



# Nitrogen addition shapes soil enzyme activity patterns by changing pH rather than the composition of the plant and microbial communities in an alpine meadow soil

Xiaochen Liu · Shiting Zhang

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## Abstract

**Aims** Increasing nitrogen (N) deposition has considerable effects on soil organic matter (SOM) decomposition mediated by soil enzyme activities. Few studies, however, have explored how N addition shapes soil enzyme activity patterns by changing plants, soils and microbes.

**Methods** We conducted a five-year field fertilization experiment (0, 5, 10, and 15 g N m<sup>-2</sup> yr<sup>-1</sup>) to study how N addition affected soil enzyme activity patterns in the topsoil (0–20 cm) and subsoil (20–40 cm) in a Tibetan alpine meadow. Enzyme activity patterns were calculated by the percentage of the sum of all measured enzyme activities. The composition of the plant and microbial communities were evaluated through measuring the abundance of plant functional groups and quantifying microbial phospholipid fatty acids (PLFAs), respectively. Soil pH and available N were also measured.

**Results** We found that soil N availability primarily controlled plant community composition, but pH controlled the composition of the microbial community, irrespective of soil depth. Soil enzyme activity patterns differed

between two soil depths and among N addition rates. Importantly, N addition shaped soil enzyme activity patterns through the changes in soil pH rather than via the composition of the plant and microbial communities.

**Conclusions** Our findings indicate that N addition can affect components of plant-soil system and, in particular, weaken the linkages between plant and microbial communities and enzyme activity patterns. The work suggests that N enrichment-induced soil acidification plays a key role in SOM decomposition and nutrient cycling in the Tibetan meadow ecosystem.

**Keywords** N addition · Soil enzyme activity patterns · Plant community · Soil microbial community · Soil depth · Soil pH

## Abbreviations

C	Carbon
N	Nitrogen
P	Phosphorus
SOM	Soil organic matter
SEM	Structural equation model
PLFAs	Phospholipid fatty acids
Control	0 g N m <sup>-2</sup> yr <sup>-1</sup>
N5	5 g N m <sup>-2</sup> yr <sup>-1</sup>
N10	10 g N m <sup>-2</sup> yr <sup>-1</sup>
N15	15 g N m <sup>-2</sup> yr <sup>-1</sup>
G <sup>+</sup> :G <sup>-</sup> ratio	Gram-positive bacteria:gram-negative bacteria
F:B	Fungi:bacteria
NMDS	Nonmetric multidimensional scaling

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X. Liu · S. Zhang (✉)  
State Key Laboratory of Grassland and Agro-Ecosystems, School of Life Science, Lanzhou University, Lanzhou 730000, China  
e-mail: zhangsht@lzu.edu.cn

PERMANOVA	Permutational multivariate analysis of variance
PCA	Principal component analysis
BG	$\beta$ -1,4-glucosidase
UREA	Urease
AP	Acid phosphomonoesterase
PHO	Phenol oxidase

## Introduction

Nitrogen (N) addition impacts many ecosystem functions (Manning et al. 2006), for example, productivity and diversity of the plant and microbial communities (Bassin et al. 2007; LeBauer and Treseder 2008; Jing et al. 2016; Riggs and Hobbie 2016; Wardle et al. 2004), which have cascading effects on soil enzyme activities (Ge et al. 2017; Kjoller et al. 2012; Treseder 2008). Soil enzyme activity plays a vital role in understanding the biochemistry of decomposition and nutrient cycling, owing to decomposing soil organic matter (Treseder 2008). Although the influence of N addition on soil enzyme activities has been studied (Riggs and Hobbie 2016; Stone et al. 2014), the direct and indirect roles of N enrichment in modulating soil enzyme activities through the plant-soil system is poorly understood (Schnecker et al. 2015).

There is a complex relationship among N availability, acidification, the resulting composition of the plant and microbial communities and soil enzyme activity. Elevated N availability can stimulate hydrolytic enzyme activities, but depress oxidative enzyme activities directly by modifying stoichiometry of substrates for enzymes (Burns et al. 2013). N addition also indirectly affects soil enzyme activities through the changes in the composition of plant and microbial communities (Sinsabaugh et al. 2008; Kjoller et al. 2012). Furthermore, supplemental N can cause shifts in biomass of different plant functional groups (Bai et al. 2010), which may mediate soil enzyme activities differently, such as increased activity of cellulose enzymes in the presence of legumes (Sanaullah et al. 2011). A global scale meta-analysis study suggests that soil microbial communities are constrained by C and P limitation under N addition, which can change composition of microbial community and then produce more related soil hydrolytic enzyme activities to relax the limitation (Jian et al. 2016). Additionally, N enrichment generally reduces soil fungi

biomass, which can suppress activity of soil oxidases (Stone et al. 2014; Jian et al. 2016). It is well-established that N enrichment leads to soil acidification due to increased nitrification (Wei et al. 2013; Riggs and Hobbie 2016), which can directly affect enzyme activities by altering the enzyme molecular conformation (Speir et al. 1999). As a consequence of increased toxic ions (e.g.  $\text{NH}_4^+$ ,  $\text{Al}^{3+}$ ) and decreased base cations (e.g.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Na}^+$ ), soil acidification also can affect the composition of plant and microbial communities through the effect on biomass and diversity (Chen et al. 2013; Liu and Greaver 2010). Therefore, the interrelated effects of N availability, acidification and plant and microbial community composition on soil enzyme activities following N addition are important but unresolved issues.

Many biotic and abiotic properties change with soil depth (Rumpel and Kögel-Knabner 2011; Stone et al. 2014), and the soil microenvironment and substrates for soil enzymes vary accordingly. For example, the biomass of plant roots and soil microbes decreases (Fierer et al. 2003; Lamb et al. 2010) and their composition changes with soil depth (Jackson et al. 2009; Schnecker et al. 2015). In addition, soil abiotic properties depend on the soil depth. Indeed, factors such as oxygen, temperature, and pH can decrease from the topsoil to the subsoil, whereas moisture and complex organic matter can increase with depth (Davidson et al. 2012; Wallander et al. 2003). Owing to the above factors, soil depth may affect distribution, production and activity of soil enzymes (Bending et al. 2002; Sinsabaugh et al. 2008). To date, the majority of studies on soil enzyme activities have focused exclusively on topsoil (Eskelinen et al. 2009; van der Putten et al. 2016).

The overarching goal of this study was to better understand how changes in N availability affected patterns in enzyme activity patterns in Tibetan meadows, we conducted a five-year N addition field experiment. Tibetan alpine meadows are characterized by cold temperatures, high SOM content and low soil N and P content (Cao and Zhang 2001). Large-scale N deposition ( $1.0\text{--}1.5 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) has occurred during recent decades in the Tibetan grasslands (Jia et al. 2014). We intended to explore the following hypotheses: (1) Supplemental N will result in a change in the composition of the plant and microbial communities. Specifically, there will be a shift in biomass of different plant functional groups, increased bacterial biomass, and decreased fungal biomass; (2) N addition will increase soil hydrolytic

enzyme activities associated with C, N and P acquisitions while also depressing oxidative enzyme activities, and thus affect soil enzyme activity patterns; (3) All biological production (i.e. plant belowground biomass, soil microbial biomass and enzyme activities) will be greater in topsoil compared to subsoil; (4) Soil enzyme activity patterns in topsoil and subsoil are both related to a set of key parameters (e.g. N availability, pH and composition of plant and microbial communities), and will be mainly driven by microbial community composition, because enzyme activities are generally related to microbial community composition (Sinsabaugh et al. 2008; Nannipieri et al. 2012; Riggs and Hobbie 2016). Structural equation modeling (SEM) is used to reveal the pathway through which N addition influences soil N availability, pH, the composition of plant and microbial communities and enzyme activity patterns.

## Materials and methods

### Study site

The study was conducted at the Alpine Meadow and Wetland Ecosystems Research Station of Lanzhou University (Azi branch station), in Maqu, Gansu Province, China (101°51'E, 33°40'N, 3500 m a.s.l). The study site has a mean annual temperature of 2.75 °C and precipitation of 601 mm for the period of 2011–2015 (Fig. S1). The soil type is defined as typical alpine meadow soil. Species richness is relatively high, with 25–40 species per 0.25 m<sup>2</sup>. The plant community is dominated by *Kobresia capillifolia*, *Carex kansuensis*, *Elymus nutans*, *Anemone rivularis* and *Saussurea nigrescens*, and the mean aboveground biomass is 440 g m<sup>-2</sup> (dry mass) (Zhang et al. 2015).

### Experimental design

The experimental site (100 × 100 m) has been fenced since April 2011 with grazing allowed during the winter. We used a randomized block design with five replicates, and four 10 × 20 m plots composed of four fertilization treatments distributed in each block (60 × 80 m). Nitrogen fertilizer (NH<sub>4</sub>NO<sub>3</sub>) was applied annually in early May at the following rates: 0 g N m<sup>-2</sup> yr<sup>-1</sup> (Control), 5 g N m<sup>-2</sup> yr<sup>-1</sup> (N5), 10 g N m<sup>-2</sup> yr<sup>-1</sup> (N10), and 15 g N m<sup>-2</sup> yr<sup>-1</sup> (N15).

### Plant sampling and measurements

In late July 2015, a 0.5 × 0.5 m subplot was harvested for each treatment within a block. We clipped all aboveground vegetation and separated it into four functional groups: grasses, legumes, sedges and forbs. After clipping, we collected topsoil (0–20 cm) and subsoil (20–40 cm) samples in each subplot with three soil cores (4 cm inner diameter). The roots in the two soil depths were separated from soil by running water. Aboveground plant vegetation and roots were both dried at 70 °C for 48 h and weighed as aboveground biomass (biomass of each functional group and total biomass of the four functional groups) and belowground biomass (root biomass in topsoil and subsoil), respectively (g m<sup>-2</sup>).

### Soil sampling and measurements

For each subplot, five additional soil cores (4 cm inner diameter) were collected and pooled together for topsoil and subsoil, respectively. After removal of visible organic debris, each soil sample was sieved (< 2 mm) and divided into three subsamples: 1) the first was air-dried at room temperature for soil physicochemical analysis; 2) the second was stored at –20 °C for analysis of soil enzyme activities within one month; 3) the third was freeze-dried and stored at –80 °C for phospholipid fatty acid (PLFA) analysis.

Soil pH was measured with a soil:water (1:2.5) mixture using a pH meter. Soil NO<sub>3</sub><sup>-</sup>-N and NH<sub>4</sub><sup>+</sup>-N were extracted with 2 M KCl and then analyzed by a flow injection analyzer (San++, Skalar, Netherlands). The composition of the soil microbial community was evaluated by PLFA analysis according to Bossio and Scow (1998). Qualitative and quantitative PLFA analysis were performed by GC-MS (a gas chromatograph combined with a mass spectrometer, Thermo Scientific, Bremen, Germany). An internal standard of 19:0 methyl ester was used for assurance of quantification. The PLFAs i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0 were used as indicators of gram-positive (G<sup>+</sup>) bacteria; 16:1ω7c, 17:1ω8c, 18:1ω7c, cy17:0 and cy19:0 were used as indicators of gram-negative (G<sup>-</sup>) bacteria; the PLFAs 10Me16:0, 10Me17:0 and 10Me18:0 were used as indicators of actinomycetes; the PLFAs 16:1ω5c and 18:2ω6,9c were used as indicators of fungi (Frostegård and Bååth 1996; Frostegård et al. 1993; Zelles 1999). Bacteria consisted of G<sup>+</sup> and G<sup>-</sup> bacteria in this study.

The PLFAs for soil microbes were expressed as  $\text{nmol g}^{-1}$  dry soil.

We measured the activities of  $\beta$ -1,4-glucosidase (EC 3.2.1.21), urease (EC 3.5.1.5), acid phosphomonoesterase (EC 3.1.3.1) and phenol oxidase (EC 1.10.3.2) in topsoil and subsoil. The  $\beta$ -1,4-glucosidase activity was measured using the substrate of  $\rho$ -nitrophenyl- $\beta$ -D-glucopyranoside (Tabatabai 1994). The product  $\rho$ -nitrophenol (PNP) was measured colorimetrically at 410 nm with a UV-VIS spectrophotometer (Specord 200 Plus, Analytic Jena, Germany). The acid phosphomonoesterase assay was similar to the  $\beta$ -1,4-glucosidase assay, except that  $\rho$ -nitrophenyl phosphate was the substrate (Tabatabai and Bremner 1969). The activity of  $\beta$ -1,4-glucosidase and acid phosphatase was expressed as  $\mu\text{mol PNP h}^{-1} \text{g}^{-1}$  dry soil. For the urease assay, 4 mM urea was used as the substrate. The  $\text{NH}_4^+$  produced was measured at 578 nm, and urease activity was expressed as  $\mu\text{mol NH}_4^+ \text{h}^{-1} \text{g}^{-1}$  dry soil (Kandeler and Gerber 1988). The assay for phenol oxidase activity used 50 mM pyrogallol as a substrate and was measured at 410 nm, and the phenol oxidase activity was expressed as  $\mu\text{mol PYGL h}^{-1} \text{g}^{-1}$  dry soil (Sinsabaugh 2010).

#### Data analysis

Prior to analysis, all variables were log-transformed to mitigate non-normality and linearity except for soil pH. One-way analysis of variance followed by Duncan's multiple range test ( $P < 0.05$ ) was used to evaluate the effects of N addition and soil depth on the measured variables. We also used mixed linear models with N addition and soil depths as fixed factors and block as a random factor to compare the responses of soil N availability, pH, the composition of microbial community and enzyme activities. Second, we calculated enzyme activity patterns (as described by Schneckner et al. 2015), and then obtained Euclidean distance matrixes. We used these matrixes to create Nonmetric Multidimensional Scaling (NMDS) plots to visualize the effects of N addition and soil depth on enzyme activity patterns. We used Permutational Multivariate Analysis of Variance (PERMANOVA) with Euclidean distance matrixes (Anderson 2001), to evaluate the differences in soil enzyme activity patterns between different depths and N addition rates. Third, we classified all variables into the following four groups (Table S1): 1) soil N availability, evaluated by PC1 scores based on  $\text{NH}_4^+$ -N and

$\text{NO}_3^-$ -N; 2) plant community composition, using PC1 scores based on the abundance of the four plant functional groups and root:shoot ratio; 3) microbial community composition, evaluated by PC1 scores using PLFAs of three microbial groups (bacteria, fungi and actinomycetes),  $\text{G}^+:\text{G}^-$  ratio and F:B ratio; 4) soil enzyme activity patterns, derived from PC1 scores using the community-weighted activities of  $\beta$ -1,4-D-glucosidase, urease, acid phosphatase and phenol oxidase. Principal component analysis (PCA) summarized each variable group for each soil depth (Table S1), and four PC1 scores explained 50.5–97.8% of the total variance for each group. SEM was performed to analyze hypothetical pathways that may explain how N availability affects soil enzyme activity patterns following N addition. The initial structural equation model was shown in Fig. S2. Most variables or categories examined in our N addition experiment were correlated with one another, making these data sets appropriate for SEM analysis (Table S2).

Statistical analysis was performed using R version 3.3.2 (R Core Team 2016). The mixed linear model was implemented using the “lme4” package (Bates et al. 2018). The PCA, NMDS and PERMANOVA were conducted using the “vegan” package (Oksanen et al. 2018). The SEM analysis was performed using AMOS 21.0 (Amos Development, Spring House, Pennsylvania, USA).

## Results

### Plant biomass, soil N availability, pH and PLFAs

Aboveground biomass responded to N addition but the pattern varied among functional groups. The total aboveground biomass increased in the N5 and N10, but there was no significant difference in N15 compared to control (Fig. S3a). N addition increased sedge biomass, but there was no significant difference between N addition rates (Fig. S3a). Legume biomass declined in both N10 and N15 (Fig. S3a). Forb biomass tended to decrease with N addition rates and there was a significant difference between N5 and N10. In contrast, grass biomass did not vary significantly with N supplementation (Fig. S3a).

The topsoil biomass followed the same pattern as the total aboveground biomass with increasing N addition rates. The subsoil biomass decreased significantly in all N addition treatments compared to control (Fig. S3b).

Additionally, the biomass in topsoil was far higher than that in subsoil (Fig. S3c).

N addition and soil depth had significant effects on pH,  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  individually, but their interaction only affected  $\text{NO}_3^-\text{-N}$  (Table 1). N addition strongly increased soil  $\text{NO}_3^-\text{-N}$  and  $\text{NH}_4^+\text{-N}$ , and decreased pH (Table 1). Soil pH was lower in topsoil than in subsoil, while soil  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were higher in topsoil than subsoil (Table 1).

The mixed linear model showed that N addition and soil depth affected PLFAs of bacteria,  $G^+$ ,  $G^-$ , actinomycetes, fungi and total PLFAs. The F:B ratio was affected by N addition but not by soil depth (Table 2). N15 significantly increased PLFAs of bacteria,  $G^+$ ,  $G^-$ , actinomycetes and total PLFAs, but significantly decreased PLFAs of fungi and F:B ratio at both soil depths compared to control. For all measured microbial parameters, there was significant difference between N15 and N10 (Fig. 1a–c). All the soil PLFAs decreased with soil depth (Fig. 1d).

### Enzyme activity patterns

The activities of  $\beta$ -1,4-D-glucosidase, urease, phenol oxidase were affected by soil depth and N addition, whereas acid phosphomonoesterase activity was only affected by soil depth (Table 2). In the topsoil,  $\beta$ -1,4-glucosidase activity markedly increased compared to the control and there was no significant difference among N addition rates (Fig. 2a). Urease activity significantly increased with increasing N addition rates, and the phenol oxidase activity decreased in N15 compared to control (Fig. 2a). The effects of N addition on all of the enzyme

activities in the subsoil were very similar with those in the topsoil (Fig. 2b). Generally, soil enzyme activity was lower in subsoil than in topsoil (Fig. 2c).

Soil enzyme activity patterns differed more among N addition rates than between soil depths (Fig. 3). Indeed, PERMANOVA pairwise comparisons of enzyme activity patterns between N addition rates in each depth showed significant differences, except that there was a similar pattern between N5 and N10 in the topsoil and between N10 and N15 in the subsoil (Table 3). In contrast, PERMANOVA pairwise comparisons between soil depths for each N addition rate showed significant differences (Table 3).

The SEMs implied by our data supported some of the hypothesized relationships among soil availability, pH, the composition of plant and microbial community and soil enzyme activity patterns (Fig. 4). N availability explained 84–86% of the variation in soil pH, 61–64% of the variation in plant community composition, and 61–74% of the variation in the composition of the soil microbial community through soil pH at both depths (Fig. 4). Surprisingly, the topsoil and subsoil model explained 82% and 87% of the variation in the enzyme activity patterns only by soil pH, respectively, but not by the other measured properties (Fig. 4). This suggests that soil pH played an important role in determining the soil enzyme activity patterns following N addition.

### Discussion

Our study represents an attempt to how N addition affects soil enzyme activity patterns through plant-soil system.

**Table 1** Results of one-way ANOVAs testing for effects of soil depth at each N addition level and N addition rate in each soil depth on pH,  $\text{NO}_3^-\text{-N}$  and  $\text{NH}_4^+\text{-N}$ , respectively, and mixed liner model results of effects of soil depth and N addition on pH,  $\text{NO}_3^-\text{-N}$  and  $\text{NH}_4^+\text{-N}$

Variables	Soil depth	Nitrogen addition rates ( $\text{N g m}^{-2} \text{ yr}^{-1}$ )				<i>F</i>		
		0	5	10	15	Nitrogen addition	Depth	Nitrogen addition $\times$ Depth
pH	topsoil	6.67 $\pm$ 0.04 (Aa)	6.04 $\pm$ 0.05 (Bb)	5.78 $\pm$ 0.03 (Ac)	5.56 $\pm$ 0.03 (Bd)	244.78***	23.6***	2.40 <sup>ns</sup>
	subsoil	6.70 $\pm$ 0.04 (Aa)	6.22 $\pm$ 0.04 (Ab)	5.89 $\pm$ 0.05 (Ac)	5.79 $\pm$ 0.06 (Ac)			
$\text{NO}_3^-\text{-N}$ ( $\text{mg.kg}^{-1}$ )	topsoil	3.24 $\pm$ 0.08 (Ac)	3.56 $\pm$ 0.05 (Ab)	3.99 $\pm$ 0.05 (Aa)	4.22 $\pm$ 0.04 (Aa)	105.91***	406.59***	10.29***
	subsoil	2.77 $\pm$ 0.06 (Bc)	3.05 $\pm$ 0.07 (Bb)	3.28 $\pm$ 0.04 (Ba)	3.28 $\pm$ 0.03 (Aa)			
$\text{NH}_4^+\text{-N}$ ( $\text{mg.kg}^{-1}$ )	topsoil	2.65 $\pm$ 0.12 (Ac)	3.07 $\pm$ 0.09 (Ab)	3.66 $\pm$ 0.04 (Aa)	3.75 $\pm$ 0.05 (Aa)	79.69***	77.19***	2.16 <sup>ns</sup>
	subsoil	2.35 $\pm$ 0.10 (Ac)	2.75 $\pm$ 0.05 (Bc)	3.10 $\pm$ 0.04 (Bb)	3.18 $\pm$ 0.05 (Ba)			

Values are means ( $\pm$  SE) of five replicates. The contrasting lowercase letters indicate significant differences among N addition rates, and the contrasting capital letters indicate significant differences between two soil depths. ns, no significant; \*\*\*,  $P < 0.001$

**Table 2** The results of mixed linear model of effects of N addition and soil depth on soil PLFAs and enzyme activities

Factors	Totalsoil PLFAs	Bacterial PLFAs	Fungal PLFAs	G <sup>+</sup> PLFAs	G <sup>-</sup> PLFAs	Actinomycetic PLFAs	F: B ratio	BG activity	UREA activity	AP activity	PHO activity
N addition	9.29 <sup>***</sup>	31.06 <sup>***</sup>	7.99 <sup>***</sup>	6.75 <sup>**</sup>	27.03 <sup>***</sup>	9.23 <sup>***</sup>	33.64 <sup>***</sup>	52.36 <sup>***</sup>	108.61 <sup>***</sup>	0.03 <sup>ns</sup>	6.07 <sup>**</sup>
Soil depth	118.26 <sup>***</sup>	75.90 <sup>***</sup>	39.77 <sup>***</sup>	11.30 <sup>**</sup>	53.59 <sup>***</sup>	12.93 <sup>***</sup>	0.62 <sup>ns</sup>	51.62 <sup>***</sup>	365.22 <sup>***</sup>	70.51 <sup>***</sup>	6.40 <sup>**</sup>
N addition × Soil depth	0.33 <sup>ns</sup>	0.17 <sup>ns</sup>	0.16 <sup>ns</sup>	0.29 <sup>ns</sup>	0.05 <sup>ns</sup>	0.05 <sup>ns</sup>	0.10 <sup>ns</sup>	1.49 <sup>ns</sup>	2.27 <sup>ns</sup>	0.47 <sup>ns</sup>	2.89 <sup>ns</sup>

The values are *F* values. G<sup>+</sup>, gram positive bacteria; G<sup>-</sup>, gram negative bacteria; F: B ratio, Fungi:Bacteria ratio; BG, β-1,4-D-glucosidase; UREA, urease; AP, acid phosphomonoesterase; PHO, phenol oxidase. ns, no significant; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001

We demonstrated soil pH rather than soil N availability and the composition of the plant and microbial communities was main predictor of changes in soil enzyme activity patterns in a Tibetan alpine meadow. Thus, our results provide empirical evidence of the importance of N enrichment-induced acidification for soil nutrient cycling.

#### Influence of N addition on the composition of plant and microbial community

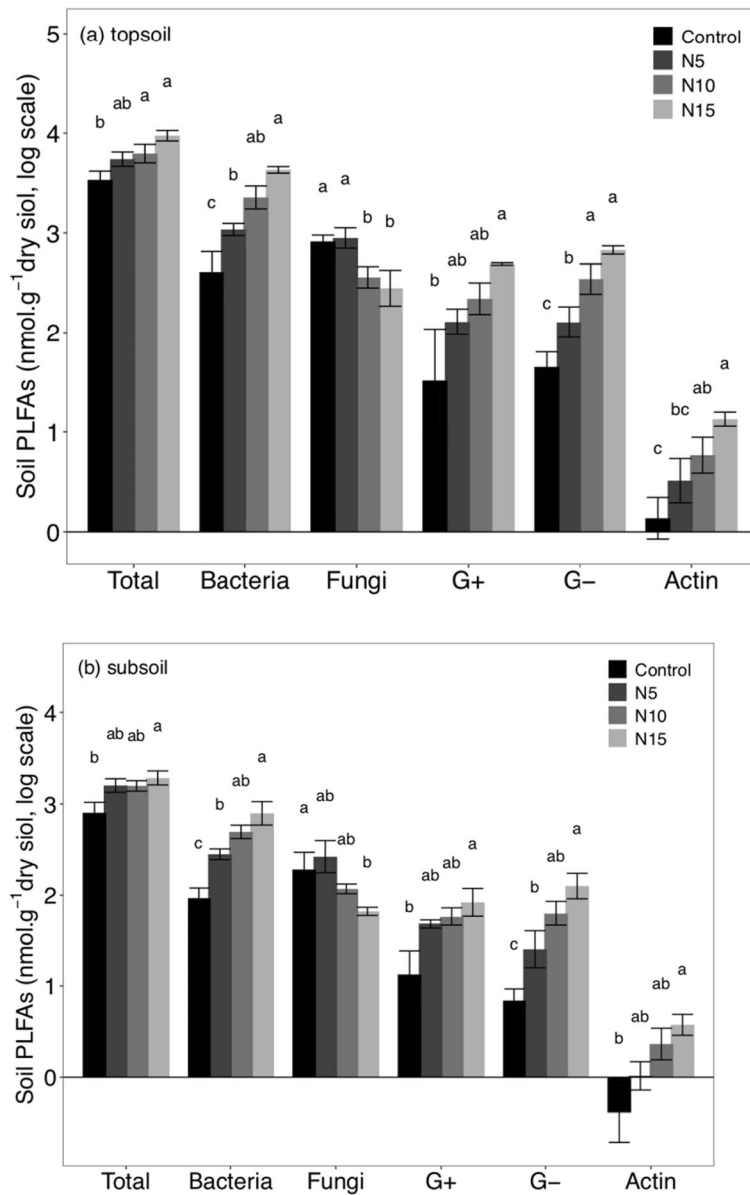
In support of our first hypothesis, we found N addition altered the plant community composition with sedge biomass increasing and forbs biomass decreasing (Fig. S3a), probably because sedges are better adapted to high N availability and have higher N-use efficiency than forbs, which are more sensitive to acidification (Bai et al. 2010; van den Berg et al. 2005; Yang et al. 2011). For total aboveground biomass, our results showed that higher biomass occurred in N5 and N10 compared to control (Fig. S3a), which is consistent with previous reports (LeBauer and Treseder 2008; Xi et al. 2014; Xia and Wan 2008).

In our third hypothesis, we speculated that belowground biomass would be higher in topsoil than in subsoil. Consistent with our hypothesis, belowground biomass declined with soil depth (Fig. S3b, c). Indeed, most roots are present in the top 20 cm of alpine meadow soils (Tao et al. 2006), probably because the soil particle size increases and soil nutrient decreases with soil depth (Li et al. 2011). It is interesting to note that the topsoil biomass showed the same trend as the total aboveground biomass with increasing N addition rates (Fig. S3a, b), confirming what has already been reported in alpine grasslands (Yang et al. 2009). In contrast, the subsoil biomass declined with increasing N addition rates (Fig. S3c), probably because sedges, whose biomass increased significantly with N addition, had

fibrous root systems, which cannot reach deeper soil layers. In contrast, the tap roots of forbs, such as *Anemone rivularis* and *Saussurea nigrescens*, can reach deeper soil layers than fibrous root systems. Moreover, the fibrous root system of sedges experiences rapid cycles of renewal and decay within the growing season, which also limits the access to deep soil layers (Vameralli et al. 2003).

In agreement with our first hypothesis, N addition increased soil PLFAs of gram-positive and gram-negative bacteria, actinomycetes and total PLFAs, but decreased fungal PLFAs and the F:B ratio, regardless of soil depth (Fig. 1a–c; Table 2), changing the composition of soil microbial community. Our findings confirmed the observations of Zhou et al. (2017), but contradicted the report of Canarini et al. (2016), who observed that fertilization did not significantly affect total soil PLFAs and the PLFAs of the microbial groups. Increased N availability can stimulate microbial growth (Zhou et al. 2017), but it can also inhibit microbial growth due to the decreased pH (Chen et al. 2015). Therefore, divergent responses of soil PLFAs to N addition can be the result of the relative role of soil N availability and soil pH. The decline in the F:B ratio coincided with the decline in soil pH (Fig. 4), confirming previous reports (Wei et al. 2013; Xiong et al. 2016; Zhou et al. 2017). This result arises likely because fungi are more sensitive to N addition and have lower nutrient demands than bacteria (Kjoller et al. 2012). Thus, N addition could cause asymmetric changes in the composition of soil microbial community (Zhao et al. 2014).

Consistent with our third hypothesis, we found all of the soil PLFAs declined with depth (Fig. 1d; Table 2), confirming previous reports (Heitkötter et al. 2017; Stone et al. 2014), probably due to the decline in energy sources from root exudation and to the decrease in nutrient availability with soil depth (Fierer et al. 2003;



**Fig. 1** Responses of soil microbial community to N addition (a–c) and to soil depth (d), respectively. Values are means  $\pm$  SE. Different letters above- and below- bars/dots denote significant differences along N addition rates and between soil depths. Actin, G<sup>+</sup>,

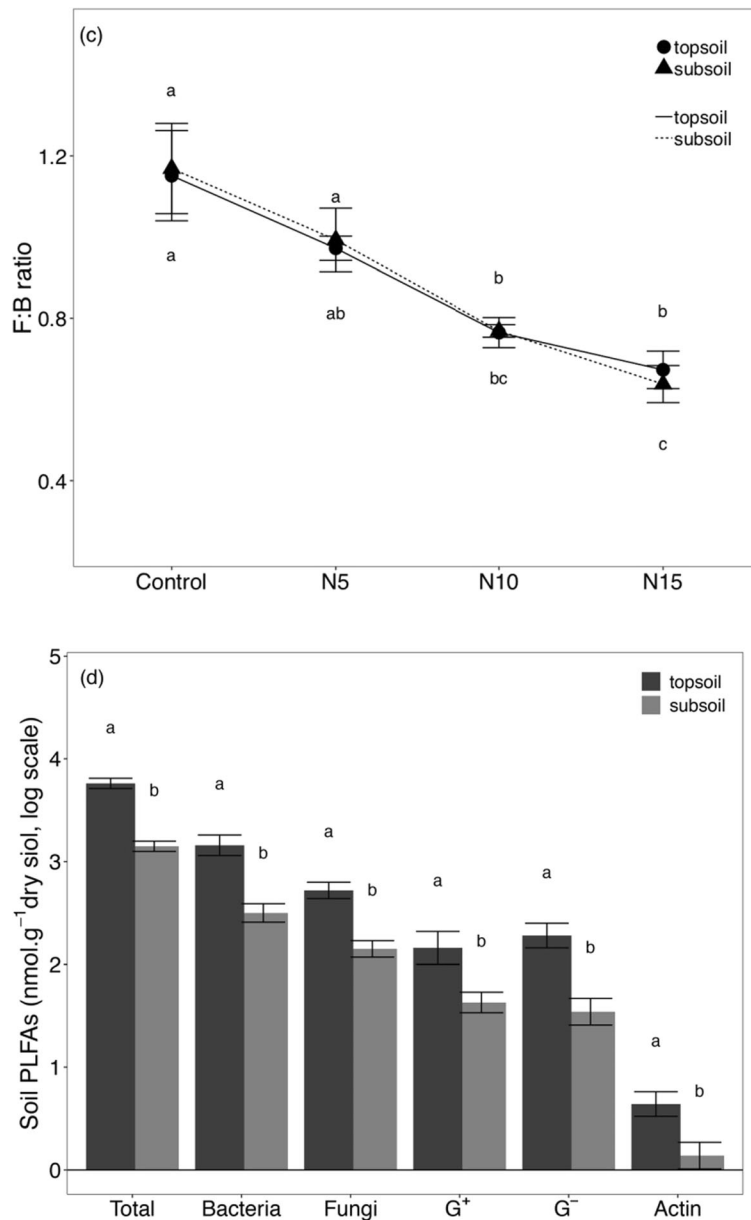
G<sup>-</sup>, Total and F:B represent actinomycetes, gram-positive bacteria, gram-negative bacteria, total microbes and fungi:bacteria ratio, respectively. Control, 0 g N m<sup>-2</sup> yr<sup>-1</sup>; N5, 5 g N m<sup>-2</sup> yr<sup>-1</sup>; N10, 10 g N m<sup>-2</sup> yr<sup>-1</sup>; N15, 15 g N m<sup>-2</sup> yr<sup>-1</sup>

Lamb et al. 2010). In contrast to observations of the F:B ratio in forest soil (Jackson et al. 2009; Stone et al. 2014), the F:B ratio did not change with soil depth in our alpine meadow soil (Fig. 1d; Table 2). Changes in the F:B ratio are due to changes in soil micro-environmental conditions (Hu et al. 2014; Schneckner et al. 2015), and these changes probably did not occur in the alpine meadow because the biomass of plant litter

is lower than in a forest (van der Heijden et al. 2008). It is possible that changes in the F:B ratio may occur in deeper soil layers than those we analyzed.

Response of soil enzyme activity patterns to N addition

In our second hypothesis, we speculated that N addition would increase soil hydrolytic enzyme activities



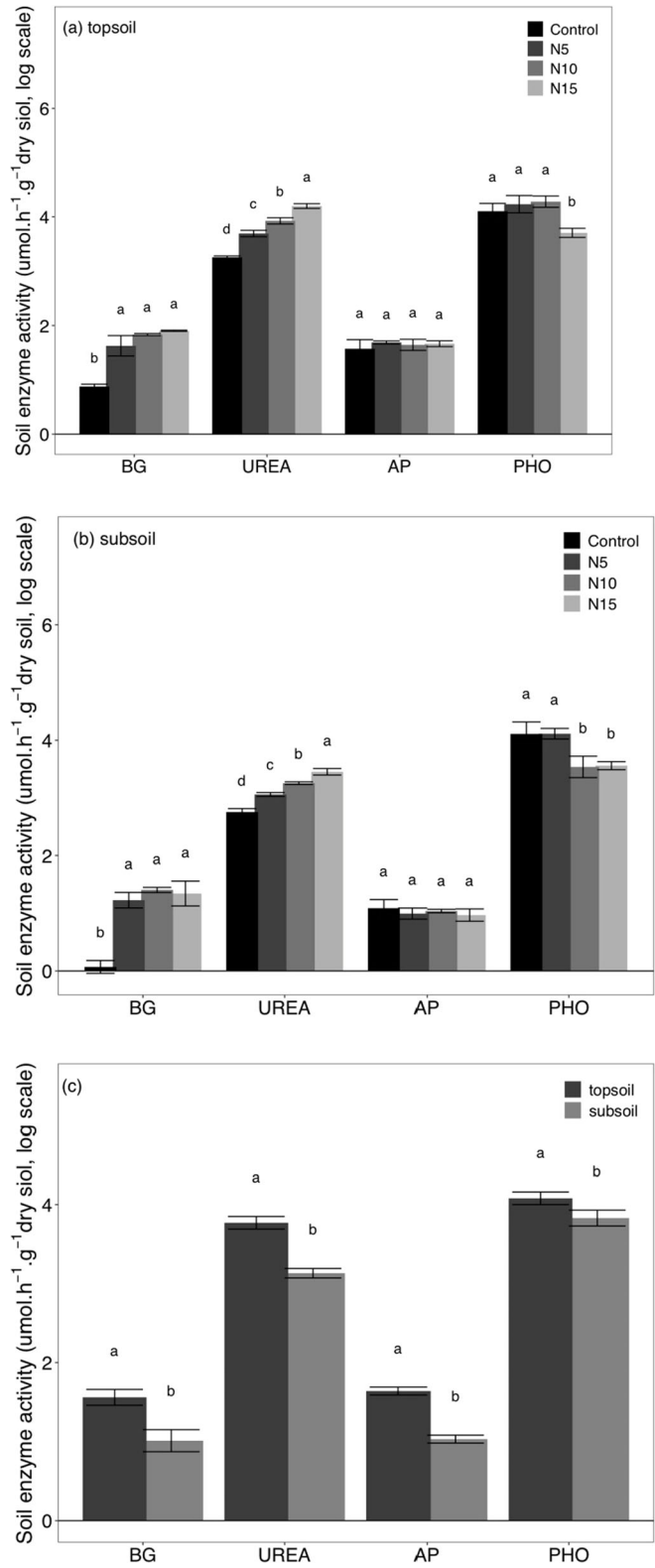
**Fig. 1** (continued)

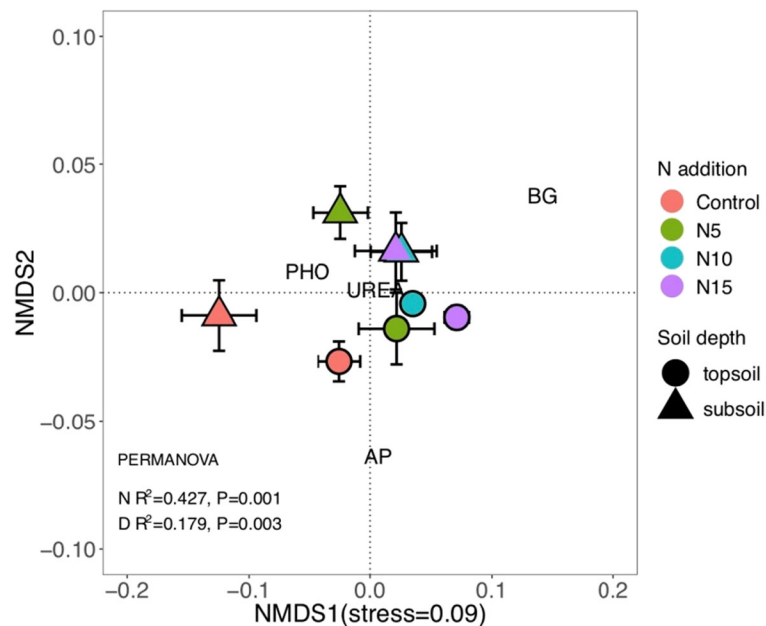
associated with C, N and P acquisitions but depress oxidative enzyme activities, and thus influence soil enzyme patterns. Our results partially supported this expectation. Both  $\beta$ -1,4-glucosidase and urease activities increased, whereas acid phosphomonoesterase activity did not change in both soil depths, and phenol oxidase activity decreased at higher N addition rates (Fig. 2a, b). According to the resource allocation theory, sufficient N supply can stimulate C-acquisition enzyme activities, leading to faster decomposition of cellulose-rich litter

(Allison et al. 2011; Carreiro et al. 2000). The increase in urease activity with increasing N addition rates confirmed the findings of Jian et al. (2016) and Shi et al. (2018), because soil microorganisms prefer low molecular weight organic N compounds, such as urea, to inorganic N (Dunn et al. 2006; Schimel and Weintraub 2003). The lack of a significant effect of N addition on acid phosphomonoesterase activity is in agreement with previous reports (Geisseler and Scow 2014; Jing et al. 2016). This result depends on the fact that this activity is



**Fig. 2** Responses of soil  $\beta$ -1,4-glucosidase (BG), urease (UREA), acid phosphomonoesterase (AP) and phenol oxidase (PHO) activities to N addition (**a, b**) and to soil depth (**c**), respectively. Values are means  $\pm$  SE. Different letters above bars denote significant differences along N addition rates





**Fig. 3** Non-metric Multidimensional Scaling (NMDS) ordination of soil enzyme activity patterns. NMDS plot of enzyme activity patterns calculated from distance matrices of standardized individual enzyme activities. Colors represent N addition rates (0 gN m<sup>-2</sup>yr.<sup>-1</sup>, pink; 5 gN m<sup>-2</sup>yr.<sup>-1</sup>, purple; 10 gN m<sup>-2</sup>yr.<sup>-1</sup>, green; 15 gN m<sup>-2</sup>yr.<sup>-1</sup>, blue), and different symbols represent

different soil depths (circles are topsoil; triangles are subsoil). The results of PERMANOVA show the effects of nitrogen addition (N) and soil depth (D), indicated by R<sup>2</sup> and P value. Error bars represent standard error. N, N addition; D, soil depth; BG, β-1,4-D-glucosidase activity; UREA, urease activity; AP, acid phosphomesterase activity; PHO, phenol oxidase activity

not related to soil N reactions but to P reactions, given that enzyme synthesis is repressed by inorganic P (Nannipieri et al. 2011; Nannipieri et al. 2012). The phenol oxidase activity decreased at higher N addition

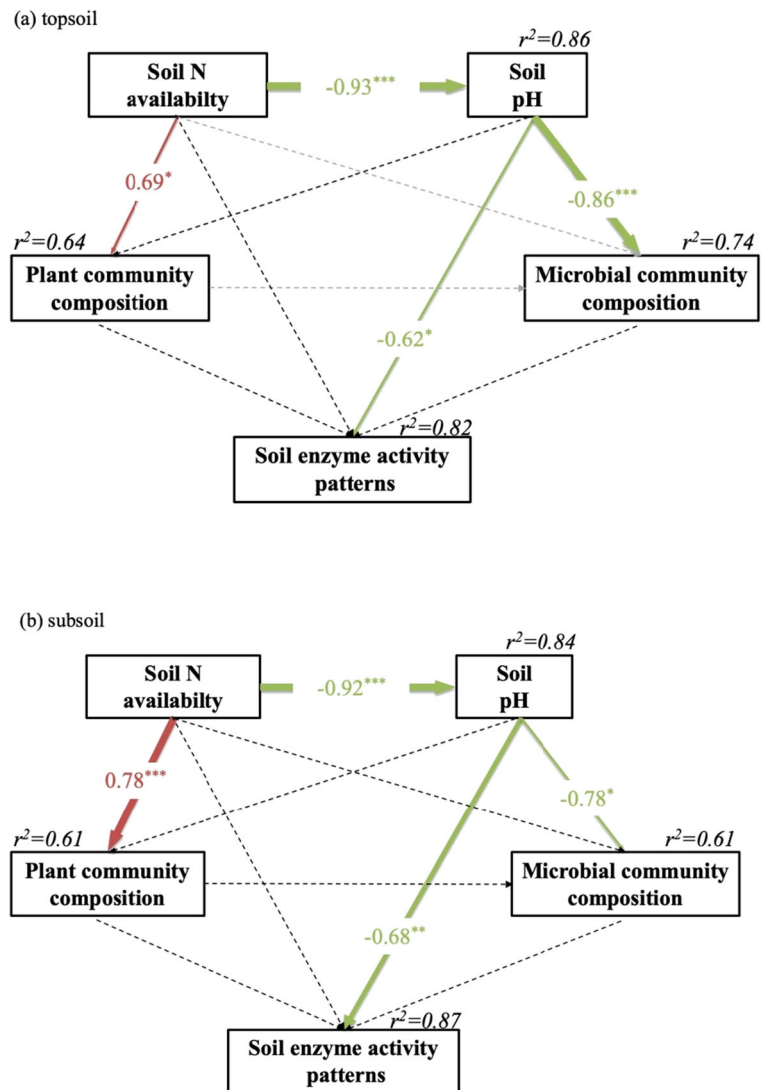
rates in our study, possibly because the growth of some fungal species producing ligninolytic enzymes is depressed (Fog 1988). It is worth noting that most soil enzyme activity patterns were significantly different

**Table 3** Pairwise comparisons of soil enzyme activity patterns within one soil depth among different N addition rates and between two soil depths at each N addition rate, respectively, using PERMANOVA with Euclidean distance matrixes

Factors		Control		N5		N10		N15	
		<i>r</i> <sup>2</sup>	<i>P</i>	<i>r</i> <sup>2</sup>	<i>P</i>	<i>r</i> <sup>2</sup>	<i>P</i>	<i>r</i> <sup>2</sup>	<i>P</i>
Control	topsoil	–	–	–	–	–	–	–	–
	subsoil	–	–	–	–	–	–	–	–
	topsoil vs subsoil	0.587	0.006	–	–	–	–	–	–
N5	topsoil	0.424	0.032	–	–	–	–	–	–
	subsoil	0.624	0.011	–	–	–	–	–	–
	topsoil vs subsoil	–	–	0.458	0.019	–	–	–	–
N10	topsoil	0.649	0.012	0.066	0.823	–	–	–	–
	subsoil	0.780	0.008	0.395	0.015	–	–	–	–
	topsoil vs subsoil	–	–	–	–	0.325	0.044	–	–
N15	topsoil	0.816	0.004	0.470	0.010	0.644	0.011	–	–
	subsoil	0.726	0.009	0.382	0.011	0.090	0.518	–	–
	topsoil vs subsoil	–	–	–	–	–	–	0.489	0.013

Control, 0 gN m<sup>-2</sup>yr.<sup>-1</sup>; N5, 5 g N m<sup>-2</sup> yr.<sup>-1</sup>; N10, 10 g N m<sup>-2</sup> yr.<sup>-1</sup>; N15, 15 g N m<sup>-2</sup> yr.<sup>-1</sup>

**Fig. 4** Structural equation model (SEM) of the relationships between soil N availability, pH, plant and microbial community and enzyme activity patterns following N addition in both soil depths. (a) represents topsoil, and model fit statistics for the model were  $\chi^2 = 3.799$ ,  $P = 0.150$ , RMSEA = 0.021. (b) represents subsoil, and model fit statistics for the model:  $\chi^2 = 1.203$ ,  $P = 0.273$ , RMSEA = 0.013. Gray dashed arrows show the paths removing from the conceptual model. Solid and dashed arrows indicate significant ( $P < 0.05$ ) and non-significant ( $P > 0.05$ ) relationships between the variable of onset arrow and the variable of terminated arrow, respectively. The thickness of the arrows reflects the degree of relationships, and red and green arrows indicate positive and negative relationships. Numbers at arrows are standardized path coefficients.  $r^2$  values indicate the variation of response variables explained by the model



among N addition rates at both soil depths (Fig. 3; Table 3), possibly resulting from the obvious responses of phenol oxidase and urease to N addition rates (Fig. 2).

In support of our third hypothesis, our study revealed all enzyme activities decreased with soil depth (Fig. 2d; Table 2), which is consistent with numerous reports (Heitkötter et al. 2017; Šnajdr et al. 2008; Stone et al. 2014; Venkatesan and Senthurpandian 2006). Overall, the contribution of plant roots and litter to subsoil C pools is small, causing the reduction in soil enzyme activities with soil depth (Hernández and Hobbie 2010). We also found that topsoil enzyme activity patterns differed from subsoil enzyme activity patterns (Fig. 3; Table 3) due to the decrease in the activity of each enzyme with soil depth, as discussed above. Future

studies that focus on detecting the expression of the relative enzyme-encoding genes are needed to better understand the underlying mechanisms of soil location-specific patterns of enzyme activity.

The role of abiotic and biotic factors in determining soil enzyme activity patterns following N addition

In contrast to our fourth hypothesis, we found that only soil pH but not N availability and the composition of plant and microbial communities affected enzyme activity patterns in both soil depths (Fig. 4). Manning et al. (2006) suggested that abiotic effects of N deposition on ecosystem functions could be more important than the changes in the composition of the plant community.

Indeed, our results showed the variation of plant community following N addition did not affect enzyme activity patterns, which may be partially explained by removing substantial litter owing to severe winter grazing in our study. Another explanation might be that impact of N addition on the overall composition of the plant community was relatively small in our study. Although soil pH affected the composition of microbial community (Fig. 4), as indicated by other studies (Delgado-Baquerizo et al. 2016; Sun et al. 2018), Surprisingly, we found no effect of microbial community composition on soil enzyme activity patterns, which was conflicted with many previous reports (Nannipieri et al. 2012; Riggs and Hobbie 2016). In fact, the relative importance of microbial community composition vs environmental factors in regulating enzyme expression remains unclear (Allison et al. 2007; Frossard et al. 2012). Notably, some recent reports showed variation in soil enzyme activities was better accounted for with soil physicochemical characteristics than microbial community composition (Bowles et al. 2014; Schneckner et al. 2014). In this study, N availability did not directly affect soil enzyme activity patterns, but indirectly through soil pH. The probable explanation is that the primary controlling factor for soil microbes shifts from resource availability (e.g. soil N availability) to soil acidification following N addition (Wei et al. 2013). Among our measured variables, soil pH significantly negatively affected enzyme activity patterns, indicating soil pH was a dominant controller of soil microbial enzyme activities following N addition in alpine meadow soil.

## Conclusion

In conclusion, our results showed that N addition shaped soil enzyme activity patterns mediated by increasing soil acidification but not the changes in the composition of the plant and microbial communities. Our study provides a new mechanistic understanding of how N addition affects soil enzyme activities through the plant-soil system in the Tibetan meadow. More research involving the detection and the expression of the enzyme-encoding genes and combined with the determination of the relative enzyme activities is needed to help expand our understanding of nutrient cycling in this high altitude ecosystem.

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