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SHORT COMMUNICATION

Phosphorus addition enhances gross microbial N cycling in phosphorus-poor soils: a ^{15}N study from two long-term fertilization experiments

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Abstract

The tight coupling between nitrogen (N) and phosphorus (P) suggests that P availability may affect soil microbial N dynamics in terrestrial ecosystems. However, how P addition affects the internal N transformations in P-deficient agricultural soil remains poorly understood. We hypothesized that an increase in gross microbial N rates in P-deficient soil should occur after long-term P inputs in agricultural soils. We thus conducted a ^{15}N pool dilution experiment to quantify the gross microbial N transformation rates after long-term mineral fertilizer applications in an upland fluvo-aquic soil (from Fengqiu with pH 8.55) and upland red soil (from Qiyang with pH 5.49) in China. We found that P addition significantly enhanced the gross N mineralization and immobilization rates when N and K were also applied, probably due to the increased soil total C and N concentrations at both soils. Also, gross nitrification rate was stimulated by P addition, perhaps because of enhanced gross N mineralization rates and associated NH_4^+ substrate availability. Our results showed that long-term P addition may stimulate soil gross N dynamics and hence increase overall N availability for crops in P-deficient agricultural soils.

Keywords Phosphorus deficiency · P availability · N dynamics · Gross N mineralization · ^{15}N recovery

Introduction

Nitrogen (N) and phosphorus (P) are key nutrients often limiting crop production in agricultural ecosystems. The N and P cycles are tightly coupled, so that P availability may directly affects N dynamics (de Groot et al. 2003). In contrast to the well-known effects of N availability on soil N dynamics, the response of soil N cycling to P addition remains poorly understood. Furthermore, the effects of P addition upon soil N dynamics have mainly focused on net N dynamics and N_2O emissions, particularly in forest and grassland ecosystems (Bauhus and Khanna 1994; He and Dijkstra 2015; Mehnaz

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and Dijkstra 2016; Mori et al. 2016; Chen et al. 2017), whereas the effects of P addition on soil N transformation rates are largely unknown in P-deficient agricultural soils.

Short-term P addition can cause large gaseous N loss from P-poor soils in grassland ecosystems, most likely by directly stimulating denitrification by increasing denitrification gene abundance (He and Dijkstra 2015; Wei et al. 2017), whereas the effects of P addition on soil net N dynamics were found dependent on the initial N status (N-saturated or -limited) in forest ecosystems (Chen et al. 2017). This suggests that P availability stimulates soil N dynamics only when the ecosystem is saturated with N, or when there is already considerable N deposition, just because P becomes the main limiting factor when N is larger or P addition alleviates soil acidification caused by N addition. Agricultural ecosystems, especially in China, are highly N-fertilized and so they often are characterized by an N surplus (Ju et al. 2009; Cui et al. 2013). Therefore, we hypothesized that an increase in gross microbial N rates in P-poor soil should occur after long-term P inputs in agricultural soils.

To empirically test this hypothesis, we collected soil samples from various fertilization treatments after 17 years from upland fluvo-aquic soil at Fengqiu, and after 25 years from upland red soil at Qiyang, where both soils are P-deficient (Bai et al. 2013; Jing et al. 2017). A ^{15}N dilution study was carried out to quantify soil gross microbial N transformation rates and to obtain a process-based understanding of the mechanisms driving the internal N cycle in response to P addition (Murphy et al. 2003; Lang et al. 2016). Based on ^{15}N pool dilution method, the application of $^{15}NH_4^+$ enables the measurement of gross mineralization and the sum of NH_4^+ consumption, and application of $^{15}NO_3^-$ enables the measurement of gross nitrification and NO_3^- consumption.

Materials and methods

Study sites

Two long-term field experiments were located at the Fengqiu State Key Agro-Ecological Experimental Station (35°00′N, 114°24′E) in Henan Province (FQ) and the Qiyang Red Soil Experimental Station (26°45′N, 111°52′E) in Hunan Province (QY). Both sites are representative of the typical regional agriculture that uses a wheat–maize rotation system. The FQ site has a warm temperate continental monsoon type, with an annual rainfall of 596 mm and annual average temperature of 13.9 °C; its soil is classified as an aquic inceptisol with a sandy loam texture. The QY site has a subtropical monsoon climate, with an annual rainfall of 1300 mm and annual average temperature of 18 °C; its soil is classified as a Ferralic Cambisol with a silty clay texture.

Experimental setup

Both experiments were set up in a randomized block design with three replicates. The FQ site had five treatments: control without fertilizers (CK); chemical fertilizers N and P (NP); chemical P and potassium (PK); N and K (NK); and N, P, and K (NPK). In the FQ site, urea was applied at 300 kg N ha^{-1} $year^{-1}$ for all N treatments, and superphosphate and potassium sulfate (K_2SO_4) were applied at 59 kg P and 248 kg K ha^{-1} $year^{-1}$, respectively, for all P or K treatments. The QY site also included five treatments: control without fertilizers (CK); chemical fertilizer N (N); chemical fertilizers N and P (NP); N and K (NK); and N, P, and K (NPK). In the QY site, urea was applied at 300 kg N ha^{-1} $year^{-1}$ for all N treatments, and superphosphate and potassium chloride (KCl) were applied at 53 kg P and 100 kg K ha^{-1} $year^{-1}$, respectively, for all P or K treatments. Detailed information on the management and fertilization of both field experiments is given by Zhang et al. (2012) and Cai et al. (2015), respectively. Fresh soil samples from the plow layer (0–20 cm) were collected after maize harvest from each replicated plot and pooled together to form a composite sample. Then, soil sample was sieved (2 mm) and stored at 4 °C for 1 week for the incubation studies. The properties of the soil under the various fertilization treatments at both sites are shown in Table 1. Gross N transformation rates were determined by the ^{15}N dilution technique with a paired labeling experiment (Kirkham and Bartholomew 1954; Murphy et al. 2003). Half of each pair was labeled with $^{15}NH_4NO_3$, while the other half was labeled with $NH_4{}^{15}NO_3$.

Sample preparations

Each fresh soil sample (20 g of fresh soil with an oven-dried basis) was placed inside a 250-mL flask and sealed. These flasks were then pre-incubated in the dark at 25 °C in the laboratory for 24 h. After pre-incubation, 2 mL of either the ^{15}N-enriched $^{15}NH_4NO_3$ or the $NH_4{}^{15}NO_3$ solution (20 and 10 at.% ^{15}N excess for the FQ and QY sites, respectively) were applied to each soil sample by pipetting the solutions uniformly over the soil surface; this was equivalent to adding 50 mg of NH_4^+-N and 50 mg of NO_3^--N kg^{-1} to the soil. Next, the final moisture content of each labeled sample was adjusted to 60% WHC by adding deionized water. Flasks were then sealed with rubber stoppers and incubated at 25 °C in the dark for 3–6 days. During this incubation period, the flasks were opened for 30 min each day to refresh the atmosphere inside each flask. The moisture content of the incubated soil samples was maintained by adding water every 3 days, as needed, to compensate for the amount of water lost through evaporation. Soil samples were extracted destructively at 0.5 h, 1, 2, and 3 days for the FQ site, and 0.5 h, 2, 4, and 6 days for the QY site, after the ^{15}N labeling by using a 100-mL solution of 2 M KCl to determine the concentrations and isotopic compositions of exchangeable

Table 1 Soil properties for the various fertilized soils at Fengqiu (FQ) and Qiyang (QY) sites

	Treatment	pH	Total C g kg^{-1} soil	Total N	C/N ratio	Available P (mg kg^{-1})	Relative P status
FQ	CK	8.55 (0.03)a	4.43 (0.06)e	0.40 (0.02)d	11.1	1.56c	P-deficient
	NP	8.39 (0.02)a	5.43 (0.03)b	0.65 (0.02)b	8.35	7.00b	P-sufficient
	PK	8.49 (0.01)a	5.11 (0.13)c	0.51 (0.02)c	10.0	19.9a	P-sufficient
	NK	8.46 (0.03)a	4.71 (0.05)d	0.41 (0.02)d	11.5	1.49c	P-deficient
	NPK	8.38 (0.02)a	5.61 (0.14)a	0.70 (0.03)a	8.01	7.10b	P-sufficient
QY	CK	5.49 (0.07)a	7.75 (0.04)d	1.03 (0.03)d	7.52	12.7c	P-deficient
	N	4.00 (0.01)b	8.25 (0.05)c	1.05 (0.03)cd	7.86	18.0c	P-deficient
	NK	3.97 (0.00)b	7.93 (0.03)cd	1.11 (0.03)c	7.14	14.4c	P-deficient
	NP	4.08 (0.10)b	10.1 (0.03)b	1.31 (0.03)b	7.71	122a	P-sufficient
	NPK	4.19 (0.00)b	11.8 (0.03)a	1.71 (0.03)a	6.90	99.0b	P-sufficient

FQ treatments: control without fertilizers (CK), and with mineral fertilizers NP, PK, NK, and NPK. The QY treatments: control without fertilizers (CK), and with mineral fertilizers N, NK, NP, and NPK. Different letters within the same column indicate significant differences between treatments for each site at $P < 0.05$ (Duncan test). Values in parentheses denote the standard deviation ($n = 3$). Available P data of FQ site cited from Xin et al. (personal communication). Soil in FQ was P-limited, as crops receiving no P did not respond to nitrogen (N) and potassium (K) fertilization (Hu et al. 2009). Soil from the NP and NPK treatments was considered to be relatively P-sufficient, although the available P content was only 7.00 mg kg^{-1} after 21 years of the mineral P fertilizer application, and still unable to satisfy the crop requirements due to a low rate of mineral P input and a low basal available P content. In the province of Hunan, where QY is located, 74% is below the critical Olsen-P level (20 mg kg^{-1}) for crop production (Bai et al. 2013)

NH_4^+ and NO_3^-. Different incubation periods between FQ and QY sites were used to ensure sufficient NH_4^+ availability for nitrification, thereby avoiding inconsistent results from restraining gross nitrification rates (Figs. S1 and S2). After the KCl extraction, the residual soil was washed with 150 mL deionized water three times, oven-dried at 60 °C to a constant weight, and ground to pass through a 0.15-mm sieve for the ^{15}N analysis of insoluble organic N (Fig. S3).

Analyses

Olsen-P was extracted by 0.5 mol L^{-1} $NaHCO_3$ (2.5 g soil, 50 mL solution, 25 °C, shaken for 30 min), followed by the colorimetric measurement of inorganic P using the molybdate–ascorbic acid method (Murphy and Riley 1962). The isotopic compositions of NH_4^+, NO_3^-, and organic N were measured by an automated C/N analyzer isotope ratio mass spectrometer (Europa Scientific Integra, Sercon 20–22, UK). NH_4^+ and NO_3^- were separated for ^{15}N measurements by distillation with magnesium oxide and Devarda's alloy, respectively (Bremner 1996). Specifically, a portion of the extract was steam-distilled with MgO to separate NH_4^+ on a steam distillation system. The sample in the flask was distilled again after the addition of Devarda's alloy to separate out the NO_3^-. Liberated NH_3 was trapped using boric acid solution. To prevent isotopic cross-contamination between samples, 25 mL of reagent-grade ethanol were added to the distillation flasks and steam-distilled for 3 min between each distillation. Trapped N was acidified and converted to $(NH_4)_2SO_4$ using 0.005 mol L^{-1} H_2SO_4 solution. The H_2SO_4 solution (containing NH_4^+) was then evaporated to dryness at 60 °C in an oven and analyzed for ^{15}N abundance.

Gross rates of N mineralization, nitrification, and NH_4^+ and NO_3^- consumption were calculated for various time intervals with the analytical equations of Kirkham and Bartholomew (1954) and Murphy et al. (2003), based on the amounts and isotopic excesses of NH_4^+ and NO_3^- (Table S1; Figs. S1 and S2). The potential gross NH_4^+ immobilization rate was calculated by subtracting the gross nitrification rate from the NH_4^+ consumption rate (immobilization + gaseous loss and nitrification), assuming that NH_4^+ consumption through volatilization was zero. However, NH_3 volatilization might have occurred in the FQ soils with pH above 7.0, and thus gross NH_4^+ immobilization rates in the FQ soils may be overestimated. We also assumed that NO_3^- consumption via denitrification was negligible; therefore, the gross NO_3^- immobilization rate was equivalent to the gross NO_3^- consumption rate under aerobic incubation (Murphy et al. 2003; Vervaet et al. 2004). Gross NH_4^+ and NO_3^- immobilization and nitrification rates may be overestimated due to stimulation by ^{15}N substrate addition; however, any such stimulation would be consistent among all treatments thus permitting comparisons. One-way ANOVA was used to compare the difference in the time-weighted average gross N transformation rates among the different fertilization treatments for each site.

Results and discussion

The long-term field experiment has demonstrated that the FQ soil is P-limited since the crops unfertilized with P did not respond to N and K fertilizations (Hu et al. 2009). In Hunan province, where QY is located, 74% of the arable soils show

lower values than the critical Olsen-P level (20 mg kg^{-1}) recommended for crop production (Bai et al. 2013). According to the available P content, after long-term field fertilization at the FQ site, the treatments which received no P fertilizer (i.e., CK and NK) were considered P-deficient (available P < 1.6 mg P kg^{-1}) whereas those that received P fertilizer (NP, PK, and NPK) were relatively P-sufficient (available P 7.0–19.9 mg P kg^{-1}) (Table 1). Similarly, at the QY site, compared with the NP and NPK treatments that had an available P content of 99–122 mg P kg^{-1}, the CK, N, and NK treatments were relatively P-deficient (available P < 18.0 mg P kg^{-1}). The Olsen P method may have overestimated available P in acidic soil because the high pH (8.5) of $NaHCO_3$ extract may solve non-bioavailable P, such as Fe–P and Al–P (Bai et al. 2013), resulting in a relatively higher available P content in acidic QY soils. In addition, a relatively low critical value in carbonate-rich soils (i.e., FQ site) might be due to the release of proton and carboxylate exudation by maize roots, as a mechanism to acidify the rhizosphere and mobilize soil inorganic P in calcareous soil (Neumann and Römheld 2002; Zhang et al. 2010). The pH values of all treated FQ soils fluctuated between 8.38 and 8.55, indicating that field fertilization with NP, PK, NK, and NPK for 17 years did not greatly alter soil pH (Table 1). By contrast, those of the QY soils decreased from 5.49 to values of 3.97–4.19 due to the input of N-containing fertilization (N, NK, NP, and NPK), indicating that high rates of N fertilization for 25 years led to severe soil acidification. Significant acidification in major Chinese croplands due to high-N fertilizer inputs and the uptake and removal of base cations by plants is a general phenomenon with the exception of the FQ soils, which were resistant to acidification probably because of their relatively high $CaCO_3$ content (5 to 10%; Guo et al. 2010). Soil total C concentration ranked as CK < NK < PK < NP < NPK and CK ≤ NK ≤ N < NP < NPK, and soil total N concentration ranked as CK ≤ NK < PK < NP < NPK and CK ≤ N ≤ NK < NP < NPK, at the FQ and QY sites, respectively (Table 1). This indicated that long-term field fertilization increased soil total C and N concentrations at both sites. The comparison of data among the NP, NK, PK, and NPK treatments at FQ with those among the N, NK, NP, and NPK treatments at QY suggest that the soil total C and N accumulations were limited more easily by the availability of P, followed by N, and least by K at FQ site, and by P followed by K availability at QY site.

The gross N mineralization rates at FQ soils were significantly enhanced by the N-fertilized treatments (NP, NK, and NPK) but not affected by the PK treatment (Fig. 1a), whereas those of the QY soils were not significantly affected by any of the N fertilizer treatments (Fig. 2a). The gross N mineralization rates were probably limited most by N availability when comparing the PK and NPK treatment, followed by P availability in the FQ soils and by P availability in the QY soils when comparing the NK and NPK treatment. P addition increased plant residue decomposition by stimulating microbial activity (Chen et al. 2016), and probably thus increased the soil organic C and N concentrations in the P-limited soil (Table 1). The basic importance of substrate availability for controlling soil N mineralization has been reviewed by Booth et al. (2005). In addition, enhanced rate of litter decomposition due to P addition may stimulate extra mineralization of organic N in the soil (the priming effect). In contrast, without the P addition effect on gross N mineralization has been reported in a P-limited grassland soil and attributed to the non-limited microbial activity by P (Mehnaz et al. 2018).

Gross nitrification rates could be ranked as CK < PK < NK < NP ≤ NPK and NK < N ≤ NP ≤ NPK < CK in the FQ and QY soils, respectively (Figs. 1b and 2b), indicating that gross nitrification was significantly stimulated by the N-fertilized treatments and by the PK-fertilized treatment at FQ site, but it was significantly inhibited by the N-fertilized treatments at QY site. Generally, long-term N application can stimulate soil nitrification, while soil acidification caused by nitrification in turn can inhibit nitrification (Cheng et al. 2015). It was, therefore, likely that the stimulatory effect of long-term N application on the nitrification rate might have been completely counteracted by the inhibitory effect of soil acidification in the QY soils. In contrast, after 17 years of N fertilizer application, the FQ soils did not exhibit soil acidification, and thus soil nitrification often increased. The gross nitrification was probably limited most easily by the availability of N, followed by P, and least by K in the FQ soils, whereas it was only limited by P availability in the QY soils when N and K were also applied. Reduced gross nitrification was probably due to the reduced gross N mineralization rates under P-deficient conditions (Figs. 1a and 2a). Our results are consistent with those reported for a purple soil, where a proper rate of P addition (18–26 mg P kg^{-1}) significantly stimulated both the growth of nitrifying bacteria and potential nitrification rates under P-deficient conditions (Zhao et al. personal communication). Moreover, Chen et al. (2016) found that P addition, at lower levels than 25 mg P kg^{-1}, accelerated soil net nitrification by increasing activity of ammonia-oxidizing bacteria (AOB) rather than that of the ammonia-oxidizing archaea (AOA) in a P-deficient acid red soil. A 21-year fertilization experiment showed that P addition can increase the potential nitrification rate and may affect the responses of community composition of AOB more than AOA to fertilization in a purple soil (Zhou et al. 2014), while it was previously assumed that AOA might dominate environments having low P bioavailability (Erguder et al. 2009). The addition of P also promoted heterotrophic nitrification in two acid forest soils (Bauhus and Khanna 1994). Heterotrophic nitrification is predominantly carried out by fungi (Landi et al. 1993). P addition may affect soil nitrification pathways probably through reducing fungal species richness and changing fungal community composition (He et al. 2016). However, the relationship

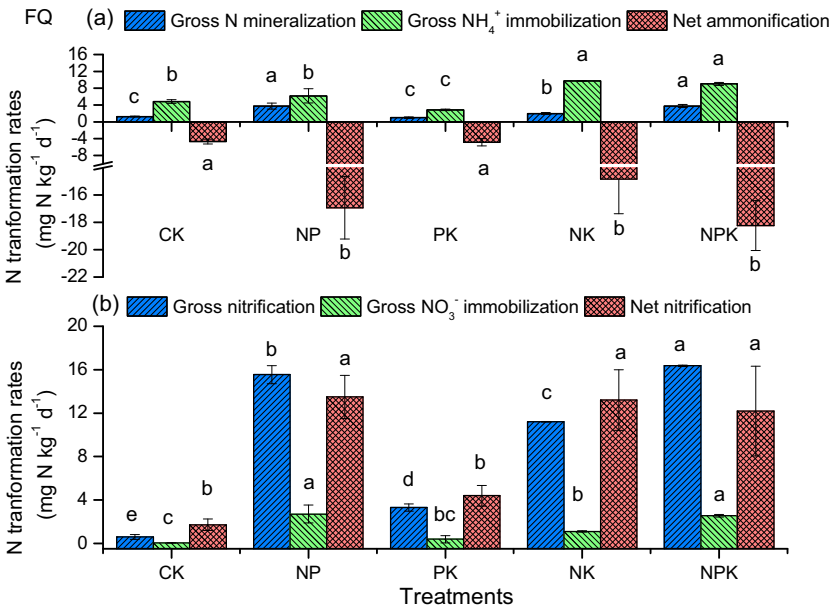

Fig. 1 Soil gross N mineralization and NH_4^+ immobilization rates and net ammonification rates (**a**), and gross nitrification and NO_3^- immobilization rates and net nitrification rates (**b**) in the differently fertilized FQ soils. Control without fertilizers (CK); mineral fertilizers are NP, PK, NK, and NPK. Gross N transformation rates refer to the weighted average gross N transformation rates over the 3 days of incubation. Different letters (a, b, c, d, and e) indicate significant differences between treatments for the same N transformation rate at $P < 0.05$ (Duncan test). Error bars are standard deviations of the means ($n = 3$)

between P availability and both autotrophic and heterotrophic nitrification and their associated microbial driven mechanisms are poorly known and thus they should be further investigated in both soils studied.

Gross NH_4^+ and NO_3^- immobilization rates ranked as PK < CK ≤ NP < NPK ≤ NK and CK ≤ PK < NK < NPK ≤ NP in the FQ soils, respectively (Fig. 1). This indicated that the NH_4^+ and NO_3^- immobilization rates were probably limited by N and K availability and by N and P availability, respectively. It should be noted that the calculated gross NH_4^+ and NO_3^- immobilization rates must be considered as potential rates because gross NH_4^+ and NO_3^- immobilization may be overestimated due to stimulation by ^{15}N substrate addition. Since generally low gross and net N transformation rates would introduce error into rate calculations when using the ^{15}N dilution method in the QY soils, we calculated the rate of ^{15}N recovery in the organic N pool in the NH_4^+-^{15}N and NO_3^--^{15}N labeled treatments as the gross NH_4^+ and NO_3^- immobilization rates, respectively (Fig. S3). The ^{15}N recovery rate in the organic N pool in the NH_4^+-^{15}N and NO_3^--^{15}N labeled treatments ranked as N ≤ NK ≤ NP < CK ≤ NPK and N ≤ NK ≤ NP < CK ≤ NPK, respectively (Fig. 2c), indicating that the gross NH_4^+ and NO_3^- immobilization rates were probably limited by P and K availability in the QY soils. Our

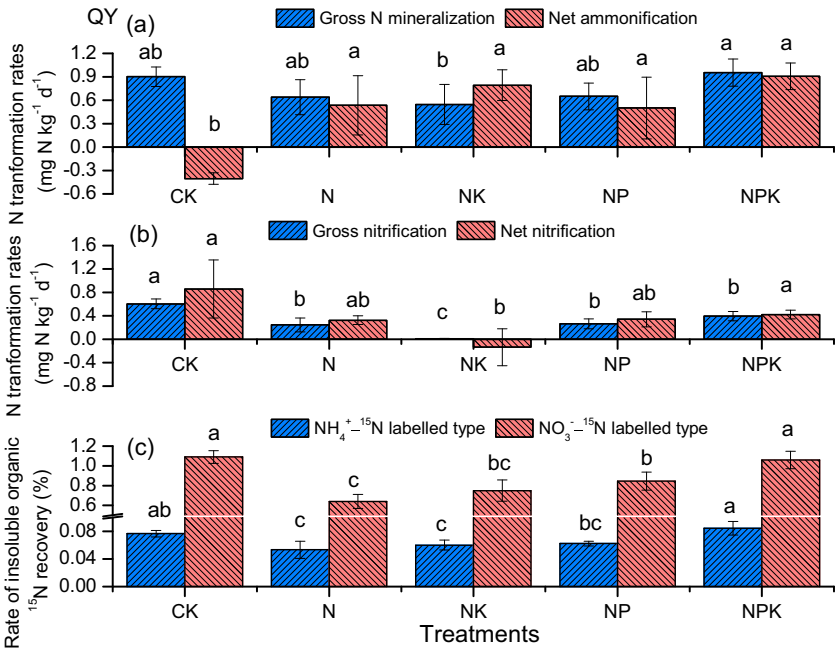

Fig. 2 Soil gross N mineralization and net ammonification rates (**a**), gross nitrification and net nitrification rates (**b**), and rate of ^{15}N recovery in the insoluble organic N pool (**c**) in the differently fertilized QY soils. Control without fertilizers (CK); mineral fertilizers are N, NK, NP, and NPK. Gross N transformation rates refer to the weighted average gross N transformation rates over the 6 days of incubation. Different letters (a, b, and c) above the bars indicate significant differences between treatments for the same N transformation rate at $P < 0.05$ (Duncan test). Error bars are standard deviations of the means ($n = 3$)

results regarding P-limited gross N immobilization rates in both soils agreed with Li et al. (2015), who found that microbial biomass was increased by P addition over 3 years of fertilization in a secondary tropical forest soil. An increase in microbial biomass N and gross NH_4^+-N immobilization rates also has been observed by a short-term incubation of P-treated temperate forest soils (Zhou et al. 2017) and grassland soil (Mehnaz et al. 2018), respectively. Yet, our results contrasted with what reported by He and Dijkstra (2015), who showed that short-term P additions reduced microbial biomass N and ^{15}N recovery, and thus enhanced the potential N loss in a P-poor grassland soil. In addition, Shi et al. (2012) reported no significant changes upon soil microbial biomass after 17 years of P fertilization in an agriculture soil. Even P addition to a tropical forest soil increased microbial biomass and altered the composition of microbial community (Liu et al. 2012), but this was a transient effect that disappeared after 4 years of fertilization (Liu et al. 2013). Therefore, it is likely that the response of soil microbial N immobilization to P fertilization is generally complex and associated with soil types, duration of P applications, as well as other possible factors.

In conclusion, our data of the two long-term fertilized soils supported our hypothesis that P addition increased the gross N mineralization and immobilization rates when N and K were also applied, probably due to enhanced soil total C and N accumulations. Similarly, gross nitrification rates were also enhanced by P addition, possibly due to the enhanced gross N mineralization rates. Thus, gross N transformation rates are likely affected by the P status in the studied agricultural soils. This study thus provides a process-based explanation for how P addition affects the internal mineralization–immobilization–turnover in fertilized agricultural soils even if the behavior of the only P-treated soil should be also investigated to support these conclusions. Further research is needed to elucidate the community structure and composition of microbial populations related to gross N transformation processes under long-term P deficiency and enrichment.

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