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Responses of soil microbial communities and functions associated with organic carbon mineralization to nitrogen addition in a Tibetan grassland

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ABSTRACT

Alpine grasslands with a high soil organic carbon (SOC) storage on the Tibetan Plateau are experiencing rapid climate warming and anthropogenic nitrogen (N) deposition; this is expected to substantially increase the soil N availability, which may impact carbon (C) cycling. However, little is known regarding how N enrichment influences soil microbial communities and functions relative to C cycling in this region. We conducted a 4-year field experiment on an alpine grassland to evaluate the effects of four different rates of N addition (0, 25, 50, and 100 kg N ha^{-1} $year^{-1}$) on the abundance and community structure (phospholipid fatty acids, PLFAs) of microbes, enzyme activities, and community level physiological profiles (CLPP) in soil. We found that N addition increased the microbial biomass C (MBC) and N (MBN), along with an increased abundance of bacterial PLFAs, especially Gram-negative bacterial PLFAs, with a decreasing ratio of Gram-positive to Gram-negative bacteria. The N addition also stimulated the growth of fungi, especially arbuscular mycorrhizal fungi, reducing the ratio of fungi to bacteria. Microbial functional diversity and activity of enzymes involved in C cycling (β-1,4-glucosidase and phenol oxidase) and N cycling (β-1,4-*N*-acetyl-glucosaminidase and leucine aminopeptidase) increased after N addition, resulting in a loss of SOC. A meta-analysis showed that the soil C/N ratio was a key factor in the response of oxidase activity to N amendment, suggesting that the responses of soil microbial functions, which are linked to C turnover relative to N input, primarily depended upon the soil C/N ratio. Overall, our findings highlight that N addition has a positive influence on microbial communities and their associated functions, which may reduce soil C storage in alpine grasslands under global change scenarios.

Key Words: alpine grassland, C cycling, C turnover, community level physiological profiles (CLPP), enzyme activity, microbial community composition, microbial function, N input

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INTRODUCTION

Reactive nitrogen (N) addition to terrestrial ecosystems through fertilization or atmospheric deposition has increased by three- to five-fold over the past century and is now considered to be one of the most widespread drivers of global change (Galloway *et al*., 2008; Fowler *et al*., 2013). A growing body of evidence suggests that an elevated N input increases the production of plant biomass, which then benefits the carbon (C) uptake, particularly in N-limited natural terrestrial ecosystems (Xia and Wan, 2008; Wieder *et al*., 2015). In many cases, N enrichment also causes a change in the plant community composition and loss of plant diversity due to size asymmetry and subsequent light competition (DeMalach *et al*., 2017). Nevertheless, how the projected global change will influence soil microbial communities remains unclear.

Microbes play a prominent role in soil nutrient cycling, organic matter (SOM) decomposition, and other ecosystem functions (Fierer *et al*., 2009). The responses of microbes to increasing N deposition may have profound consequences for global C cycling (García-Palacios *et al*., 2015). Recently, a few meta-analysis attempts have been made to assess how microbial biomass would respond to N addition, and found that N addition tends to suppress microbial biomass (Zhang *et al*., 2018), with variation in the effects among different ecosystems (Treseder, 2008). For example, Zhou *et al*. (2017) suggested that N addition could suppress microbial biomass in temperate grasslands and forests. In contrast, some studies have shown that N addition has a positive effect on microbial biomass, particularly in lower montane tropical forests (Cusack *et al*., 2011). Microbial community composition is also sensitive to N enrichment. In general, N addition reduces the abundance of fungi, particularly arbuscular mycorrhizal fungi (Wei *et al*., 2013), and the ratio of fungi to bacteria, as a result of the relatively low

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nutrient demands of fungi compared to bacteria (Fierer *et al*., 2007; Gutknecht *et al*., 2012). The N-induced shifts in plant biomass and/or composition may alter the C/N ratio of the substrate, which stimulates the growth of bacteria more than fungi (De Deyn *et al*., 2008). Soil acidification caused by N addition is likely to enhance the ratio of fungi to bacteria (Chen *et al*., 2016). There are many interacting factors at play during N deposition and C cycling, each with a unique and variable impact on the affected microbes within an ecosystem.

Shifts in the abundance and composition of a soil microbial community are generally accompanied by changes in its functions (Cusack *et al*., 2011). Extracellular enzymes play a key role in the decomposition of SOM (Sinsabaugh *et al*., 2008) and may be used to assess microbial nutrient demands and predict their functional feedbacks in response to global change (Xiao *et al*., 2018). Nitrogen addition tends to increase glycosidase activity with application rate, particularly when applied as an organic fertilizer (Zeglin *et al*., 2007; Chen *et al*., 2017), but also has the potential to suppress activity of typical lignin-degrading enzymes (Frey *et al*., 2004; Xiao *et al*., 2018). The effects of increased N availability on the activity of N acquisition enzymes are inconsistent (Chen *et al*., 2018). For instance, Wang *et al*. (2015) observed increases in the activity of β-1,4-N-acetyl-glucosaminidase (NAG) in temperate grasslands under N amendment, while Fatemi *et al*. (2016) recorded decreases in the NAG activity in hardwood and softwood forests that were subjected to chronic N enrichment. However, N deposition caused no apparent impacts on the activity of leucine aminopeptidase (LAP) and NAG in tropical, subtropical, and temperate forest ecosystems (Jing *et al*., 2017). These different experimental results highlight that uncertainties remain in our understanding of the responses of certain enzymes to N addition and the responses could be ecosystem- and site-specific.

The Tibetan Plateau is the world's highest (4 000 m above sea level on average) and largest (2.5×10^6 km^2) plateau, comprised of more than 60% alpine grassland that contains 4.4 Pg of C in the top 30 cm layer of soil (Yang *et al*., 2009). Thus, it has great potential to shift between acting as a C sink and a C source under N enrichment. This region has experienced rapid climate warming at a rate of 0.2 °C per decade over the past 50 years (Chen *et al*., 2013), which is assumed to have increased the nutrient availability in soil by enhancing SOM mineralization. Atmospheric N deposition is obvious on the Tibetan Plateau, particularly in the northeastern region, ranging from 4 to 13.8 kg N ha^{-1} year^{-1} (Lü and Tian, 2007; Fang *et al*., 2012). Extensive evidence shows that N addition can affect the diversity and/or composition of the aboveground plant community in these alpine grasslands (Fang *et al*., 2012; Luo *et al*., 2019). Although the microbial community is as important as the plant community for ecosystem functioning (Fierer *et al*., 2009), the responses of soil microbial communities and functions relative to C cycling in the context of increasing N availability remain unclear in this alpine ecosystem. Here, a 4-year field experiment, with four N addition rates, was conducted on an alpine grassland of the Tibetan Plateau. The objectives of this study were to: i) evaluate the influence of N addition on soil microbial community composition, enzyme activity, and functional diversity and ii) elucidate the factors affecting the microbial functions that are associated with organic C mineralization in response to N addition, through a synthetic analysis of literature data.

MATERIALS AND METHODS

Study site and experimental design

This study was conducted at the Haibei Alpine Grassland Ecosystem Research Station on the northeastern Tibetan Plateau in Qinghai Province, China (37°37′ N, 101°12′ E, 3 220 m above sea level). This area has a continental monsoon climate with a mean annual temperature of −1.2 °C and a mean annual precipitation of 489 mm, 80% of which is concentrated within the plant growing season from May to September. The soil is classified as a Gelic Cambisol (WRB, 1998). The alpine grassland is dominated by perennial grasses, sedges, legumes, and forbs, including *Kobresia humilis, Stipa aliena, Festuca ovina, Elymus nutans, Poa pratensis, Carex scabrirostris, Scirpus distigmaticus, Gentiana straminea, Gentiana farreri, Leontopodium nanum, Blysmus sinocompressus*, and *Potentilla nivea*.

The N enrichment experiment was initiated in 2011 by fencing off an area of 80 m × 60 m to exclude grazers. Four N addition rates as treatments were designed: a control without N addition (CK), 25 kg N ha^{-1} year^{-1} (N25), 50 kg N ha^{-1} year^{-1} (N50), and 100 kg N ha^{-1} year^{-1} (N100). Sixteen 6 m × 6 m plots were arranged in a randomized block design. Blocks were separated by a 2-m-wide buffer strip, with plots within each block separated by a 1-m-wide buffer strip to minimize disturbance from neighboring plots. The N fertilizer in the form of urea was applied to the plots in three equal monthly applications per year at the beginning of June, July, and August.

Plant and soil sampling

The aboveground vascular plant biomass was sampled from a 0.5 m × 0.5 m quadrat in each plot in mid-August 2015, when plant growth had peaked. All living vascular plants were clipped at the soil surface, sorted into species, oven dried at 65 °C for 48 h, and weighed. The belowground plant biomass was estimated by extracting roots from three soil cores collected using a 7-cm diameter soil auger, with the root material being dried and weighed as before.

After harvesting the plant biomass, five soil cores (5-cm diameter, 0–10 cm depth) were collected randomly from each plot and combined to form a single composite sample, from which the root and stone material was removed. The soil sample was passed through a 2-mm mesh sieve and thoroughly mixed. A subsample of the fresh soil was stored at 4 °C, and the rest was air dried for the analysis of the soil properties.

Soil physicochemical property analysis

Soil samples were dried at 105 °C for 24 h to measure the moisture content. The soil pH was determined using a glass electrode in a 1:2.5 soil/water suspension. The contents of the soil organic C (SOC) and total N (TN) were analyzed using the wet oxidation-redox titration and micro-Kjeldahl methods, respectively (Lu, 2000). The available phosphorous (AP) was extracted using $NaHCO_3$ and then analyzed using the molybdenum blue method (Lu, 2000). For dissolved organic C (DOC) extraction, fresh moist soil samples in deionized water at a soil/solution ratio of 1:5 were shaken for 30 min, centrifuged for 10 min at 10 000 r min^{-1}, and filtered through a 0.45-μm polyethersulfone membrane filter. Then, the organic C content was measured using a TOC analyzer (Multi N/C 3100, Analytik Jena, Germany). Ammonium (NH_4^+), nitrate (NO_3^-), and dissolved N (DN) were extracted from fresh moist soil using a 2 mol L^{-1} KCl solution at a 1:5 soil/solution ratio, and their contents were measured with a continuous-flow autoanalyzer (San^{++}, Skalar, the Netherlands). Then, the dissolved organic N (DON) content was calculated:

$$\mathrm{DON} = \mathrm{DN} - \mathrm{NH_4^+\text{-}N} - \mathrm{NO_3^-\text{-}N} \quad (1)$$

Soil microbial biomass and community composition measurements

Microbial biomass C (MBC) and N (MBN) were determined using a chloroform fumigation-extraction method (Vance *et al*., 1987); their contents were calculated as the difference in the organic C and TN between fumigated and nonfumigated samples divided by a factor of 0.45 (Vance *et al*., 1987) and 0.54 (Brookes *et al*., 1985), respectively . The organic C and TN in the extracts were analyzed with a Multi N/C 3100-TOC/TN Analyzer (Analytik Jena, Germany).

Soil microbial community composition was evaluated using phospholipid fatty acid (PLFA) (Bossio and Scow, 1998). The total lipids were extracted from 1 g of freeze-dried soil using a chloroform:methanol:citrate buffer (1:2:0.8, volume/volume/volume), and then the extracted fatty acid methyl esters were analyzed with a gas chromatograph (Agilent 7890, Agilent Technologies Inc., USA) and a MIDI Sherlock Microbial Identification System (MIDI Inc., USA). The content of each PLFA (nmol g^{-1}) was calculated based on the 19:0 internal standard content, where PLFAs are representative markers of specific groups: Gram-positive (G^+) bacteria (iso- and anteiso-branched PLFAs) (Zelles, 1999); Gram-negative (G^-) bacteria (cyclopropyl bacteria and unsaturated PLFAs) (Zelles, 1999); general bacteria (12:0, 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0) (Frostegård and Bååth, 1996); arbuscular mycorrhizal fungi (AMF, 16:1ω5c) (Olsson, 1999); and saprotrophic fungi (18:1ω9c, 18:2ω6c, and 18:3ω6c) (Frostegård and Bååth, 1996). The ratio of fungal to bacterial PLFAs (F/B) was calculated using the respective PLFAs, and the ratio of G^+ bacterial to G^- bacterial PLFAs (G^+/G^-) was evaluated based on the PLFA biomass of each group.

Soil enzyme activity assays

The measurements of β-1,4-glucosidase (BG), NAG, and LAP activities were performed on the basis of *p*-nitrophenol (PNP) release following the cleavage of enzyme-specific synthetic substrates (*p*-nitrophenyl-β-D-glucopyranoside for BG, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide for NAG, and L-leucine-*p*-nitroanilide for LAP), according to the methods of Tabatabai (1994) and Sinsabaugh *et al*. (2002). Briefly, 1 g of fresh soil (on the oven-dry basis) was mixed with 5 mmol L^{-1} substrate and 50 mmol L^{-1} acetate buffer (pH 5) and incubated in the dark at 37 °C for 1 h. Then, 0.5 mol L^{-1} $CaCl_2$ and 0.1 mol L^{-1} tris(hydroxymethyl)aminomethane (pH 12) were added to terminate the reaction. A control was prepared for each sample by mixing the buffer with either a soil or substrate solution (the same as below). The resulting products were filtered through Whatman No. 2V filter paper and determined with a UV-VIS spectrophotometer (UV-1800, Mapada Instruments Co., China) at 410 nm. The activities of BG, NAG, and LAP were expressed in μmol PNP g^{-1} h^{-1}.

The activity of phenol oxidase (POX) was measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Sinsabaugh *et al*., 2002). Here, 1 g of fresh soil (on the oven-dry basis) with 5 mmol L^{-1} L-DOPA plus 50 mmol L^{-1} acetate buffer (pH 5) was incubated in the dark at 25 °C for 1 h. Then, the reaction was terminated by immediate centrifugation at 5 000 r min^{-1} for 5 min at 4 °C, and the absorbance of the supernatant was measured at 460 nm. The activity of POX was expressed in μmol 2,3-dihydroindole-5,6-quinone-2-carboxylate (DICQ) g^{-1} h^{-1}.

Soil community level physiological profile (CLPP) analysis

The CLPPs of the soil microbial communities were determined using BIOLOG 96-well Eco-Microplates (Biolog Inc., USA), with three replicates of 31 different sources of

organic C in each microplate, which included carbohydrates, carboxylic acids, polymers, phenolic acids, amino acids, and amines. Microbes were extracted from 10 g of soil (on the oven-dry basis), to which 90 mL of sterile (0.85%, weight/volume) saline solution was added (Classen *et al*., 2003). The mixture was shaken for 30 min at 70 r min^{-1} and left to stand for 30 min. Then, 1 mL of the supernatant was diluted to 10 mL, twice, using sterile saline solution, before each sample (150 μL) was inoculated into each well. The plates were incubated at 25 °C in the dark, and the color development in each well was recorded as the optical density (OD) at 590 nm in 24 h intervals over a period of 168 h, using a plate reader (Thermo Scientific Multiskan MK3, USA). Plate readings after 96 h of incubation were used to calculate the CLPPs. Microbial functional diversity of organic C metabolism, recorded as the average well color development (AWCD), was calculated:

$$\text{AWCD} = \sum(C_i - R)/n \quad (2)$$

where C_i is the absorption value of the sample substrate well i, R is the control absorption well, and n is the number of plates ($n = 31$).

Several indexes were used to analyze the diversity and richness of soil microbial communities, including Shannon-Wiener index (H),

$$H = -\sum P_i \times \ln P_i \quad (3)$$

where P_i is the ratio of the relative absorption of the well i divided by the sum of all the relative color development of the plates, McIntosh index (U),

$$U = \sqrt{\sum(C_i - R)^2} \quad (4)$$

and richness index (S), $i.e.$, the number of wells with $C_i - R > 0.25$ (Gomez *et al*., 2006).

Meta-analysis of soil cellulase and oxidase activities

We derived cellulase and oxidase activity data from international, peer-reviewed journal articles. Data collection was limited to the results where the i) cellulase or oxidase activity and SOC content were reported in the control and N-amended treatments and ii) records of the N rates, TN, and C/N ratio were shown. If the results were presented graphically, data were extracted from figures using Engauge Digitizer software. Cellulase denotes a group of hydrolytic enzymes that degrade cellulose and hemicellulose, including BG, β-1,4-xylosidase, and β-D-cellobiosidase. Oxidative enzymes produced by microbes decompose recalcitrant substrates ($i.e.$, lignin). The most frequently assayed oxidases include peroxidase, phenol oxidase, and polyphenol oxidase. If a paper reported the activities of multiple kinds of cellulase or oxidase, their sum values were used as an overall response of the respective enzyme activity. In total, 58, 80, and 75 paired measurements for the activities of cellulase, oxidase, and SOC, respectively, were selected from 20 published papers (Table SI, see Supplementary Material for Table SI).

Statistical analysis

Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity (Levene's test) of variance prior to testing for treatment differences using one-way analysis of variance (ANOVA), followed by a least significant difference test (LSD) at $P < 0.05$. The response ratio (RR) of cellulase activity, oxidase activity, or SOC content to N addition was calculated as the ln-transformed ratio of the value in the N-amended treatment to that of the control, based on the literature data. We used linear regression analysis to explore the relationships between soil MBC (or MBN) and plant aboveground biomass (AGB) or DON and between the RR of cellulase/oxidase activity or SOC and soil properties or N rates. Statistical analyses were conducted in SPSS 19.0 software package for Windows (SPSS Inc., USA).

R software (version 3.3.3) was utilized to conduct the following analysis using the vegan package. To determine the effects of N addition on the soil microbial community composition, contents of 29 individual PLFAs of soil samples were subjected to non-metric multidimensional scaling (NMDS) analysis based on the Bray-Curtis dissimilarity matrix. We evaluated the relationships between the soil microbial community composition, enzyme activities, and environmental parameters (soil properties and plant biomass) using redundancy analysis (RDA) combined with Pearson's correlation analysis. A forward selection of environmental factors, with a Monte Carlo permutation test, was performed to test their impact on enzyme activity, and the position and length of the arrows in the figures indicate the direction and strength, respectively, of the effect of an environmental parameter on enzyme activity.

RESULTS

Soil physicochemical properties and plant biomass

The contents of soil NO_3^- and DON in the 0–10 cm layer increased with the added N rates, while the content of soil NH_4^+ only exhibited a significant increase in the N50 treatment (Table I). In contrast, the contents of SOC and soil TN significantly decreased following N addition, while soil pH and contents of AP and DOC did not differ between the CK and N-amended treatments. The plant AGB increased with N addition *via* increases in the biomass of grasses and sedges; in contrast, the plant belowground biomass was not affected by N addition ($P > 0.05$) (Fig. S1, see

Supplementary Material for Fig. S1).

Soil microbial community composition

Soil MBC and MBN increased with N addition ($P < 0.01$) (Table I) and showed linear relationships with plant AGB and soil DON (Fig. 1). The NMDS of the PLFA profiles showed that soil samples under N addition harbored a microbial community that was distinct from that in the CK plot (Fig. 2). In detail, the abundance of total bacterial, G^- bacterial, and fungal PLFAs increased with N rates ($P < 0.05$) (Fig. 3). However, no statistically significant differences were found in the G^+ bacterial PLFAs between N-amended and CK soils. In addition, N addition enhanced the AMF abundance, but reduced the F/B ratio, and the ratio of G^+/G^- bacteria decreased by 14.5%–43.4% in the N-amended treatments ($P < 0.01$).

Pearson's correlation analysis showed that the abundances of soil fungal, total bacterial, and G^- bacterial PLFAs were positively correlated with soil NO_3^- ($P < 0.05$) and DON ($P < 0.01$), while soil AMF biomass was only positively correlated with soil DON ($P = 0.014$) (Table SII,

TABLE I

Effects of different rates of N amendment on soil properties[a)]

Treatment[b)]	pH	SOC	TN	C/N ratio	DOC
		g C kg^{-1}	g N kg^{-1}		mg C kg^{-1}
CK	7.21 ± 0.10[c)] ab[d)]	66.7 ± 1.0a	6.7 ± 0.1a	9.9 ± 0.1a	88.7 ± 1.1a
N25	7.45 ± 0.02a	63.4 ± 0.8b	6.5 ± 0.1ab	9.8 ± 0.1a	86.1 ± 7.8a
N50	7.31 ± 0.01a	63.6 ± 0.5b	6.4 ± 0.1b	9.9 ± 0.1a	80.8 ± 1.3a
N100	7.11 ± 0.18a	59.9 ± 0.9c	6.1 ± 0.3c	9.8 ± 0.1a	96.7 ± 8.4a

Treatment	NH_4^+	NO_3^-	DON	AP	MBC	MBN
	mg N kg^{-1}			mg P kg^{-1}	mg C kg^{-1}	mg N kg^{-1}
CK	3.7 ± 0.1b	22.3 ± 1.1b	33.8 ± 0.6c	7.0 ± 0.4a	1 113 ± 83c	124 ± 8b
N25	3.7 ± 0.1b	31.5 ± 1.8ab	35.4 ± 2.9bc	6.7 ± 0.3a	1 194 ± 83bc	134 ± 8b
N50	4.1 ± 0.1a	33.9 ± 2.8a	40.6 ± 1.7ab	6.9 ± 0.5a	1 377 ± 50ab	162 ± 1a
N100	3.5 ± 0.1b	40.5 ± 5.1a	44.6 ± 1.5a	6.6 ± 0.5a	1 502 ± 82a	181 ± 7a

[a)] SOC = soil organic C; TN = total N; C/N ratio = ratio of SOC to TN; DOC = dissolved organic C; DON = dissolved organic N; AP = available P; MBC = microbial biomass C; MBN = microbial biomass N.
[b)] CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}.
[c)] Means ± standard errors ($n = 4$).
[d)] Different letters within the same column indicate significant differences among treatments at $P < 0.05$.

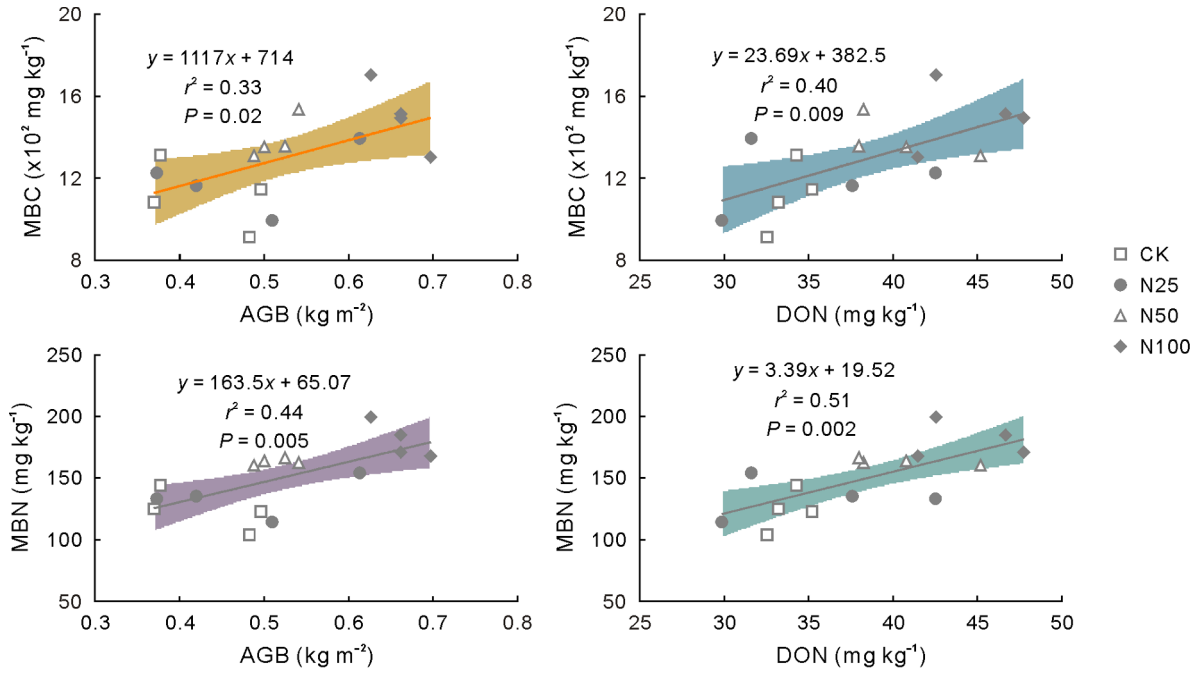

Fig. 1 Relationships of soil microbial biomass C (MBC) and microbial biomass N (MBN) with plant aboveground biomass (AGB) and soil dissolved organic N (DON) in the treatments of different N addion rates. Shaded sections indicate the 95% confidence intervals of the regression models. CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}.

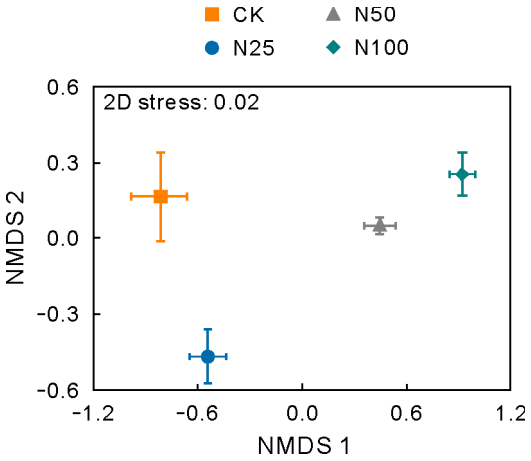

Fig. 2 Non-metric multidimensional scaling (NMDS) plot of the soil microbial community composition based on the contents of individual phospholipid fatty acids (PLFAs) in the treatments of different N addion rates. CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}.

see Supplementary Material for Table SII). Significant negative relationships were observed for soil F/B ratio with soil DON and plant AGB ($P < 0.05$). Meanwhile, soil G^+/G^- ratio was positively correlated with soil TN ($P = 0.005$) and SOC ($P = 0.007$), but negatively correlated with soil NO_3^- and DON and plant AGB ($P < 0.05$).

Soil enzyme activities

Soil enzyme activities generally increased with N addition compared to the CK (Fig. 4). For BG activity, a significant increase was only found for the N100 treatment ($P < 0.05$). The increases in POX activity ranged from 3.7% to 25.0% ($P < 0.001$). For N acquisition enzymes, NAG activity showed increasing trends with N rates ($P < 0.01$), while a significant increase in LAP activity was found only in the N50 treatment ($P < 0.05$).

Redundancy analysis (Fig. 5) revealed that soil enzyme

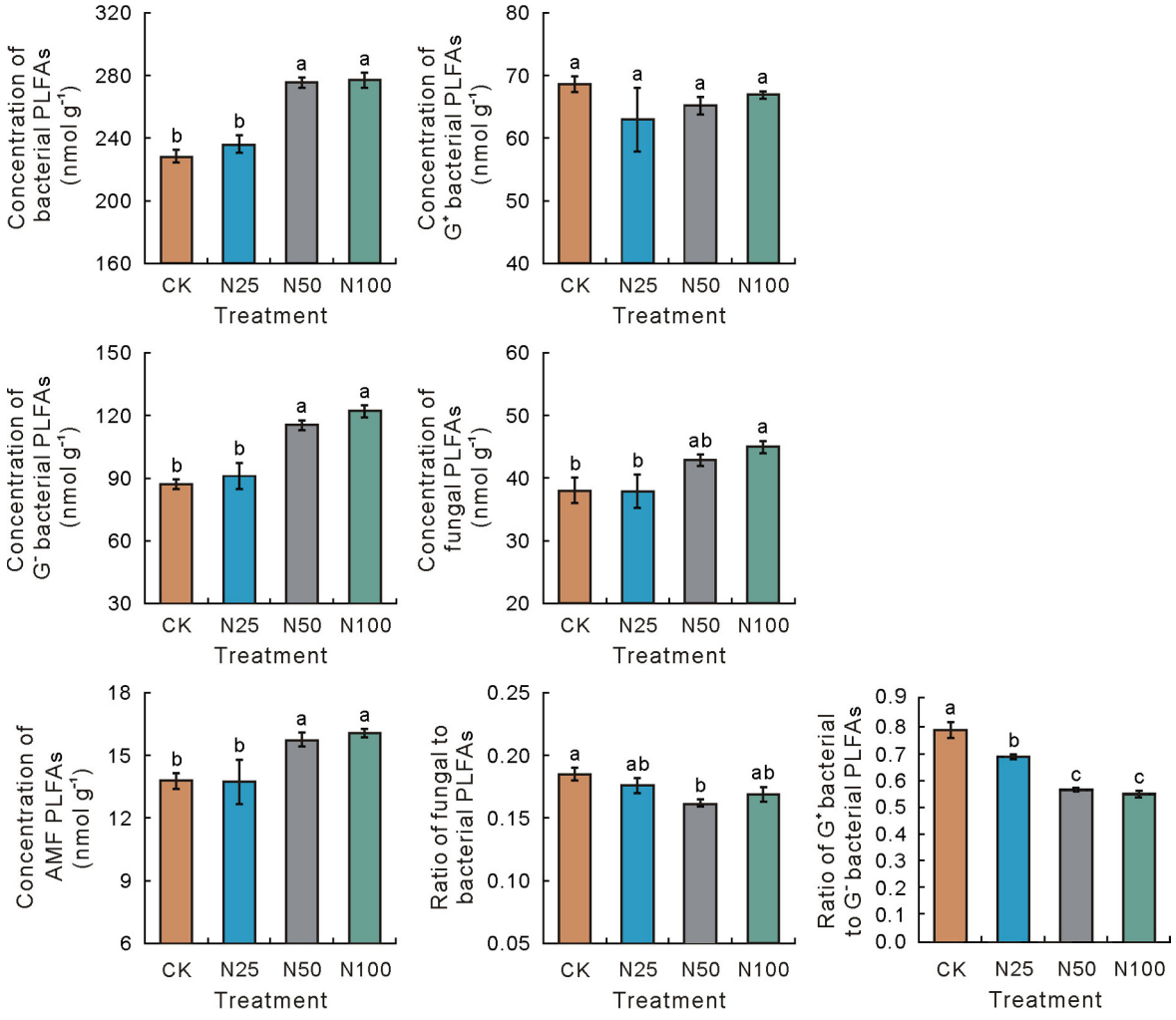

Fig. 3 Effect of N addition on the abundance and ratios of soil microbial groups, as indicated by phospholipid fatty acids (PLFAs), in the treatments of different N addion rates. Error bars are standard errors of the means ($n = 4$). Bars with different letters indicate significant differences among treatments at $P < 0.05$. CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}; G^+ = Gram-positive; G^- = Gram-negative; AMF = arbuscular mycorrhizal fungal.

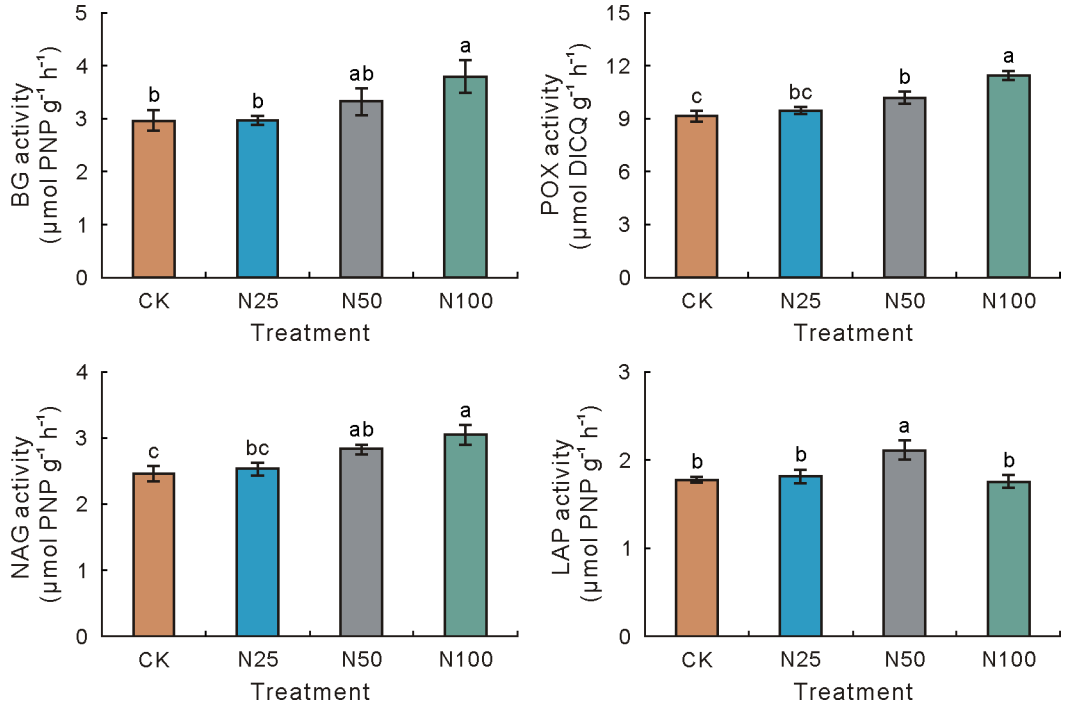

Fig. 4 Activities of soil β-1,4-glucosidase (BG), phenol oxidase (POX), β-1,4-*N*-acetyl-glucosaminidase (NAG), and leucine aminopeptidase (LAP) in the treatments of different N rates. Error bars are standard errors of the means ($n = 4$). Bars with different letters indicate significant differences among treatments at $P < 0.05$. CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}; PNP = *p*-nitrophenol; DICQ = 2,3-dihydroindole-5,6-quinone-2-carboxylate.

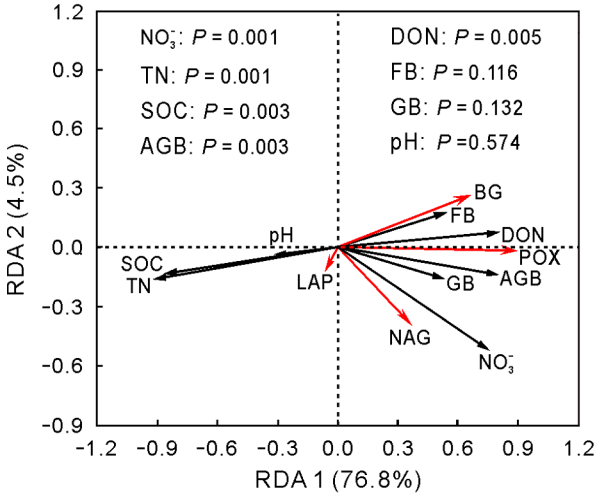

Fig. 5 Redundancy analysis (RDA) between soil enzyme activities and soil physicochemical properties or plant biomass. BG = β-1,4-glucosidase; POX = phenol oxidase; NAG = β-1,4-*N*-acetyl-glucosaminidase; LAP = leucine aminopeptidase; SOC = soil organic C; TN = total N; DON = dissolved organic N; AGB = aboveground biomass; GB = grass biomass; FB = forb biomass.

activities were correlated with SOC, soil TN, NO_3^-, and DON, and plant AGB. More specifically, the activities of soil BG and POX were positively correlated with soil DON and plant AGB, and inversely correlated with soil TN and SOC (Table SIII, see Supplementary Material for Table SIII). Soil NAG activity showed a positive correlation with soil NO_3^- ($P = 0.001$), plant AGB ($P = 0.005$), and grass biomass ($P = 0.018$), but had a negative relationship with soil TN ($P = 0.039$).

From the synthesized literature data, we found that the responses of oxidase activity to N addition were positively correlated with soil C/N ratio ($r^2 = 0.303$, $P < 0.001$) (Fig. 6) and inversely correlated with N rates ($r^2 = 0.050$, $P = 0.046$) (Fig. S2, see Supplementary Material for Fig. S2), but not correlated with SOC ($r^2 = 0.026$) or soil TN ($r^2 = 0.027$). Moreover, a mean soil C/N ratio of 12.4 (8.9–17.3, 95% confidence interval) was found to be the break-point threshold between negative and positive responses of oxidase activity to N amendment. The responses of the cellulase activity and SOC content to N addition were positively correlated with N rates ($r^2 = 0.376$ and 0.060, $P < 0.001$ and $= 0.034$, respectively), but not correlated with SOC ($r^2 = 0.046$ and 0.021, respectively), soil TN ($r^2 = 0.001$ and 0.012, respectively), or soil C/N ratio ($r^2 = 0.056$ and 0.022, respectively) (Figs. S3 and S4, see Supplementary Material for Figs. S3 and S4).

Organic C utilization profiles of soil microbial communities

The diversity and richness of soil microbial communities increased with N addition (Fig. 7). The AWCD and U values increased with N rates, with significant effects found in the N50 and N100 treatments. The S value slightly increased, but

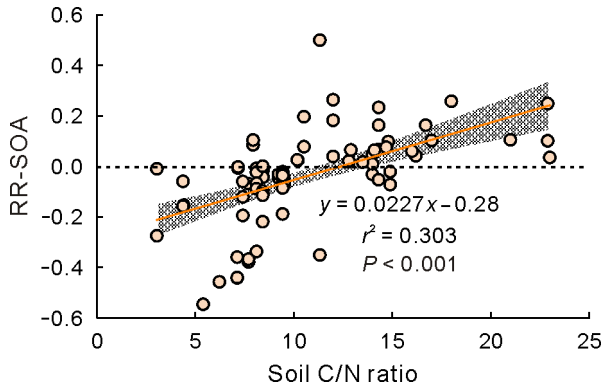

Fig. 6 Correlation between the response ratio (RR) of soil oxidase activity to N addition (RR-SOA) and soil C/N ratio based on collected literature data. Shaded section indicates the 95% confidence intervals of the regression model.

the H value did not. For the different sources of organic C, N addition significantly increased the microbial utilization of phenolic acids, amino acids, and amines ($P < 0.05$), whereas metabolisms of carbohydrates, carboxylic acids, and polymers weakly increased with N addition rates (Fig. S5, see Supplementary Material for Fig. S5).

DISCUSSION

Effect of N addition on microbial communities

We found that N addition increased the microbial biomass, which is different from the results previously reported by Treseder (2008) and Zhang *et al*. (2018). They suggested that N enrichment inhibited microbial growth *via* base cation limitation and toxicity of Al^{3+} as a result of soil acidification (Chen *et al*., 2016). This may not be the case in our study, as the soil pH was not affected by N addition (Table I). A previous study showed that the studied grassland soil was N limited (Fang *et al*., 2012), and the alleviation of N deficiency with N input resulted in an increased microbial biomass (Fig. 1). Similarly, a global meta-analysis study by Zhou *et al*. (2017) suggested that N rates < 100 kg N ha^{-1} year^{-1} stimulate microbial growth in various biomes.

Soil microbial community composition was affected by N addition, with the PLFA profiles of the communities under N amendment clearly distinct from those in the control soil (Fig. 2). Previous studies have shown that the F/B ratio increase with N rates, especially in N-rich ecosystems (Cusack *et al*., 2011), because fungi are better able than bacteria to adapt to soil with high H^+ contents induced by N addition (Rousk *et al*., 2010; Wang *et al*., 2015). However, we observed a negative relationship between the F/B ratio and N rate (Fig. 3), possibly as a result of the increased availability of N especially for DON, which favored the proliferation of bacteria that have higher N demands than fungi in this N-limited ecosystem (Table SII) (Fierer *et al*., 2007). It is also possible that N-mediated shifts in the plant community structure towards dominant grasses improved the

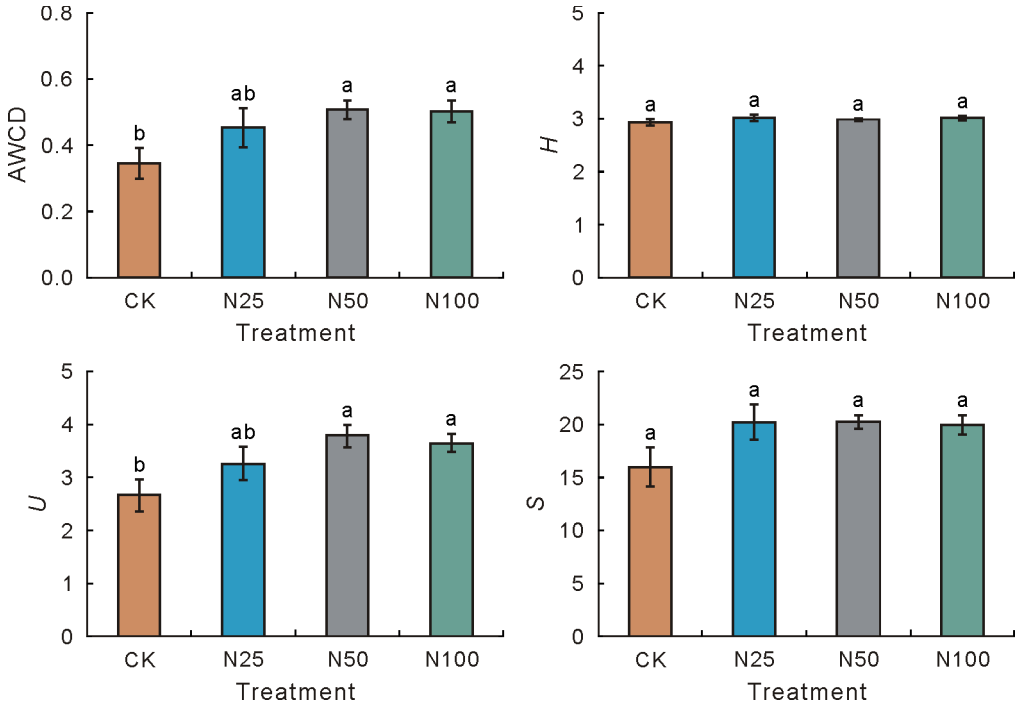

Fig. 7 Changes in the average well-color development (AWCD), Shannon-Wiener index (H), McIntosh index (U), and richness index (S) of soil microbial communities along the N addition gradients of different treatments. Error bars are standard errors of the means ($n = 4$). Bars with different letters indicate significant differences among treatments at $P < 0.05$. CK = control without N addition; N25 = 25 kg N ha^{-1} year^{-1}; N50 = 50 kg N ha^{-1} year^{-1}; N100 = 100 kg N ha^{-1} year^{-1}.

chemical quality of litter with a low C/N ratio, as previously reported in a Tibetan alpine meadow (Li *et al*., 2018), which benefits bacterial growth over fungal growth (Rousk and Bååth, 2007).

The increased abundance in AMF after N amendment was contrary to our expectations (Fig. 3) and differed from previous findings in temperate grasslands (Wei *et al*., 2013) and tropical/subtropical forests (Tian *et al*., 2017; Wang *et al*., 2018). In these previous studies, suppresion effects of N addition were attributed to reductions in photosynthetic C allocation to AMF when nutrient limitation was relieved (Gutknecht *et al*., 2012). However, this did not appear to have occurred in our studied grassland, because N addition did not reduce the belowground biomass (Fig. S1b). Deng *et al*. (2017) suggested that N addition induces P limitation in terrestrial ecosystems; therefore, it is likely that plants depend on AMF for other nutrient acquisitions, such as P, under N addition.

Among the bacterial communities, G^- bacteria became dominant in response to N addition (Fig. 3). The G^- bacteria associated with copiotrophic microbial communities tend to thrive on easily degradable substrates, while the more oligotrophic G^+ bacteria are better adapted to poor organic C conditions (Fierer *et al*., 2007; Kramer and Gleixner, 2008). Furthermore, G^+ bacteria are characterized by strong, thick, and interlinked peptidoglycan cell walls, whereas G^- bacteria have a single-layer cell wall and outer membrane (Schimel *et al*., 2007). Although these characteristics confer greater stress tolerance to G^+ bacteria than G^- bacteria, N addition did not reduce soil pH (Table I), which may have weakened the advantage of G^+ bacteria over G^- bacteria in the present study. Thus, it is likely that the increased plant AGB following N addition supplied more labile C, which favored G^- bacterial growth (Table SII).

Effect of N addition on microbial functions and use of SOC

The meta-analysis of the data collected from the literature showed that the response of SOC content increased with N rates (Fig. S4a). In contrast, N addition reduced the SOC in our studied grassland. We found that N amendment increased the activity of cellulose-degrading enzymes in the alpine grassland, similar to previous reports from a variety of grasslands (Zeglin *et al*., 2007; Cenini *et al*., 2015). Against our expectations, the addition of N also enhanced the activity of phenol oxidase. This finding differs from other observations in forest ecosystems, where the activity of oxidative enzymes involved in lignin degradation is depressed, due to declines in the abundance of fungi, especially of white-rot basidiomycetes (Frey *et al*., 2004; Cusack *et al*., 2010). Our meta-analysis showed an approximate negative relationship between the response of oxidase activity and N rate. In N-limited ecosystems, oxidative enzyme activity increases to facilitate scavenging of N occluded in complex compounds and structural plant tissues, such as from SOM decomposition (Sinsabaugh and Moorhead, 1994). Thus, N input potentially relieved the microbial N demand, thereby reducing the need to invest in the additional energy and resources required to produce oxidase by microbes in N-amended soils (Sinsabaugh *et al*., 2008). Entwistle *et al*. (2018) found that experimental N deposition had no effect on the abundance of lignolytic fungi with low-lignin substrates, but it reduced those with high-lignin substrates due to increased competition between cellulolytic and lignolytic fungi, which inhibited fungal lignolytic enzyme activity, slowed lignin decay, and increased soil C storage. Cline *et al*. (2018) suggested that increases in dominant C_4 grasses under N amendment suppresses the proliferation of saprotrophic fungi, such as *Penicillium* and *Aspergillus*, due to increasing quantities of recalcitrant compounds in litter. Thus, it is likely that the responses of oxidase activity and SOC to N amendment were dependent upon the vegetation type and that the increased stabilization and storage of C in soil was partly due to increased inputs of lignin-rich plant litters under N amendment (Dijkstra *et al*., 2004). However, this may be not applicable in our grassland, since the dominant plants were classified as C_3 grasses (Yi *et al*., 2003).

Our meta-analysis showed a positive correlation between N-induced changes in soil oxidase activity and soil C/N ratio (Fig. 6), which suggests that the responses of oxidase activity to added N were strongly dependent upon the soil C/N ratio. The responses were negative at low C/N ratio and positive at high C/N ratio. In the soil of the present study, the C/N ratio was located within the 95% confidence interval of the break-point threshold between the negative and positive response of oxidase activity to N addition. In contrast, the CLPP analysis, which showed that N addition improved the microbial utilization of recalcitrant C sources, such as phenolic acids, and functional diversity of microbial metabolism (Figs. 7 and S5), was found to differ from those reported in previous studies (Zhang *et al*., 2008). For example, Dalmonech *et al*. (2010) observed that microbial functional diversity (H value) was lower in N-fertilized than unfertilized plots in a Mediterranean forest. However, Zhang *et al*. (2008) reported that AWCD, H, and S values increased at low rates of N, but decreased at > 160 kg N ha^{-1} year^{-1}. Hence, low rates of N, along with relatively high soil C/N ratio, may have been less likely to suppress oxidative enzyme activity, but rather increase the activities of enzymes, such as phenol oxidase, and accelerate the SOC losses in the studied N-limited grassland.

Bragazza *et al*. (2012) suggested that in a wetland bog under N enrichment, the increased activity of phenol oxidase was attributable to the amelioration of peat chemistry *via* decreasing the ratio of lignin to lignin + cellulose. Accordingly,

it is likely that N amendment reduced the lignin in litter (Zhu *et al*., 2016), which enhanced the activity of phenol oxidase (Fig. 4). Based on measurements from 25 grasslands worldwide, Leff *et al*. (2015) found that N addition promoted the growth of the dominant fungi Ascomycota. Thus, it is possible that there was proliferation of Ascomycota rather than Basidiomycota in our grassland soil, as has been observed in forests (Frey *et al*., 2004), which led to an increase in the oxidase activity.

Resource allocation theory suggests that N enrichment decreases the activity of N-acquisition enzymes (Allison and Vitousek, 2005). In contrast, the activity of NAG, which is involved in the degradation of the organic compound chitin, increased with N addition (Fig. 4), as did the soil available N. We also found a positive correlation between the NAG activity and fungal biomass ($r^2 = 0.291$, $P = 0.031$). This supports the results reported by Miller *et al*. (1998) and Chung *et al*. (2007), who confirmed that NAG activity was primarily affected by a diverse group of fungi. Furthermore, plants, especially grasses, have been reported to outcompete microbes for soil available N (Kuzyakov and Xu, 2013), which causes microbial N limitation and increased NAG activity (Fig. 5). However, the activity of LAP increased with low rates of N addition, but decreased with high rates (Fig. 4), indicating that a large amount of available N in the soil could have substantially relieved the N limitation for microbes and caused a more conservative production of N-acquisition enzymes (Sinsabaugh *et al*., 2008). Considering that the molecular structure of organic matter in soil has chemical features of both proteins and microbial cell walls (Miltner *et al*., 2012), it is possible that the enhanced activities of LAP and NAG could be responsible for the reduction in SOC, through mineralization of organic N following N addition in the present study.

CONCLUSIONS

Over 4 years, N addition to an alpine grassland increased soil microbial biomass and induced shifts in soil microbial community composition towards a bacteria-dominated one, mainly due to an increase in the abundance of G^- bacteria. Moreover, N amendment promoted the microbial utilization of organic C and functional diversity and increased the activity of enzymes associated with organic C and N turnover, probably as a result of the relatively high soil C/N ratio under low rates of N. This in turn stimulated the decomposition of SOC. Our results contribute towards improving the understanding of microbial functions in mediating soil C cycling in alpine grasslands under conditions of global N enrichment.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article can be found in the online version.

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