, 1993; Lin et al., 2018), was clearly detected in the treatments with microorganisms, while its concentrations were below the detection limit in the control treatments without microorganisms (Fig. 1). Clearly, cHCl-Po in our experiments is microbially-derived P including microbial necromass P and live biomass P. Hence, on the one hand, these results indicated that for P released from microorganisms, not all of them can be easily transformed into available P under soil environmental conditions. Obviously, this result is important for the calculations of net mineralization of soil total Po. If we ignore the fact and instead use the above viewpoint (i.e., microbial P is easily degradable), the net mineralization of total Po is likely to be overestimated. For example, Achat et al. (2010) directly used changes in microbial P pool sizes combined with gross mineralization of P in dead soil organic matter and then likely overestimated the net mineralization of total Po. On the other hand, in our experiment, the emergence of cHCl-Po also suggested that soil microorganisms played a role in the stabilization of soil P and may be driving the long-term sequestration of available P in the young soil. Two explanations for the stabilization are: (1) stabilization of Po in soils has traditionally been assumed to occur through incorporation into soil organic matter (e.g., soi humus) (McGill and Cole, 1981). Soil organic matter is derived from plant and microbial residual material, and in this experiment the accumulation of stable P indicates that microbial residual material contributes not only to the accumulation of stable C and N (Liang et al., 2011; 2019, Wang et al., 2020), but also stable P. For example, microbial communities are able to not only produce some stable compounds (e.g., chitin, amino sugars and melanin) (Bai et al., 2013; Wang et al., 2020),.but also to synthesize some recalcitrant Po (e.g., inositol phosphates) (Turner, 2007). Moreover, the crushed stones (<2 mm) that constitute the model soil contained considerable amounts of Fe and Al in our experiment (Table 1). Fe and Al can be mobilized by microbial communities (Sun et al., 2012; Shi et al., 2016). Therefore, in an environment that is composed of complex organic compounds and metal ions, it is likely that complex organic compounds adsorbed P via metal (e.g., Al and Fe) bridges and then formed stable organic-metal-P complexes (Gerke, 2010). (2) The stabilization of Po may be caused by encapsulation in stable substances (e.g., microbial cell wall substances and clay mineral components). In the model soil, large amounts of Po existed within microbial cells. After cell death, these Po tended to be encapsulated by cell walls or other microbial materials, for example, amino sugars and some microbial proteins, which have been reported to be very stable in soils (Fan et al., 2004). As emphasized by Miltner et al. (2012), when examining the stabilization of microbial compounds in

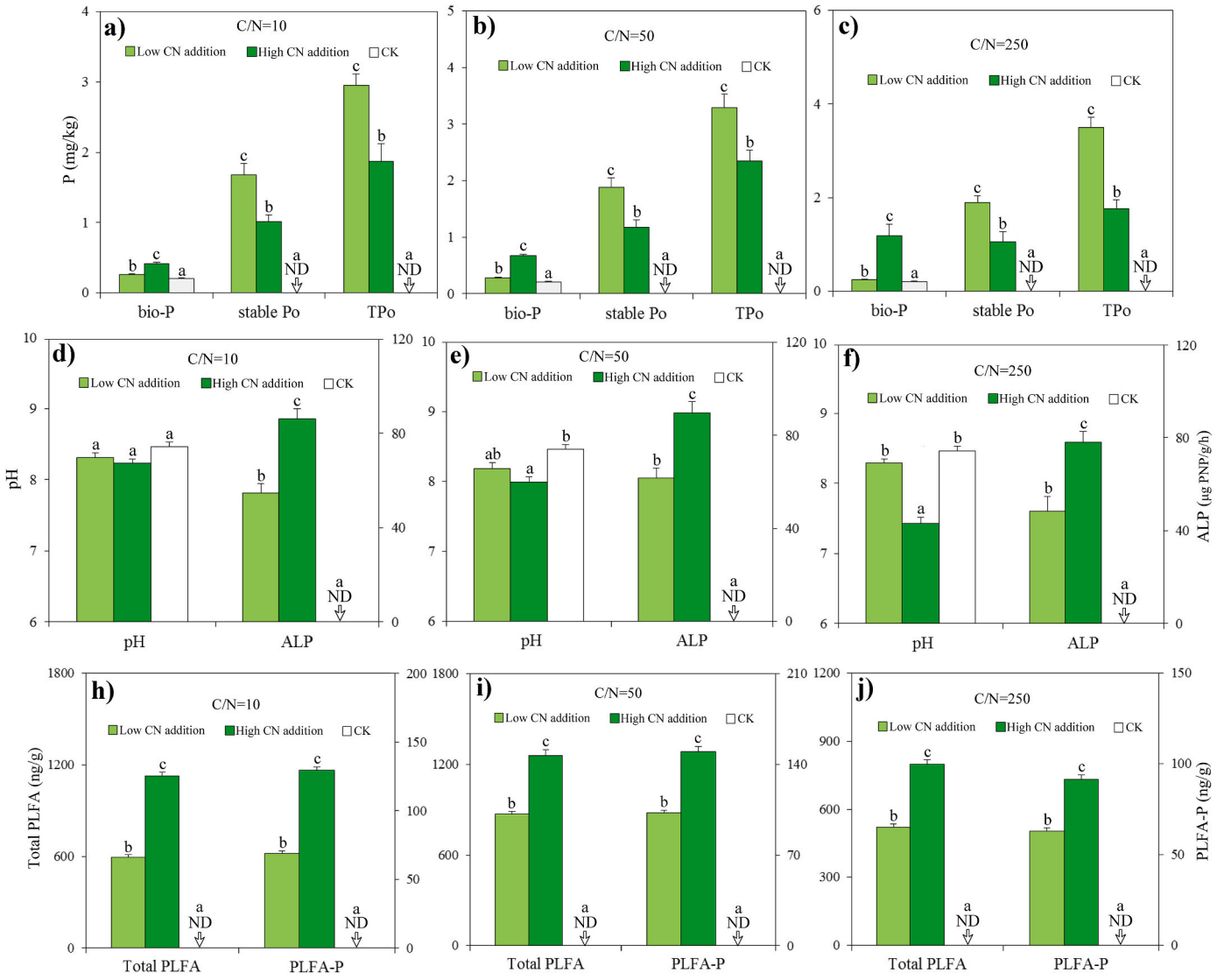

Fig. 4. Results from experiment 3 (the C:N ratios were maintained but levels of C and N additions were changed in experimental treatments, see Table 4 for the treatments). Columns show means and the error bars indicate the standard errors (n = 6). 'ND' means that the measured value is below the detection limit, and the 'ND' is defined as a value of 0 in statistical analysis. Significant differences ($p < 0.05$) by a one-way ANOVA followed by a post-hoc test (Tukey or Games-Howell) were indicated by different letters above bars. The control treatments containing code 'CN-CK3' (i.e., only soil extracts, see Table 4) are used as the CK in the figure. ALP: alkaline phosphomonoesterase activity. The PLFA-P means the P in phospholipids, and it is calculated by the mole of each phospholipid (i.e, the mole of each PLFA).

soil, we must keep in mind that upon entering the soil, these compounds are embedded in complex structures. Although some components are chemically labile, they are stable because they are surrounded by stable compounds that mays protect them from biodegradation (Miltner et al., 2012).

4.3. Microbial release of phosphorus with carbon and nitrogen addition

Our study shows the driving effect of C and N nutrition on the microbial release of phosphorus from soil minerals. Although the same initial gradient of C/N ratios was used in experiments 1 and 2, the changes in bio-P displayed very different patterns along the same gradient in the two experiments (Fig. 2a vs Fig. 3a). These results disagree with our second hypothesis, in which we expected the same change pattern of bio-P under the same initial gradient of C/N ratios. According to our experimental designs, this unexpected result was caused by different manipulation methods that set the same initial gradient of C/N ratios in experiment 1 (changes in the C/N ratio dominated by N addition) and experiment 2 (changes in the C/N ratio dominated by C addition). The two experiments indicate that the microbial release patterns of bio-P can represent two contrasting nutrition-driven modes: 1) the N-driven mode, in which the release of bio-P is driven by N availability via microbial enzyme metabolism and 2) the C-driven mode, in which the release of bio-P is driven by C availability via microbial acid metabolism. We assume that the N-driven mode is more similar to microbial organic P mineralization (from dying/dead microorganisms) by N stimulation, and that the C-driven mode allows bio-P release from mineral P dissolution with C stimulation (Fig. 5).

For the N-driven mode, phosphatase plays a key role in the release of bio-P. With increasing N additions, the significant changes in phosphatase activity and similarity of pH changes (Fig. 2b) indicate that N addition stimulates microbial enzyme metabolism to drive organic P mineralization followed by an increase in bio-P. Phosphatase is an N-rich protein with 15–20% N on a mass basis (Wang et al., 2007), and thus, a greater N supply (i.e., a lower C/N ratio in the experiment 1) provides favourable conditions for phosphatase synthesis under the

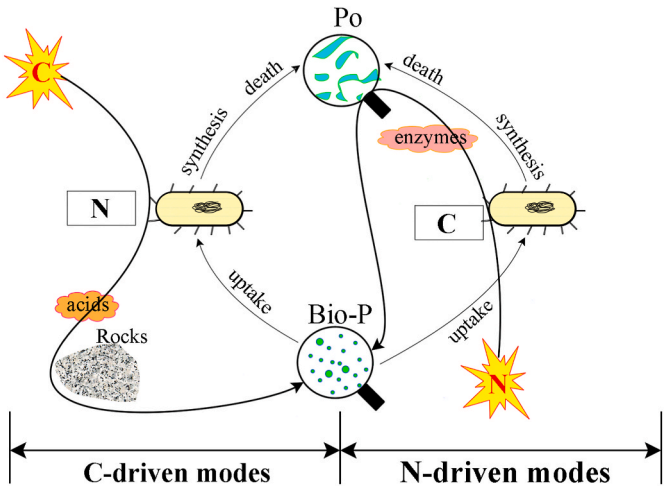

Fig. 5. A conceptual model diagram illustrating the two dominant bio-P release modes: i) the C-driven mode, in which that release of bio-P from rocks is driven by C availability via microbial acid metabolism; ii) the N-driven mode, in which the release of bio-P from the Po is driven by N availability via microbial enzyme metabolism.

same conditions of C availability. Thus, N addition can induce microbial enzyme metabolism and enhance phosphatase activity (Marklein and Houlton, 2012) to accelerate microbial organic P mineralization. For experiment 1 with a high N supply under the same C addition (i.e., C/N = 10 in Fig. 2b), the high phosphatase activity is the most likely cause of the low organic P accumulation (i.e., C/N = 10 in Fig. 2a). The substantial increase in bio-P reflects that the Pi from organic P mineralization replenished and increased the bioavailable Pi pool (i.e., bio-P for C/N = 10 in Fig. 2a). In addition, the input of NH_4^+ could cause a decrease in pH to dissolve mineral phosphates since the uptake of NH_4^+ requires microorganisms to release H^+ to maintain electroneutrality (i.e., no extra charge is generated) (Brucker et al., 2020). Although there were no significant pH differences among the experimental treatments of experiment 1, to an extent, the decrease in pH that was induced by the input of NH_4^+ contributed to the N-driven mode for the release of bio-P, because significant differences in ammonium N concentrations were detected at the end of incubation (Fig. S2a). We believe that the N-driven mode works through the following pathways (Fig. 5): bio-P uptake, microbial growth and reproduction, microbial synthesis of organic P, microbial death, and organic P mineralization.

Experiment 2, with C addition increases under the same N addition (i.e.,Fig. 3), showed the C-driven mode based on microbial acid metabolism for microbial release of phosphorus. A significant decrease in pH values with increasing C additions clearly indicates that organic C promoted microbial acid metabolism (Fig. 3b). Previous studies have experimentally proven that microbial production of organic acids and carbon dioxide (formation of carbonic acid) from microbial respiration was boosted by organic C addition (Chen et al., 2019; Brucker et al., 2020). These acid metabolisms produce large numbers of protons and organic ligands, which improve of orthophosphate solubility and chelate metal cations (Ca, Fe) to convert mineral phosphates into soluble forms (Chen et al., 2006; Brucker et al., 2020). The addition of organic C drives microbial acid metabolism via providing material and energy supplies. Further evidence for the C-driven mode is based on the observation that with C increasing addition under the same N condition, similar phosphatase activities occurred ($p > 0.05$, Fig. 3b) along the gradient of C/N ratios. These similar phosphatase activities suggest that significant changes in available P are due to microbial acid metabolisms rather than microbial mineralization of Po. For the effect of NH_4^+ on pH, the significant differences in pH changes among the different treatments of experiment 2 were unlikely to be caused by the above uptake process of NH_4^+ because the same NH_4^+ (in the form of NH_4NO_3) was added in the treatments, and no significant differences in ammonium N concentrations were found at the end of incubation (Fig. S2b).

Although we distinguished the two modes by the incubation experiments with controllable factors, they are simultaneous and interact in natural soil processes to drive the soil P cycle. In most cases, the natural soil usually receives both C and N, such as litter input, rhizodeposition (i.e, roots release organic compounds containing C and N) and atmospheric C and N deposition. Moreover, microorganisms are the most diverse and abundant group, having staggering biochemical diversity (Woese, 1994). Therefore, it is reasonable to think that these two modes exist simultaneously in natural soil. Due to the complexity of natural soil (e.g., interference of acids and enzymes from roots), it is difficult to distinguish these two modes only through the investigation of natural soil. However, these two modes summarized in our experiments may provide alternative explanations for the complex mechanism of P cycling in natural soil.

In addition, under different C/N ratio settings, our results consistently showed that high CN addition levels led to low Po accumulations (Fig. 4a, b and c). The results differed from our third hypothesis, in which we were expecting higher Po accumulations under high CN levels. There may be several mechanisms that caused this unexpected effect. First, high CN availability has been shown to shorten P retention time in microbial biomass, and moreover, adding C and N did not stimulate P incorporation into microbial biomass to produce Po (Chen et al., 2019). Based on Chen et al.'s (2019) whole research, it suggested that adding C and N did not increase the P content per unit microbial biomass. This was supported by our results, where the change patterns of TPo (Fig. 4a, b and c) were opposite to those of PLFAs at different CN addition levels (Fig. 4h, i and j). In addition, low P availability can lead to longer P retention times in microbial biomass (Spohn and Widdig, 2017), which are conducive to Po accumulation. Indeed, in the present study, the low CN additions were accompanied by low P availabilities (bio-P) and high Po accumulations (TPo) at the end of incubation (Fig. 4a, b and c). Second, high phosphatase activity reduced Po accumulation through hydrolysis reactions. This was supported by our results, which showed high phosphatase activity (Fig. 4d, e and f), high available P and low Po accumulation (Fig. 4a, b and c) under high CN addition levels. In addition, considering the high nutrient levels and P availability under high CN addition levels, another possible mechanism is that the increased P availability and high glucose addition promoted the synthesis of condensed Pi (e.g., polyphosphates and pyrophosphate) by microorganisms (Buenemann et al., 2008b; Mooshammer et al., 2014); thus, the P that was allocated to Po synthesis may have been reduced. The results indicate that microbial processes are not only controlled by the C/N ratio but also by the level of available CN.

4.4. Limitations and boundaries for this study

The findings of this study have to be seen in light of some limitations and boundaries. First, the conditions of our incubation experiments were based on the soil minerals properties (e.g., mineral composition and soil texture) and microbial community at the earliest stage of pedogenesis, hence it must be careful when our conclusions are applied to a long-term pedogenesis. In addition, at the earliest stage of pedogenesis, the soil in our study area originated from rocks without organic matter (including Po). However, not all soils originate from rocks without organic matter. For example, soils from sedimentary rocks or aeolian deposits. Because these rocks contain a considerable amount of organic matter (including Po), the Po accumulation by microorganisms is likely to be uncertain at the earliest stage of their pedogenesis. Second, although the method of sequential chemical extraction implied the possibility of stable Po accumulation in our incubation experiments, the P pools divided by this method have some degree of uncertainty, so it is unlikely to be used for the accurate quantitative calculation of stable Po accumulation. Unfortunately, the current other technologies always have limitations (involving the lack of reference materials, too high

detection limit and interference of pretreatment) and can not also perfectly identify the forms of soil stable Po.

5. Conclusions

Using a model soil without organic matter, we studied the release and immobilization of P in microbial biomass with respect to CN additions. Our study suggested that microorganisms were also one driver of Po accumulation at the earliest stage of pedogenesis, and that microbial biomass P could contribute to P fractions in stable residual pools. Thus, not all microbial biomass P is easily transformed into available P. Two modes of microbial release of bioavailable P were verified and illustrated, namely, the N-driven mode, that uses the microbial enzyme metabolism, and the C-driven mode, that uses the microbial acid metabolism. This study experimentally demonstrates that microbial P processes are controlled not only by the C/N ratios but also by the available CN levels.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108750.

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