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# How microbes cope with short-term N addition in a *Pinus tabuliformis* forest-ecological stoichiometry

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ABSTRACT

Global nitrogen (N) deposition can change the contents and stoichiometry of soil resources and thus of microbial communities, which can drive the flow of carbon (C) and nutrients in food webs in forest ecosystems. It is critical to understand the status of soil elements required for microbial growth, the elements of microbial growth, and how soil microorganisms would reallocate resources and release extracellular enzymes for adjusting the imbalance between resources and microorganisms due to N deposition. We chose a plantation of *Pinus tabuliformis* where N had been added across a gradient (0–9 g N $m^{-2}$ $y^{-1}$) for two years to reveal how matters flow and soil resources were reallocated by microbes to cope N addition. Our results showed that although two years of N inputs only significantly affect the ratio between the activities of β-1,4-*N*-acetylglucosaminidase and alkaline phosphatase in the 0–20 cm soil layer, activities for both organic N and organic phosphorus (P) acquisition enzymes scale with C acquisition with slope of about 1, following the global ecoenzymatic patterns. Contents and elemental ratios of soil organic carbon (SOC), total N (TN) and total phosphorus (TP) were changed after this short-term N addition. Ratios of SMBC, SMBN and SMBP were concentrated from soil and lower than ratios of soil resources. Soil microbial communities under N addition were limited by N and co-limited by P at 3 and 6 g N $m^{-2}$ $y^{-1}$. Although imbalance between soil and microbes caused by N addition, in the 0–20 and 20–40 cm layers, C: N relationship between soil and the microbial community indicated microbial community maintained homeostasis. However, C:P relationship between soil and the microbial community indicated no microbial community homeostasis. In addition, both soil resources elemental stoichiometry and contents can affect soil microbial communities. Further study of the structure of soil microbial communities, identifying the microbes that are more adaptive to high N concentrations and N deposition, is needed for a better understanding of the mechanisms of nutrient flow in the food web and how to maintain microbial homeostasis in response to N deposition in forest ecosystems.

## 1. Introduction

Nitrogen (N) is a limiting element in terrestrial ecosystems and plays a vital role in biogeochemical cycles (Isbell et al., 2013). N deposition is a major contributor to the global increasing N inputs and is gradually affecting the turnover of soil organic carbon (SOC) due to the burning of fossil fuels, the production and use of chemical fertilizers, human activities and animal husbandry. The effects of increasing N contents on ecosystems have been widely studied (Yan et al., 2018; Bobbink et al., 2010; Galloway et al., 2008), such as lowering soil pH (Phoenix et al., 2012), leaching of calcium and magnesium due to soil acidification (Lucas et al., 2011) and altering the structure and function of soil microbial communities (Zhang et al., 2017; Zhong et al., 2015). Reports of the effects of short-term N addition on the properties of soil microbial communities, however, have been inconsistent (Simonin et al., 2017; Yao et al., 2014; Zong et al., 2013), such as variable responses of microbial biomass and respiration to N addition (Wang et al., 2017; Ramirez et al., 2012; Sarathchandra et al., 2001). This discrepancy is because microbial communities in terrestrial ecosystems is mainly influenced by the duration and content of N inputs (Treseder, 2008). Inconsistent changes of microbial community can be found obviously in the first five years of N addition (Treseder, 2008). An

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appropriate amount of N addition can mitigate N limitation in a low N ecosystem, increase the activity of microorganisms (Zhang et al., 2017; Yao et al., 2014), and change community structure (Bai et al., 2010). However, if N inputs beyond saturation, it will inhibit soil microorganisms. Therefore, the mechanisms of the response of soil microbes to N addition have been difficult to identify.

Ecological stoichiometry is an approach where elements are used as a common currency to examine the scaling of trophic dynamics across all scales of organization within a system (Sterner and Elser, 2002). Ecological stoichiometric theory unifies ecology with elemental ratios and stoichiometric invariance that can predict nutrient retention and biomass production from subcellular to ecosystem scales. Sistla et al. (2015) found that N fertilization decreased biotic C:N and increased N:P. Ecological stoichiometry involves biogeochemical cycles that drive the flow of energy and nutrients among trophic levels in food webs and mediate the imbalance between resources and soil microbial communities by reallocating resources to the activity of extracellular enzymes. Numerous studies on the effect of added N on the activities of enzymes have reported inconsistent results. Jing et al. (2017) found that enzymatic activities did not differ significantly with N deposition in six Chinese forests. DeForest et al. (2004) reported similar results. Chen et al. (2017), however, found that the activity of β-1,4-glucosidase tended to increase with N addition. These enzymes were released to adapt new environment to balance the relationship between soil resources and microbial communities.

The threshold elemental ratio (TER) and stoichiometric homeostasis are central to the progress of the framework of ecological stoichiometry. TERs are critical nutrient ratios of foods of an organism where growth becomes limited and where an organism switches limitation from one element to another (Sterner and Hessen, 1994). The degree to which the fluctuations of microbial stoichiometry depending on resource stoichiometry is the standard of homeostasis. For example, strictly homeostatic microbes maintain a constant stoichiometry regardless of changes in resource stoichiometry, but the composition of a non-homeostatic organism varies with changes in the composition of its resources (Sterner and Elser, 2002) (Fig. 1). The stoichiometries of autotrophic organisms are generally plastic, changing with resources stoichiometry (Sterner et al., 1998). In contrast, variation in resource stoichiometry has little effect on the elemental composition of heterotrophs generally thought to be strictly homeostatic (Fagan et al., 2002). TERs and stoichiometric homeostasis indicate that microbes can vary ecoenzymatic activities to mediate C– and nutrient use efficiencies to adapt to imbalances between resources and themselves (relationship with TER or limited by an element) or to maintain stoichiometric homeostasis.

The amount of N deposition in China has sharply increased recently, e.g. increasing in Shaanxi Province from 16.12 kg ha$^{-1}$ in 2010 (Wei et al., 2010) to 28.89 kg ha$^{-1}$ in 2014 (Liang et al., 2014). The Chinese red pine, *Pinus tabuliformis*, in the TIELONGWAN plantation in Yichuan County was primarily deficient in N but is now receiving severe N deposition. N enrichment will have an impact on the function of microbes in the system. Previous studies mainly focused on the effect of N addition on soil microbial properties, such as microbial biomass, respiration, microbial community structures and enzymatic activities. Understanding how microbes cope the short-term N addition requires the integration of ecological stoichiometry, TER and homeostasis. Few studies revealed microbial community' coping strategy from ratios and contents of soil resources limiting microbial growth, to ratios and contents of microbial communities, and how the microbial community adjust the imbalance between them. Therefore, we designed an experiment with the addition of N along a gradient to reveal how microbial community adapt new environment and general status of microbial communities, in homeostasis status or not. We hypothesized that: 1) enzymatic activities and ecoenzymatic stoichiometries would vary along the N-addition gradient to maintain a balance between resources and microorganisms, 2) the dynamics of nutrient stoichiometry among trophic levels would be driven by N addition and 3) N addition would not affect soil microbial homeostasis even though a short-term addition would affect the microbial communities.

## 2. Material and methods

### 2.1. Site description

The experiment was conducted at the TIELONGWAN plantation in Yichuan County (36°04′N, 110°15′E) in northern Shaanxi Province, China. The region is characterized by a continental climate with a mean annual temperature of 9.7 °C. The average frost-free period is 180 days. The mean annual precipitation is 584.4 mm, with approximately 60% occurring between July and September. The soil is a gray forest soil (Gray Luvisol, FAO soil classification), and the landscape consists of rolling hills, with slopes from 20° to 25°. The elevation of the plantation ranges from 860 to 1200 m.

The artificial *P. tabuliformis* forest in our experiment has an area of

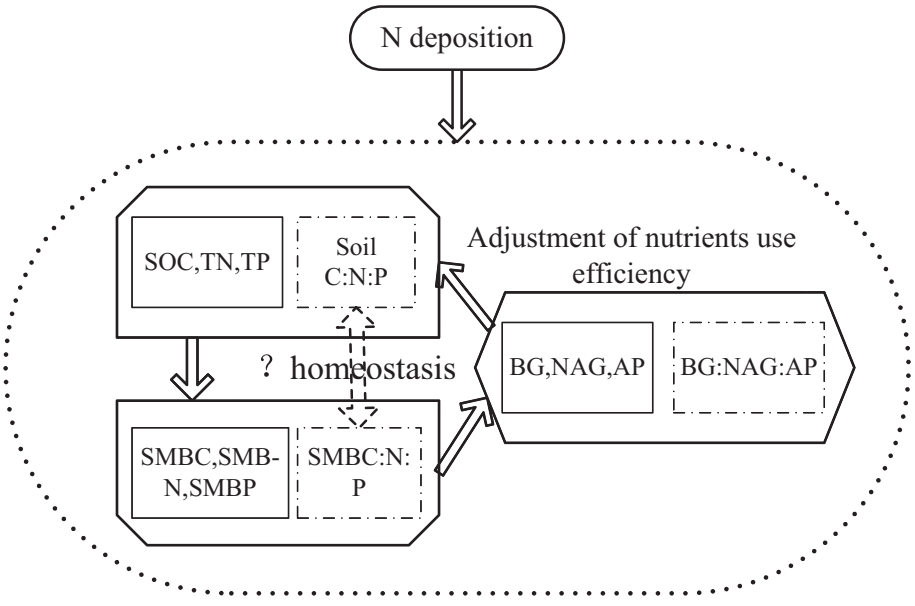


**Fig. 1.** Mutual effects between soil and soil microbes caused by N deposition. SOC, TN and TP represent soil organic carbon, soil total nitrogen and total phosphorus, respectively. MBC, MBN and MBP represent microbial biomass carbon, microbial biomass nitrogen and microbial biomass phosphorus, respectively. Soil C:N:P represents elements ratio of SOC, TN and TP. MBC:N:P represents element ratio of MBC, MBN and MBP. BG, NAG and AP represent β-1,4-glucosidase, β-1,4-*N*-acetylglucosaminidase and alkaline phosphatase, respectively. BG: NAG: AP represents ratio of BG, NAG and AP.

600 ha and was established in 1966. The zonal vegetation is temperate deciduous broad-leaved forests. The main trees are *P. tabuliformis.* And there are *Populus davidiana*, shrubs, like *Lespedeza davurica*, *Elaeagnus umbellata*, *Rcsa xanthine*, *Spri- raea salicifolia* and *Caragana korshinskii*, and herbs, *Carex lanceolate*, distributing sporadically in the forest.

### 2.2. Experimental design and soil sampling

The experiment had a randomized design with four treatments, each with four replicates. N was applied at rates of 0 g N $m^{-2}$ $y^{-1}$ (CK), 3 g N $m^{-2}$ $y^{-1}$ (N3), 6 g N $m^{-2}$ $y^{-1}$ (N6) and 9 g N $m^{-2}$ $y^{-1}$ (N9), which were based on the global N-deposition levels (Bobbink et al., 2010) and the amounts of N addition in experiments in China and other countries. Each plot had an area of 10 × 10 m, and a buffer strip 5 m wide separated the plots. N was applied in the form of urea ($CO(NH_2)_2$) four times a year in April, June, August and October from 2014. N3, N6 and N9 received urea in solution one day before a rain to decrease ammonia volatilization. CK received the same volume of water.

The soil was sampled in September 2015 after two years of fertilization. Soil cores were collected from the 0–20 and 20–40 cm layers from four randomly selected locations in each plot and were combined into composite samples for each layer. All samples were sieved through a 2-mm mesh after the stones and roots were manually removed. The sieved samples were divided into three subsamples. One subsample was air-dried and then sieved through a 0.25-mm mesh for the determination of SOC, total N (TN) and total P (TP) contents. Another subsample was stored at 4 °C for measuring soil microbial biomass C (SMBC), soil microbial biomass N (SMBN) and soil microbial biomass P (SMBP). The third subsample was stored at −80 °C for determining enzymatic activities and for extracting DNA for Illumina MiSeq sequencing.

### 2.3. Biogeochemical analyses

The chemical and physical properties of the soil were determined using standard procedures. SOC content was measured with the $H_2SO_4$-$K_2Cr_2O_7$ method. TN content was measured using the Kjeldahl method (Bremner and Mulvaney, 1982). TP content was determined colorimetrically after digestion with $H_2SO_4$ and $HClO_4$ (Schade et al., 2003). Soil microbial biomass was measured by chloroform fumigation, and SMBC was determined using a TOC analyzer (liauid TOC II, elementar, Germany), at a $K_{EC}$ (extractable part of microbial biomass C) of 0.38. SMBN was determined by ultraviolet spectrophotometric colorimetry, at a $K_{EN}$ (extractable part of microbial biomass N) of 0.54. SMBP was measured by molybdenum-antimony colorimetry with $Na(HCO_3)_2$ extracts, at a $K_{EP}$ (extractable part of microbial biomass P) of 0.4.

### 2.4. Analyses of enzymatic activity

The activities of β-1,4-glucosidase (BG), β-1,4-*N*-acetylglucosaminidase (NAG) and alkaline phosphatase (AP) are commonly measured as indicators of energy (C) demand, N demand and P demand, respectively (Schimel and Weintraub, 2003). The activities of these three enzymes were measured as described by Saiya-Cork et al. (2002) with modifications by German et al. (2011). The potential activities of BG, NAG, and AP were also quantified. Sample suspensions were prepared for all enzymes by adding 1 g of the soil stored at −80 °C to 125 ml of buffer (50 mM sodium acetate buffer, pH 8.5) for the extraction of enzymes and homogenization for 2 h. BG activity was measured using 4-MUB-β-D-glucoside as a substrate, NAG activity was measured using 4-MUB-*N*-acetyl-b-D-glucosaminide as a substrate and AP activity was measured using 4-methylumbelliferyl phosphate as a substrate. The reactions were terminated, and fluorescence was measured using a fluorometer set at 365 nm excitation and 450 nm emission. We calculated enzymatic activity as the rate of substrate converted in nmol $g^{-1}$ dry soil $h^{-1}$.

### 2.5. DNA extraction and PCR amplification

Microbial DNA was extracted from 0.25 g of soil using a TIANamp Soil DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The DNA extracts were diluted ten-fold and spectrophotometrically assessed for quality and quantity (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, USA). The integrity of the DNA extracts was confirmed by 1% agarose gel electrophoresis. The gene of bacterial V3-V4 region were amplified by PCR using primers for bacterial 16S rRNA gene. The 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGG TATCTAAT-3′) were designed to for V3-V4. The primers were tagged with unique barcodes for each sample. The PCR reactions were performed in triplicate volume of 30 μl mixtures, containing 2 μl of sterile ultrapure water, 15 μl of Phusion Master Mix (2 ×), 3 μl of 6 μM primers and 10 μl of template DNA (5–10 ng). Successful PCR amplification was verified by 2% agarose gel electrophoresis. The triplicate PCR products were mixed in equidensity ratios and were then purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). The amplicons were then sequenced on an Illumina HiSeq2500 platform, generating 250-bp paired-end reads.

### 2.6. Processing of sequencing data

The sequences were quality-filtered and chimera checked using the Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Quality filtering of raw tags was performed under specific conditions to obtain high-quality clean tags (Bokulich et al., 2013) following QIIME quality control. Sequence with the same barcode were sorted into same sample (Edgar et al., 2011). Compared with a reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (Caporaso et al., 2010), the chimeric sequences were identified and remove to obtain the effective tags. The remaining sequences were clustered by UPARSE (Edgar, 2013) and assigned to operational taxonomic units (OTUs) with a 97% similarity. Taxonomic information for each representative sequence was obtained from the GreenGene Database (De Santis et al., 2006) using the RDP classifier (Wang et al., 2007) algorithm.

### 2.7. Threshold elemental ratio and stoichiometric homeostasis

TER for C:N and C:P (Allen and Gillooly, 2009) was calculated as:

$$TER_{C:N} = \left(\frac{A_N}{GE}\right) B_{C:N} \quad (1)$$

$$TER_{C:P} = \left(\frac{A_P}{GE}\right) B_{C:P} \quad (2)$$

where $A_P$ and $A_N$ are assimilation efficiencies for P and N, using 0.9 for both $A_P$ and $A_N$, GE (microbial growth efficiency) is 0.29 (Sinsabaugh et al., 2009) and $B_{C:N}$ and $B_{C:P}$ are the C:N and C:P ratios of microbial biomass.

The degree of community-level microbial C:N and C:P homeostasis (H) by soil microorganisms can be represented as:

$$H = \frac{log_{10}(x)}{log_{10}(y) - log_{10}(c)} \quad (3)$$

where x is the resource nutrient stoichiometry (e.g. C:N or C:P), y is the microbial nutrient stoichiometry and c is a constant. Therefore, 1/H is the slope of the regression between log(y) and log(x) and should be between 0 and 1. $H \gg 1$ represents a strictly stoichiometric homeostasis, and $H \approx 1$ indicates weak or no homeostasis (Sterner and Elser, 2002). The regression slope, 1/H, could thus be used in this analysis. Data with significant regressions and $0 < 1/H < 1$ were classified as homeostatic ($0 < 1/H < 0.25$), weakly homeostatic ($0.25 < 1/H < 0.5$), weakly plastic ($0.5 < 1/H < 0.75$) or plastic ($1/H > 0.75$). We classified cases as strictly homeostatic if the least

squared regression slope was not significant ($P > 0.05$).

### 2.8. Statistical analyses

The abundances of nitrogen-fixing and nitrifying bacteria were determined by Illumina MiSeq sequencing at the genus level. All data were analyzed by one-way ANOVAs. Duncan's tests at $P < 0.05$ were used for multiple comparisons. All statistical analyses and regressions were performed using SPSS 20.0. Differences were considered significant at $P < 0.05$. Graphs were plotted using Origin 9.0.

Microbial N-use efficiency (NUE) describes the partitioning of organic N taken up during growth and the release of inorganic N to the environment (i.e. N mineralization). C-use efficiency (CUE) is the efficiency of conversion of organic matter to microbial biomass. The microbial-community CUE: NUE ratios were calculated as (Mooshammer et al., 2014):

$$\text{CUE: NUE} = B_{C:N}: R_{C:N} \quad (4)$$

where $B_{C:N}$ is the C:N ratio of the microbial biomass and $R_{C:N}$ is the C:N ratio of the soil.

A structural equation model (SEM) was used based on the effect of N addition on soil and microbial nutrient contents and stoichiometries. We first reduced the number of variables for microbial nutrient contents and stoichiometries using principal component analyses (PCAs) (Veen et al., 2010). Soil microbial nutrient (SMN) content and soil microbial nutrient stoichiometry (SMNS) were used as raw data for the PCAs. The first principal component (PC1) was used in the subsequent SEM analysis to represent SMN concentration (PC1 explained 94.14% of the variance) and SMNS (PC1 explained 76.27% of the variance). We assumed that N addition alters soil nutrient content and stoichiometry and thus SMN content and SMNS.

## 3. Results

### 3.1. Contents and ratios of SOC, TN and TP in the soils and TERs

SOC and TN contents were higher in the 0–20 cm than the 20–40 cm layer (Fig. 2A, C, E). $TER_{C:N}$ and $TER_{C:P}$ in the 0–20 cm layer were 8.75 and 87.37, respectively.

SOC, TN and TP contents in the 0–20 cm layer varied among the treatments. SOC content first increased and then decreased, was highest at the lowest level of N addition (N3) (Fig. 2A) and did not differ significantly between N3 and N6. TN and TP contents tended to increase with N addition. TN content was significantly higher at N6 and N9 than that at N3 and CK (Fig. 2C). TP content was significantly lower in CK than the other treatments but did not differ significantly between N3, N6 and N9 (Fig. 2E). SOC and TN contents in the 20–40 cm layer had a similar pattern of increase and were highest at N9, but SOC content did not differ significantly between N6 and N9, and TN content did not differ significantly between N3, N6 and N9 (Fig. 2A, C). TP content in the 20–40 cm layer did not differ significantly among the treatments (Fig. 2E).

The SOC:TN (soil C:N) and SOC:TP (soil C:P) ratios in the 0–20 cm layer had a pattern similar to the SOC concentrations, first increasing and then decreasing (Fig. 2B, D). Soil C:N and C:P were highest at N3 at 18.99 and 103.19, respectively. TN:TP (soil N:P) tended to increase and was highest at N6 but did not differ significantly between N6 and N9 (Fig. 2F). C:P and N:P in the 20–40 cm layer all tended to increase. C:N at N6, C:P at N6 and N9 and N:P at N9 were significantly higher than for CK but did not differ significantly between CK and the other treatments.

### 3.2. Contents and ratios of C, N and P in soil microbial biomass

Microbial nutrients were higher in the 0–20 cm than the 20–40 cm layer (Table 1). SMBC, SMBN and SMBP in the 0–20 cm layer were highest at the intermediate rate of N addition (N6) but decreased at the highest rate (N9). SMBC in the 20–40 cm layer was highest at N6, and SMBN was highest at N3 but did not differ significantly between N3, N6 and N9. SMBP was highest at CK, did not differ significantly between N6 and N9, was lowest at N3 and did not differ significantly between N3 and N9.

The SMBC:N ratio in the 0–20 cm layer tended to decrease with N addition, was highest for CK (2.60), and did not differ significantly between N3, N6 and N9. SMBN:P tended to first increase and then decrease and was highest at N6. SMBC:P did not differ significantly between N3 and N6 (Table 1). SMBC:N in the 20–40 cm layer was significantly higher in CK than the other treatments. SMBN:P also tended to increase and then decrease, was highest at N3 and did not differ significantly between N6 and N9. MBC: P also did not differ significantly between CK and N3 and was highest at N6.

### 3.3. Enzymatic activities and ecoenzymatic stoichiometry

The activities of BG, NAG and AP did not differ significantly among the treatments in either layer (Fig. 3A, C, E). BG:NAG and BG:AP also did not differ significantly among the treatments in either layer. NAG:AP did not differ significantly in the 20–40 cm layer but in the 0–20 cm layer was higher in N6 than CK, N3 and N9 and did not differ significantly between CK, N3 and N9.

Energy-acquisition activities relative to nutrient-acquisition activities for all treatments were analyzed (Fig. 4). The regressions indicated that ln(BG) vs ln(NAG) and ln(BG) vs ln(AP) for both layers were significantly linearly correlated and that the slopes of the regression lines were lower for the 0–20 cm than the 20–40 cm layer (Fig. 4). For example, the slope for ln(BG) vs ln(NAG) (slope = 1.0441) was lower for the 0–20 cm than the 20–40 cm layer (slope = 1.051). The slope for ln (BG) vs ln(AP) for the 0–20 and 20–40 cm layers were 0.9217 and 1.0815, respectively. All slopes were near 1.

### 3.4. Stoichiometric homeostasis

Associations between the microbial biomass elemental ratios and those for the soil resources were simulated to test the strength of the stoichiometric homeostasis (Fig. 5). When all the data for each layer were analyzed together, the slopes of $\log(C:N_B)$ vs $\log(C:N_R)$ in the 0–20 and 20–40 cm layers were near 0, indicating strong homeostasis. The correlation between $\log(C:P_B)$ and $\log(C:P_R)$, however, was significant, with slopes near 1 in both layers, indicating a plastic relationship between microbial C:P and soil C:P.

### 3.5. Integrated response of soil microbial communities to N addition

Correlation analysis (Table 2) and SEM (Fig. 6) were conducted to illustrate how microorganisms and their stoichiometry coped with varying resources and how their stoichiometry was affected by N deposition. SOC was significantly positively correlated with SMBC, SMBN and SMBP, and TN and TP contents were not significantly correlated with SMB nutrient contents (Table 2). SC:P (soil C:P) was significantly positively correlated with SMBC, SMBN and SMBP, and SC:N (soil C:N) was positively correlated with SMBC and SMBP. SN:P (soil N:P) but was not significantly correlated with SMB nutrient content or stoichiometry. Resources and soil microbial properties were not significantly correlated with enzymatic activity.

The SEM model fitted the data well, indicating an interaction of nutrient contents and stoichiometry between soil and microbes in response to N addition (Chi-square = 9.909, $P = 0.194$; standardized path coefficients are shown in Fig. 6). SC:N (soil C:N), SC:P (soil C:P) and SN:P (soil N:P) calculated using SOC, TN and TP contents explained 100% of the variance. The model explained 89% of the variance in SMN, 95% of the variance in SMNS and 59 and 80% of the variances in TN and TP, respectively. N addition increased TN and TP contents

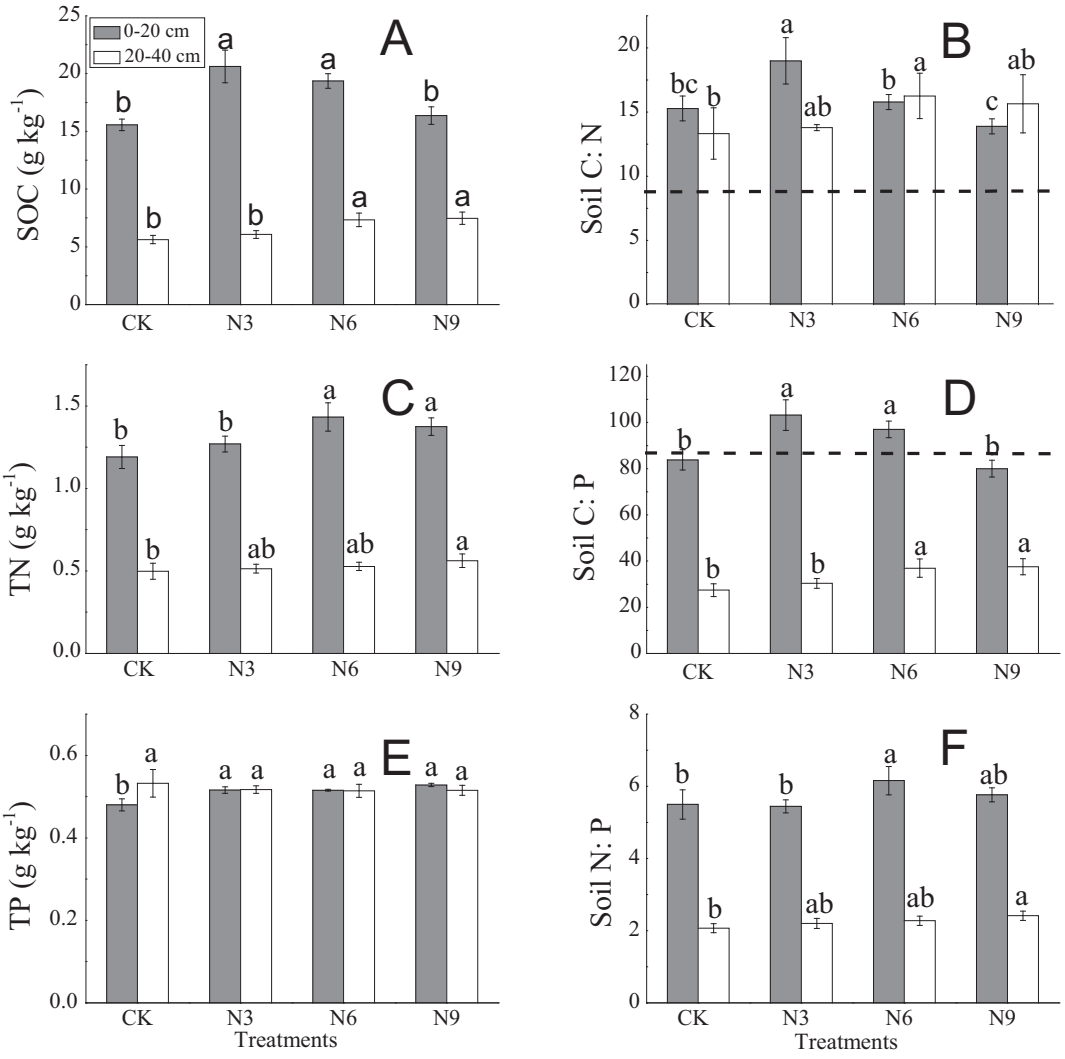


**Fig. 2.** The contents and ratios of SOC, TN and TP in the 0–20 and 20–40 cm layers. In Fig. 2A, C and E, SOC (soil organic carbon), TN (soil total nitrogen) and TP (total phosphorus) contents in 0–20 cm and 20-40 cm are described, respectively. In Fig. 2B, element ratio of SOC (soil organic carbon) and TN (soil total nitrogen) in 0–20 and 20–40 cm are expressed by soil C:N, and the dashed line in Fig. 2B represents $TER_{C:N}$ for the 0–20 cm layer. In Fig. 2D, element ratio of SOC (soil organic carbon) and TP (total phosphorus) are expressed by soil N:P, and the dashed line in D represents $TER_{C:P}$ for the 0–20 cm layer. In Fig. 2F, element ratio of TN (soil total nitrogen) and TP (soil total phoshporus) are expressed by soil N:P. Different letters represent significant differences of means for each layer ($P < 0.05$). Error bars represent standard errors.

relative to CK, consistent with the soil properties (Fig. 2). N addition both indirectly and directly affected SMN (Fig. 6). SC:N and SC:P had opposite effects on SMN. The relationships between the remaining exogenous and endogenous variables were not significant but improved the model fit.

## 4. Discussion

### 4.1. Response of ecoenzymes to N addition

Enzymatic activities are associated with both microbial metabolism

**Table 1**
Average SMBC, SMBN and SMBP and ratios of SMBC, SMBN and SMBP in the 0–20 and 20–40 cm layers.

| | Treatment | SMBC | SMBN | SMBP | SMBC:N | SMBC:P | SMBN:P | CUE:NUE |
|---|---|---|---|---|---|---|---|---|
| 0–20 cm | CK | 205.56 ± 1.28c | 93.25 ± 11.89c | 17.90 ± 0.91c | 2.60 ± 0.35a | 29.73 ± 1.55b | 11.53 ± 1.25d | 0.17 ± 0.02a |
| | N3 | 269.31 ± 2.99b | 175.11 ± 9.73b | 20.28 ± 0.28b | 1.80 ± 0.10b | 34.30 ± 0.47a | 19.13 ± 1.26b | 0.10 ± 0.00c |
| | N6 | 281.68 ± 7.23a | 206.60 ± 5.36a | 21.51 ± 0.68a | 1.59 ± 0.07b | 33.85 ± 0.81a | 21.30 ± 1.24a | 0.10 ± 0.01c |
| | N9 | 149.17 ± 3.67d | 94.73 ± 8.93c | 14.46 ± 0.51d | 1.85 ± 0.16b | 26.67 ± 1.07c | 14.50 ± 1.19c | 0.13 ± 0.01b |
| 20–40 cm | CK | 127.17 ± 4.16b | 36.02 ± 0.78b | 7.45 ± 0.28a | 4.12 ± 0.17a | 44.15 ± 1.95c | 10.72 ± 0.21c | 0.31 ± 0.05a |
| | N3 | 89.11 ± 4.19c | 55.94 ± 4.04a | 4.88 ± 0.56c | 1.86 ± 0.06d | 47.48 ± 4.21c | 25.49 ± 1.69a | 0.14 ± 0.00c |
| | N6 | 162.09 ± 17.00a | 51.94 ± 0.41a | 5.80 ± 0.63b | 3.64 ± 0.39b | 72.30 ± 2.40a | 20.02 ± 2.20b | 0.23 ± 0.05b |
| | N9 | 122.80 ± 5.57bc | 55.52 ± 3.40a | 5.56 ± 0.52bc | 2.59 ± 0.23c | 57.32 ± 4.21b | 22.24 ± 2.30b | 0.17 ± 0.02c |

Different letters represent significant differences of means for each layer ($P < 0.05$). SMBC, SMBN and SMBP represent microbial biomass carbon, microbial biomass nitrogen and microbial biomass phosphorus, respectively. SMBC:N, SMBC:P, SMBN:P represent element ratio of SMBC and SMBN, SMBC and SMBP, SMBN and SMBP. SMBC, SMBN and SMBP are in mg $kg^{-1}$.

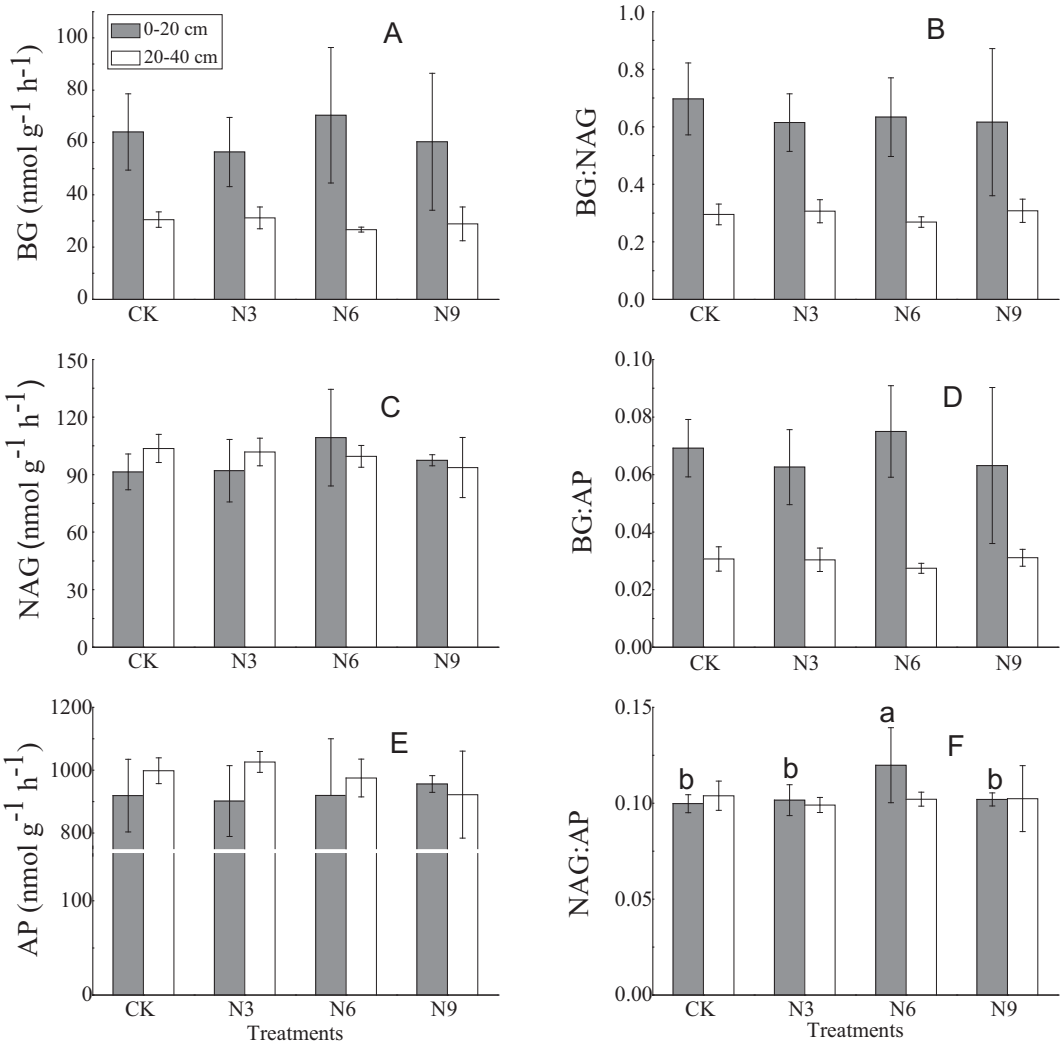


**Fig. 3.** BG, NAG and AP enzymatic activities and BG:NAG, BG:AP and NAG:AP activity ratios in the 0–20 and 20–40 cm layers. In Fig. 3A, C and E, BG (β-1,4-glucosidase), NAG (β-1,4-*N*-acetylglucosaminidase) and AP (alkaline phosphatase) enzymatic activities in 0–20 and 20–40 cm are expressed, respectively. In Fig. 3B, D, F, BG (β-1,4-glucosidase): NAG (β-1,4-*N*-acetylglucosaminidase), BG (β-1,4-glucosidase): AP (alkaline phosphatase) and NAG (β-1,4-*N*-acetylglucosaminidase): AP (alkaline phosphatase) represents ratios of BG (β-1,4-glucosidase), NAG (β-1,4-*N*-acetylglucosaminidase) and AP (alkaline phosphatase) enzymatic activities in 0–20 and 20–40 cm. Different letters represent significant differences of means for each layer ($P < 0.05$). Error bars represent standard errors.

and the availability of environmental resources, indicating the adaptation of microbes to resource stoichiometry. Microbes vary their allocations of resources to C-, N- or P-acquiring enzymes, depending on the relative demand of these resources for microbial growth (Allison et al., 2011). The activities of extracellular enzymes should therefore vary along N-addition gradients, which has been reported (Fan et al., 2018; Wang et al., 2008; Sinsabaugh et al., 2005; Michel and Matzner, 2003). The soil ecoenzymatic activities in our study (Fig. 3), however, did not differ significantly between the N-addition treatments, which was not expected but consistent with Jing et al. (2017) and DeForest et al. (2004). Tian et al. (2017) attributed their minor effect of N addition on soil enzymes to no significant differences among treatments of soil total C, N, P and soil microbial biomass C and N. We analyzed enzymatic activities because they are not only regulated by environmental or resource signals, but also determined by environmental interactions after enzymes are released from cells, such as edaphic and climatic variables (e.g. mean annual temperature and mean annual precipitation) (Sinsabaugh and Shah, 2012).

The BG:NAG and BG:AP activity ratios in both layers and the NAG:AP ratio in the 20–40 cm layer did not differ significantly, and only NAG:AP varied significantly in the 0–20 cm layer. Although overall global soil NAG:AP was 0.44 and the soil BG:AP was 0.62, which were obtained from parts of typical plots that did not represent all forests (Sinsabaugh et al., 2009). Waring et al. (2014) also reported ratios of these enzymes inconsistent with global patterns. BG:NAG in our experiment was about 0.6 and 0.3 in the 0–20 and 20–40 cm layers, respectively, lower than the global level (1.41) (Sinsabaugh et al., 2009), indicating that the sample plots were still N-limited. BG:AP in the 0–20 and 20–40 cm layers were about 0.06 and 0.03, respectively, which were much lower than 0.62 (Sinsabaugh et al., 2009) but similar to 0.026 (Ushio et al., 2010), 0.072 (Pamer et al., 2011) and 0.037 (Caruso et al., 2005), indicating that these forests are both N- and P-limited. NAG:AP was about 0.1 in both layers but was significantly higher at N6 than the other treatments in the 0–20 cm layer, consistent with Sistla et al. (2015).

Energy-acquisition activity relative to nutrient-acquisition (N, P) activities is 1:1:1 globally, indicating coupling among C, N and P cycling. The abilities of enzymes for the acquisition of soil nutrients reported in this study (Fig. 4) were < 5, which are among the lowest levels reported (Tapia-Torres et al., 2015; Sinsabaugh et al., 2009), indicating the relatively oligotrophic nature of the soil. The slopes of the regression lines for ln(BG):ln(NAG) for the 0–20 and 20–40 cm

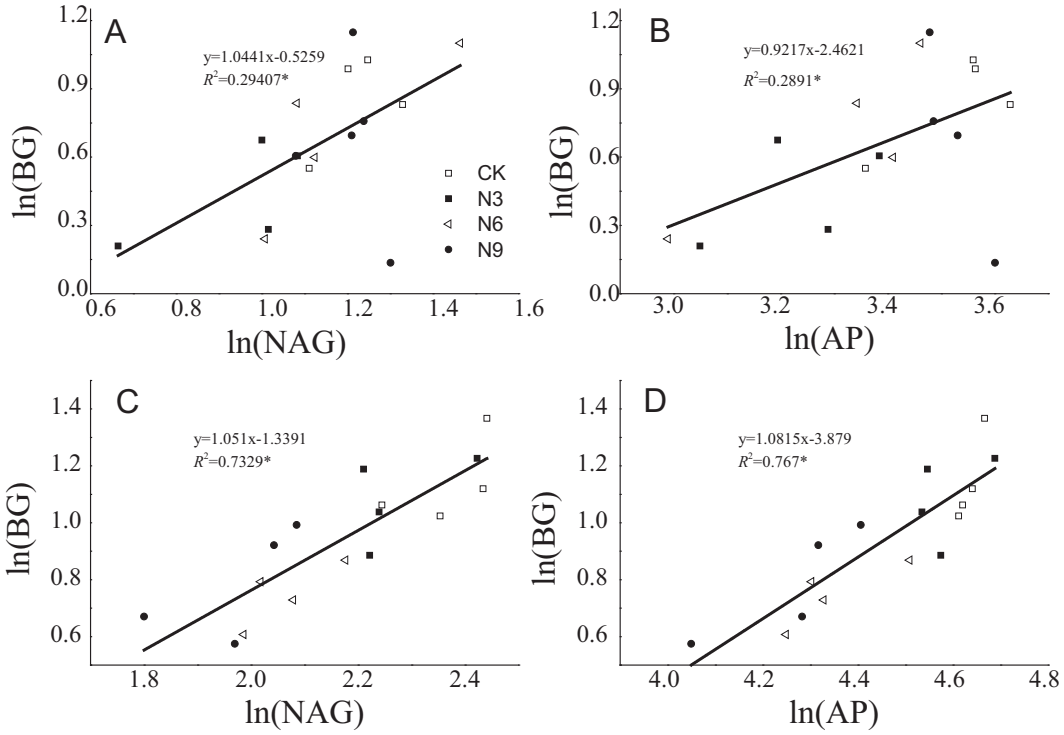


**Fig. 4.** Activities of enzymes for acquiring organic nitrogen (N) and organic phosphorus (P) relative to the activity of the enzyme for acquiring carbon (C) in the 0–20 and 20–40 cm layers. N acquisition is measured by the potential activity of β-1,4-*N*-acetylglucosaminidase (NAG) (A, C), P acquisition is measured by the potential activity of alkaline phosphatase (AP) (B, D) and C acquisition is represented by the potential activity of β-1,4-glucosidase (BG). Fig. 4A and B indicate the relationship in the 0–20 cm layer and C and D indicate the relationship in the 20–40 cm layer. Significant correlations are indicated by asterisks (*) ($P < 0.05$). Nutrient and energy acquisition are expressed as enzymatic activities in nmol $h^{-1}$ $g^{-1}$ soil organic matter.

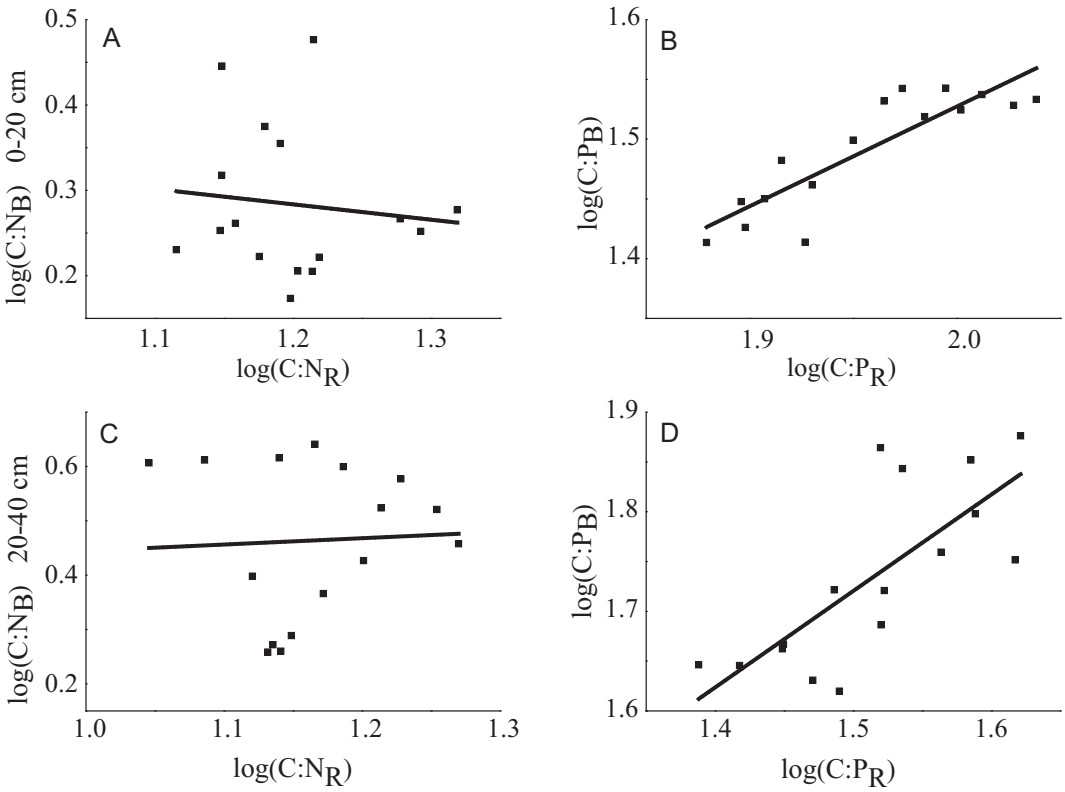


**Fig. 5.** Soil microbial community homeostasis correlated with N (left panels) and P (right panels) acquisition in the 0–20 and 20–40 cm layers. The regression equation for each panel is: A: y = −0.18004× + 0.4997 $R^2$ = 0.0133; B: y = 0.8344×-0.1414 $R^2$ = 0.7473($P < 0.05$); C: y = 0.1165× + 0.3283 $R^2$ = 0.0024 and D: y = 0.9678× + 0.2688 $R^2$ = 0.5387 ($P < 0.05$).

**Table 2**
Correlation coefficients of contents and stoichiometry between soil, microbial community and ecoenzymes.

| | SOC | TN | TP | SC:N | SC:P | SN:P | SMBC | SMBN | SMBP | SMBC:N | SMBC:P | SMBN:P | BG | NAG | AP | BG:NAG | BG:AP | NAG:AP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SOC | 1 | | | | | | | | | | | | | | | | | |
| TN | 0.271 | 1 | | | | | | | | | | | | | | | | |
| TP | 0.383 | 0.589* | 1 | | | | | | | | | | | | | | | |
| SC:N | 0.794** | −0.367 | 0.006 | 1 | | | | | | | | | | | | | | |
| SC:P | 0.957** | 0.111 | 0.100 | 0.852** | 1 | | | | | | | | | | | | | |
| SN:P | 0.123 | 0.893** | 0.164 | −0.447 | 0.084 | 1 | | | | | | | | | | | | |
| SMBC | 0.779** | 0.123 | −0.067 | 0.657** | 0.858** | 0.190 | 1 | | | | | | | | | | | |
| SMBN | 0.796** | 0.421 | 0.316 | 0.487 | 0.757** | 0.340 | 0.899** | 1 | | | | | | | | | | |
| SMBP | 0.709** | 0.136 | −0.102 | 0.581* | 0.792** | 0.226 | 0.975** | 0.861** | 1 | | | | | | | | | |
| SMBC:N | −0.553* | −0.642** | −0.828** | −0.126 | −0.336 | −0.322 | −0.354 | −0.712** | −0.315 | 1 | | | | | | | | |
| SMBC:P | 0.778** | 0.019 | −0.095 | 0.721** | 0.867** | 0.078 | 0.953** | 0.844** | 0.864** | −0.304 | 1 | | | | | | | |
| SMBN:P | 0.755** | 0.513* | 0.518* | 0.393 | 0.651** | 0.338 | 0.747** | 0.957** | 0.678** | −0.859** | 0.730** | 1 | | | | | | |
| BG | 0.143 | 0.346 | −0.044 | −0.087 | 0.166 | 0.445 | 0.109 | 0.071 | 0.112 | 0.093 | 0.092 | 0.022 | 1 | | | | | |
| NAG | 0.213 | 0.392 | 0.240 | −0.045 | 0.155 | 0.342 | 0.191 | 0.298 | 0.179 | −0.272 | 0.157 | 0.314 | 0.649** | 1 | | | | |
| AP | −0.054 | 0.192 | 0.127 | −0.158 | −0.093 | 0.164 | −0.140 | −0.100 | −0.148 | 0.041 | −0.133 | −0.063 | 0.560* | 0.698** | 1 | | | |
| BG:NAG | 0.031 | 0.163 | −0.233 | −0.078 | 0.104 | 0.328 | −0.004 | −0.134 | 0.010 | 0.332 | −0.001 | −0.211 | 0.839** | 0.133 | 0.261 | 1 | | |
| BG:AP | 0.191 | 0.323 | −0.102 | −0.033 | 0.233 | 0.448 | 0.176 | 0.113 | 0.186 | 0.100 | 0.151 | 0.039 | 0.934** | 0.446 | 0.230 | 0.887** | 1 | |
| NAG:AP | 0.325 | 0.335 | 0.201 | 0.082 | 0.284 | 0.293 | 0.395 | 0.504* | 0.405 | −0.411 | 0.320 | 0.481 | 0.228 | 0.575* | −0.170 | −0.142 | 0.323 | 1 |

Significant correlations are indicated by asterisks (*) ($P < 0.05$); Extremely significant correlations are indicated by asterisks (**) ($P < 0.01$). SC:N represents soil C:N, SC:P represents soil C:P and SN:P represents soil N:P ratios; SMBC,N, P represent soil microbial biomass C, N, P, and SMBC:N, SMBC:P and SMBN:P represent ratios of SMBC, SMBN and SMBP.

layers were 1.0441 and 1.051, respectively, similar to previous reports of 1.09 (Waring et al., 2014). The slopes of the regression lines for ln (BG):ln(AP) for the 0–20 and 20–40 cm layers were 0.9217 and 1.0815, respectively, similar to previous reports of 1.16 (Sinsabaugh et al., 2009) and 1.18 (Waring et al., 2014). The soil enzymatic activities in this study is relatively low, but the activities of the enzymes for the acquisition of organic N and organic P both scaled with the activity of the enzyme for the acquisition of C, with a slope of about 1 (Fig. 4), indicating that the soil microbial communities had similar patterns of allocation to nutrient acquisition, despite the diverse community composition and conditions of N inputs.

### 4.2. Stoichiometric dynamics of microbial community to N addition

SOC and TN contents were higher in the 0–20 cm than the 20–40 cm layer, due to litter and surface roots. Soil nutrient concentrations, microbial biomasses and microbial activities in 20–40 cm soil layer had similar change tendency with those in 0–20 cm along N addition gradients. Microbial biomasses also followed this rule, and microbial biomasses were higher in the 0–20 cm than the 20–40 cm layer. We should therefore focus on the responses of elemental cycling and microbes to N addition in the 0–20 cm layer.

We calculated a $TER_{C:N}$ of 8.75 in the 0–20 cm layer using the parameters ($A_N$ = 0.9 and GE = 0.29) provided by Sinsabaugh et al. (2009). $TER_{C:N}$ for the 0–20 cm layer was lower than soil C:N in all treatments, indicating that all plots were N-limited, consistent with the lower BG:NAG than in other studies discussed above. Soil C:N, however, first increased and then decreased, indicating that N addition alleviated N limitation at N6 and N9, similar to other studies where N addition decreased soil C:N (Sistla et al., 2015; Yang and Post, 2011). This alleviated soil condition would increase soil microbial biomasses, which is consistent with our previous results (Zhang et al., 2017). Microbial activities vary to adjust to different nutrient contents among treatments. This N alleviation was indicated by the decrease in ecoenzymatic activities per unit of microbial biomass C (data not shown). The relationships between the soil and soil microbes were also indicated by CUE and NUE. Microbes could increase C-mineralization rates to cope with disadvantageous conditions, such as high N limitation (Odum, 1985). CUE in our study was therefore higher at N9 than N6. The abundance of nitrifying bacteria did not differ significantly among the treatments (Fig. 7). Nitrifying bacteria can transform ammonium N to nitrate that can leach. The higher the N content, the higher the NUE at the same level of leaching. The abundance of nitrogen-fixing bacteria was significantly higher at N6 than the other treatments, increasing the soil N contents and NUE and decreasing CUE:NUE.

Tropical forests are P-limited, and most arid forests are N-limited, but a recent study found that arid forests can also be P-limited (DeForest et al., 2012). N addition in our study increased P concentration in the 0–20 cm layer, similar to the experiments conducted by Zhang et al. (2017). Compared with a $TER_{C:P}$ of 87.37, soil microbes at N3 and N6 were P limited, which were associated with increasing SOC content. N:P also increased, as reported by Sistla et al. (2015). SMBC, SMBN and SMBP were consistent and highest at N6 in the 0–20 cm layer (Table 1), which may be affected by roots and root exudates (Leff et al., 2015). SMBC:N and SMBC:P were lower than soil C: N and C: P, indicating that N and P were further concentrated in soil microbial biomass (Sinsabaugh et al., 2009), consistent with the soil organic matter C: N: P ratio of 186:13:1 reported by Cleveland and Liptzin (2007). N and P were further concentrated in soil microbial biomass, which had a mean C:N:P ratio of 60:7:1.

### 4.3. Analysis of microbial community homeostasis

The soil on the Loess Plateau in China are poor and N-limited (Wang et al., 2004), and soil microbes have adjusted to this situation. External factors can destabilize the relatively stable ecosystem. Soil microbial

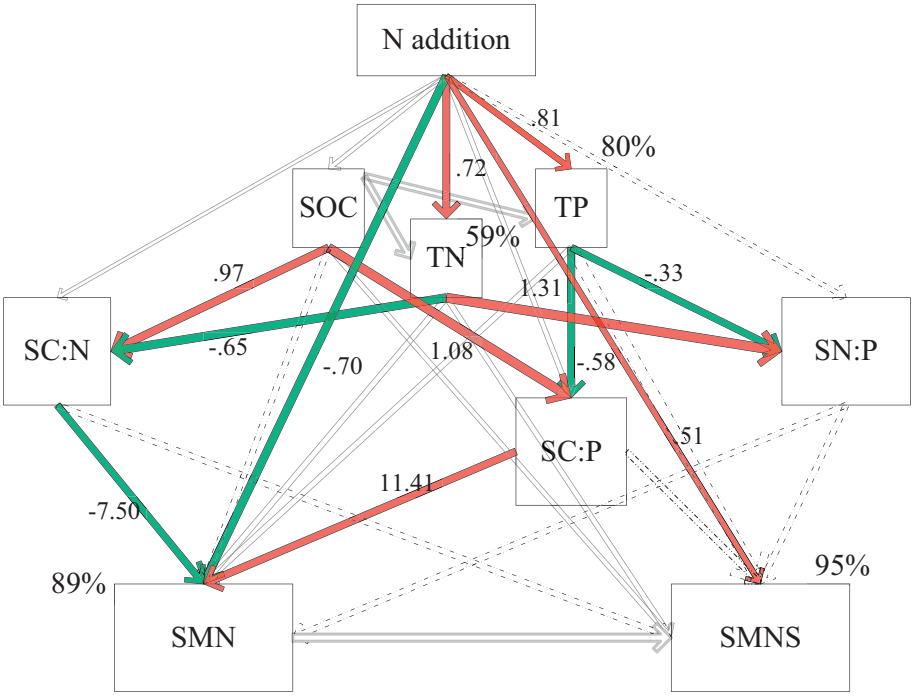


**Fig. 6.** Structural equation model of the effects of N addition on soil-microbe nutrients and nutrient stoichiometries in the 0–20 cm layer. This figure indicates the relationships between N addition (exogenous variable) and SOC, TN and TP contents; SC:N (soil C:N), SC:P (soil C:P) and SN:P (soil N:P) ratios; SMN (soil microbial nutrient) content and SMNS (soil microbial nutrient stoichiometry). The final model fit the data well: Chi-square = 9.909, $P = 0.194$, Akaike information criterion = 90.00, root mean square error of approximation = 0.166. Numbers near the arrows are standardized path coefficients. Red arrows indicate significant positive relationships, and green arrows indicate significant negative relationships ($P < 0.05$). Solid arrows indicate positive relationships, and dashed arrows indicate negative relationships. Gray arrows indicate paths removed to improve model fits. Percentages near endogenous variables indicate the variance explained by the model. SMN and SMNS represent soil microbial biomass and soil microbial biomass stoichiometry, respectively, which were processed by a PCA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

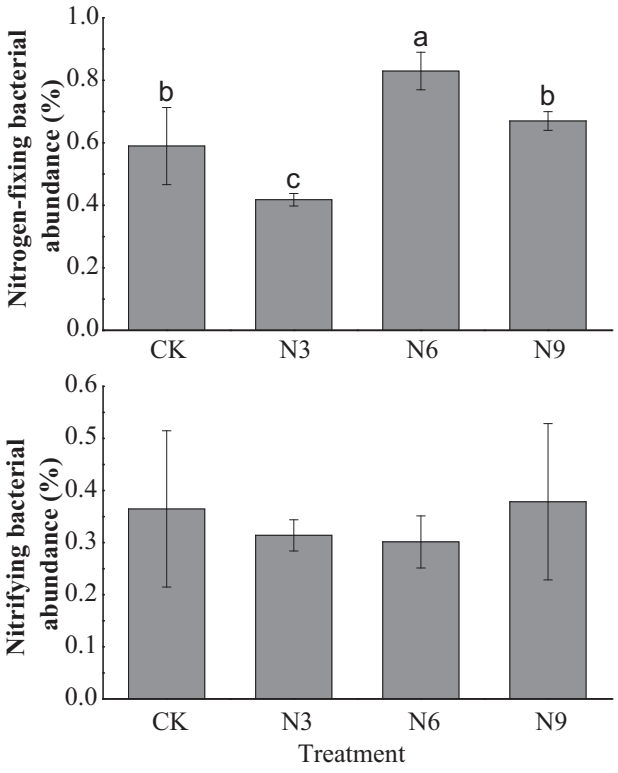


**Fig. 7.** Abundance of nitrogen-fixing and nitrifying bacteria. Different letters above the bars indicate significant differences at $P < 0.05$.

communities limited by N and co-limited by P at N3 and N6 could adjust physiologically to attain a new balance between resources and themselves, which was supported by BG:NAG and BG:AP. The relationships between log(C:$N_R$) and log(C:$N_B$) in both layers had slopes that did not differ significantly from 0 (Fig. 5), indicating that microbial communities maintained homeostasis, independent of soil resources (Fig. 7). Soil C:N, C:P and N:P did not significantly affect SMNS, and soil C:N was not significantly correlated with SMBC:N (Table 2). In addition, the relationship between log(C:$N_R$) and log(C:$N_B$) indicated that the microorganisms were heterotrophic. The slopes of the relationship between log(C:$P_R$) and log(C:$P_B$) for both layers were between 0.75 and 1, indicating no homeostasis. The microbes may therefore have been autotrophic, which would be inconsistent with the above results. P homeostasis for some heterotrophs, though, can vary from weak to strong homeostasis (DeMott and Pape, 2005), which may account for the contradiction in our study. This discrepancy should be further studied for a better understanding of the mechanism.

## 5. Conclusion

Our data showed that microbial coping strategy to adjust the imbalance between soil resources and microorganism, enzymatic activities and most ecoenzymatic stoichiometries along the N-addition gradient, did not differ significantly due to short-term N addition. However, the activities of the ecoenzymes for acquiring organic N and organic P scaled with the activity of the ecoenzyme for acquiring C, with patterns of allocation to nutrient acquisition similar to the global pattern. Soil microbial communities of this ecosystem were limited by N and co-limited by P at N3 and N6 (when SOC content was significantly higher). Ratios of soil microbial biomass were concentrated from soil resources. And finally our results N addition did not generally interfere with soil microbial community, maintaining community-level elemental homeostasis. Our findings also suggested that the contents of resources (SOC, TN and TP) and relative concentrations (stoichiometries) had an impact on the soil microbial communities. Our study contributes to a better understanding of how microbes cope and the flow of energy and nutrients in the food web due to an external factor. Further study of the structure of soil microbial communities is needed to identify the microbes that adjust to N deposition and the microbes that are heterotrophic for maintaining homeostasis, which would help to determine the mechanism.

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