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Running title: N effects on microbial communities and functions

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

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Highlights

- N addition increased microbial biomass C (MBC) and N (MBN).
- N addition stimulated growth of gram-negative bacteria and arbuscular mycorrhizal fungi.
- N amendment decreased ratios of both fungi to bacteria and gram-positive to gram-negative bacteria.
- Microbial functional diversity and activity of enzymes associated with organic C and N mineralization increased after N addition.
- Soil C/N ratio was a key factor in response of oxidase activity to N amendment.

ABSTRACT

Alpine grasslands on the Tibetan Plateau with high soil organic carbon (SOC) storage are experiencing rapid climate warming and anthropogenic nitrogen (N) deposition, which is expected to substantially increase soil N availability that may impact C cycling. However, little is known regarding how the N enrichment influences the soil microbial communities and functions relative to C cycling in this region. We conducted a 4-year field experiment to evaluate the effects of four different levels of N addition (0, 25, 50, and 100 kg N ha^{-1} yr^{-1}) on abundance and community structure (phospholipid fatty acids, PLFAs) of microbes, enzyme activities, and community level physiological profiles (CLPP) in an alpine grassland. We found that N addition increased microbial biomass C (MBC) and N (MBN), and there was an increased abundance of bacterial PLFAs, especially gram-negative bacterial PLFAs, with a decreasing ratio of gram-positive to gram-negative bacteria. N addition also stimulated the growth of fungi, especially arbuscular mycorrhizal fungi, and reduced 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 loss of soil organic C. Meta-analyses showed that soil C/N ratio was a key factor in response of oxidase activity to N amendment, suggesting that the responses of soil

microbial functions that linked to C turnover relative to N input primarily depended on soil C/N ratio. Overall, our findings highlight that N addition has positive influences on microbial communities and their associated functions, which may potentially reduce soil C storage in alpine grasslands under global change scenarios.

Key Words: alpine grassland, CLPP, enzyme activity, microbial community composition, microbial function, N addition

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 elevated N input increases production of plant biomass that then benefits 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 change in plant community composition and loss of plant diversity due to size asymmetry and subsequent light competition (DeMalach *et al*., 2017). However, it remains unclear how the projected global change influences soil microbial communities.

Microbes play a prominent role in soil nutrient cycling, organic matter (SOM) decomposition, and other ecosystem functioning (Fierer *et al*., 2009), and their responses to increasing N deposition may have profound consequences for global C-cycling (Garcia-Palacios *et al*., 2015). Recently, a few attempts of meta-analysis have been made to assess how microbial biomass would respond to N addition, and they 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 showed a positive effect of N addition 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 abundance of fungi, particularly arbuscular mycorrhizal fungi (Wei *et al*., 2013), and the ratio of fungi to bacteria (F/B), as a result of the relatively low nutrient demands of fungi compared to bacteria (Fierer *et al*., 2007; Gutknecht *et al*., 2012). N-induced shifts in plant biomass and/or composition may alter substrate C/N ratios, which stimulate the growth of bacteria more than fungi (De Deyn *et al*., 2008). Soil acidification caused by N addition is likely to enhance F/B ratios (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 abundance and composition of 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 feedback in response to global change (Xiao *et al*., 2018). N addition tends to increase glycosidase activity with application rate, particularly when applied as organic fertilizers (Zeglin *et al*., 2007; Chen *et al*., 2017), but it also has the potential to suppress activity of typical lignin-degrading enzymes (Frey *et al*., 2004; Xiao *et al*., 2018). Effects of increased N availability on N-acquisition enzyme activity are inconsistent (Chen *et al*., 2018); for instance, Wang *et al*. (2015) observed increases in activity of β-1,4-N-acetyl-glucosaminidase (NAG) in temperate

grasslands under N amendment, while Fatemi *et al*. (2016) recorded decreases in NAG activity in hardwood and softwood forests subjected to chronic N enrichment. However, no apparent impacts of N deposition occurred on 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 that any 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 million km^2) plateau, comprising 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 C source under N enrichment. This region has experienced rapid climate warming at a rate of 0.2 ℃ per decade over the past 50 years (Chen *et al*., 2013), and climate warming is assumed to increase soil nutrient availability 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} yr^{-1} (Lü and Tian, 2007; Fang *et al*., 2012). Extensive evidences show that N addition could affect diversity and/or composition of the aboveground plant community in these alpine grasslands (Fang *et al*., 2012; Luo *et al*., 2019). Although microbial community is as important as plant community for ecosystem functioning (Fierer *et al*., 2009), the responses of soil microbial communities and functions relative to C cycling to increasing N availability remain unclear in this alpine ecosystem. Here, a 4-year field experiment, with four N addition levels, was conducted in an alpine grassland on the Tibetan Plateau. The objectives of this study were to: (1) evaluate the influence of N addition on microbial community composition, enzyme activity or functional diversity; and (2) elucidate the factors affecting microbial functions associated with organic C mineralization in response to N addition combined with synthetic analysis of literature data.

MATERIALS AND METHODS

Study site and experimental design

The study site was located 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 a.s.l.). This area has a continental monsoon climate with a mean annual temperature of –1.2 ℃ and a mean annual precipitation of 489 mm, 80% of which is concentrated in the plant growing season from May to September. Soil is classified as 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*, *Blvsmus 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, and included four N addition levels: CK (without N addition), N25 (25 kg N ha^{-1} yr^{-1}), N50 (50 kg N ha^{-1} yr^{-1}), and N100 (100 kg N ha^{-1} yr^{-1}). Sixteen 6 m × 6 m plots were arranged in a randomized block design. Blocks were separated by a 2-m-wide buffer strip, and plots within each block were separated by a 1-m-wide buffer strip to minimize disturbance from neighboring plots. N fertilizers (in the form of urea) were applied to the plots in three equal monthly applications per year at the beginning of June, July and August.

Plant and soil sampling

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 ℃ for 48 h, and weighed. Belowground plant biomass was estimated by extracting roots from three soil cores collected using a 7-cm diameter soil auger; root material was 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 root and stone material was removed. The soil sample was passed through a 2-mm mesh sieve and thoroughly mixed. A subsample of fresh soil was stored at 4 ℃, and the rest of the fresh soil was air-dried for the analysis of soil properties.

Soil analysis

Soil samples were dried at 105 ℃ for 24 h to measure moisture content, and soil pH was determined using a glass electrode in a 1:2.5 soil/water suspension. Content of soil organic C (SOC) and total N (TN) was analyzed using the wet oxidation-redox titration and micro-Kjeldahl methods, respectively (Lu, 2000). Available phosphorous (AP) was extracted using $NaHCO_3$, and analyzed using the molybdenum blue method (Lu, 2000). Dissolved organic C (DOC) was extracted from fresh moist soil using deionized water in a 1:5 soil/solution ratio, shaken for 30 min, centrifuged for 10 min at 10000 rpm, and filtered through a 0.45-μm polyethersulfone membrane filter; and 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 2 M KCl solution in a 1:5 soil/solution ratio, and their concentrations were measured using a continuous-flow autoanalyzer (Skalar San^{++}, Breda, the Netherlands); and dissolved organic N (DON) was calculated as DN − NH_4^+-N − NO_3^--N.

Microbial biomass and community composition measurement

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

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

Enzyme activity assays

Measurement of β-1,4-glucosidase (BG), β-1,4-N-acetyl-glucosaminidase (NAG), and leucine aminopeptidase (LAP) activity (μmol PNP g^{-1} h^{-1}) was performed on the basis of *p*-nitrophenol (PNP) release following 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 method of Tabatabai (1994) and Sinsabaugh *et al*. (2002). Briefly, 1 g of fresh soil (on the oven-dried basis) was mixed with 5 mM substrate and 50 mM acetate buffer (pH 5), and incubated in the dark at 37 ℃ for 1 h. Then, 0.5 M $CaCl_2$ and 0.1 M trishydroxymethyl aminomethane (pH 12) were added to terminate the reaction. A control was prepared for each sample by mixing buffer with either soil or substrate solution (the same as below). Resulting products were filtered through Whatman No. 2v filter paper and determined using a UV-VIS spectrophotometer (UV-1800, Mapada Instruments Co., Shanghai, China) at 410 nm.

Activity of phenol oxidase (POX) was measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Sinsabaugh *et al*., 2002), where 1 g of fresh soil (on the oven-dried basis) with 5 mM L-DOPA plus 50 mM acetate buffer (pH 5) was incubated in the dark at 25 ℃ for 1 h; then the reaction was terminated by immediate centrifugation at 5000 rpm for 5 min at 4 ℃, and the absorbance of 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}.

Community level physiological profiles (CLPP) analysis

The CLPP of soil microbial communities was determined using BIOLOG 96-well Eco-Microplates (Biolog Inc., USA), with three replicates of 31 different sources of organic C in each microplate that included carbohydrates, carboxylic acids, polymers, phenolic acids, amino acids, and amines. Microbes were extracted from 10 g soil (on the oven-dried basis), to which 90 ml of sterile 0.85% (w/v) saline solution was added (Classen *et al*., 2003). The mixture was shaken for 30 min at 70 rpm and left to stand for 30 min; then, 1 ml of supernatant was diluted to 10 ml, twice, using sterile saline solution, before 150 μl of each sample was inoculated into each well. The plates were incubated at 25 ℃ in the dark and color development in each well was recorded as optical density (OD) at 590 nm at 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 CLPP. Microbial functional diversity of organic C metabolism, recorded as average well color development (AWCD), was calculated as $\sum(C_i - R)/n$, where C_i is the absorption value of the sample substrate well; R is the control absorption well; and n is the number of plates (n = 31).

Several indexes were used to analyze diversity and richness of the microbial communities: Shannon-Wiener index (H), $H = -\sum P_i \times (\ln P_i)$, where P_i is the ratio of the relative absorption of the well divided by the sum of all relative color development of the plate; McIntosh index (U), $U = \sqrt{\sum (C_i - R)^2}$; and richness index ($S$), S is the number of wells with $C_i - R > 0.25$ (Gomez *et al*., 2006).

Meta-analysis of cellulase and oxidase activity

We derived cellulase and oxidase activity data from international, peer-reviewed journal articles. Data collection was limited to the results that (i) cellulase or oxidase activity and SOC content were reported in the control and N-added treatments; and (ii) records of N rates, TN, and C/N ratio were shown. If results were presented graphically, data were extracted from figures using Engauge Digitizer

software. Activities of cellulase denote a group of hydrolytic enzymes that degrade cellulose and hemicellulose, including β-1,4-glucosidase, β-1,4-xylosidase, and β-D-cellobiosidase. Oxidative enzymes produced by microbes decompose recalcitrant substrates (i.e., lignin), and the most frequently assayed oxidases include peroxidase, phenol oxidase, and polyphenol oxidase. If a paper reported activities for multiple kinds of cellulase or oxidase, their sum values were used as overall response of the respective enzyme activity. In total, 58, 80, and 75 paired measurements for activities of cellulase and oxidase and SOC, respectively, were selected from 20 published papers (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 least significant difference test (LSD) at $p < 0.05$. The response ratio (RR) of cellulase or oxidase activity, and SOC content to N addition was calculated as the ln-transformed ratio of the value in the N-added treatment to that of the control based on literature data. We used linear regression analysis to explore the relationships between MBC (MBN) and aboveground plant biomass (AGB) or DON, and between response ratio 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., Chicago, IL, 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 soil microbial community composition, twenty-nine individual PLFAs concentrations of soil samples were subjected to non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis dissimilarity matrix. We evaluated the relationships between 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 direction and strength, respectively, of the effect of an environmental parameter on enzyme activity.

RESULTS

Soil properties and plant biomass

NO_3^- and DON concentrations in the 0–10 cm layer of soil increased with level of added N, while concentration of NH_4^+ significantly increased in N50 treatment only (Table I). In contrast, content of SOC and TN significantly decreased following N addition, while soil pH, or concentrations of AP and DOC did not differ between the CK and N-amended treatments. N addition increased aboveground plant biomass via increases in biomass of grasses and sedges (Fig. S1). In contrast, belowground plant biomass was not affected by N addition ($p > 0.05$).

Microbial community composition

N addition increased soil MBC and MBN ($p < 0.01$; Table I) that showed linear relationships with AGB and DON (Fig. 1). The NMDS of PLFA profile 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. N addition enhanced AMF abundance, but reduced the F/B ratio, and the ratio of G^+/G^- bacteria was reduced by 14.5–43.4% in the N-added treatments ($p < 0.01$).

Pearson correlation analysis showed that the abundances of fungal, total bacterial and G^- bacterial PLFAs were positively correlated with NO_3^- ($p < 0.05$) and DON ($p < 0.01$), while AMF biomass was only positively correlated with DON ($p = 0.014$; Table SII). Significant negative relationships were observed between the F/B ratio and DON or AGB ($p < 0.05$). Meanwhile, the G^+/G^- ratio was positively correlated with TN ($p = 0.005$) and SOC ($p = 0.007$), but negatively correlated with NO_3^-, DON, and AGB ($p < 0.05$).

Enzyme activities

N addition generally increased enzyme activities relative to the CK (Fig. 4). The N addition elevated BG activity, however significant increase was only found between CK and N100 treatment ($p < 0.05$). N addition also significantly increased POX activity by 3.7–25.0% ($p < 0.001$). 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 soil compared to the CK ($p < 0.05$).

Redundancy analysis revealed that enzyme activities were correlated with SOC, TN, NO_3^-, DON, and AGB (Fig. 5). More specifically, the activities of BG and POX were positively correlated with DON and AGB, and inversely correlated with TN and SOC (Table SIII). NAG activity showed a positive correlation with NO_3^- ($p = 0.001$), AGB ($p = 0.005$), and grasses biomass ($p = 0.018$), but had a negative relationship with soil TN ($p = 0.039$).

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

Organic carbon utilization profiles of microbial communities

The average well-color development (AWCD) and McIntosh index (*U*) values increased with N rates, and significant effects were found in the N50 and N100 plots (Fig. 7). N addition slightly increased the richness index (*S*), but not Shannon-Wiener diversity (*H*). For the types of sole organic C, N addition significantly increased microbial utilizations of phenolic acids, amino acids and amines, whereas metabolisms of carbohydrates, carboxylic acids and polymers were weakly increased with N addition rates (Fig. S5).

DISCUSSION

Effects of N addition on microbial communities

We found N addition increased 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 be not the case in our study, as 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 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} yr^{-1} stimulate microbial growth in various biomes.

Microbial community composition was affected by N addition, with the PLFA profiles of the communities under N amendment clearly distinct from those in the untreated soil (Fig. 2). Previous studies showed that F/B ratios increase with N rates especially in N-rich ecosystems (Cusack *et al*., 2011), because fungi are better able to adapt to the soil with high H^+ concentrations induced by N addition than bacteria (Rousk *et al*., 2010; Wang *et al*., 2015). However, we observed a negative relationship between F/B ratio and N rate (Fig. 3f), possibly as a result of increased availability of N especially for DON, which favored more 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 plant community structure to dominant grasses improved the chemical quality of litter with low C/N ratio, which has previously been reported in a Tibetan alpine meadow (Li *et al*., 2018), that benefited more bacterial growth than fungi (Rousk and Baath, 2007).

The increased abundance in AMF after N amendment was contrary to our expectations (Fig. 3e), 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 belowground biomass (Fig. S1b); Deng *et al*. (2017) suggested that N addition induces P limitation in terrestrial ecosystems, so it is likely that plants depend on AMF for other nutrient acquisition, such as P, under N addition.

Among the bacterial communities, G^- bacteria became dominant in response to N addition (Fig. 3g). 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) that may have weakened the advantage of G^+ bacteria over G^- bacteria in the present study. Thus, it is likely that increased aboveground biomass supplied more labile C following N addition, leading to favor G^- bacterial growth (Table SII).

Effects of N addition on microbial functions and use of SOC

Meta-analysis of the data collected from literature showed that response of SOC content increased with N rates (Fig. S4a); in contrast, N addition reduced SOC in our studied grassland. We found that N amendment increased 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). Out of expectation, addition of N also enhanced the activity of phenol oxidase. This finding differs from observations in forest ecosystems, where 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 microbial N demand, thereby reducing the need to invest 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 that 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 suppress proliferation of saprotrophic fungi, such as *Penicillium* and *Aspergillus*, due to increasing quantities of recalcitrant compounds in litter. Thus, it is likely that the response of oxidase activity and SOC to N amendment was dependent on vegetation type, and that increased stabilization and storage of C in soils 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 studied 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 oxidase activity and soil C/N ratio (Fig. 6) that suggests responses of oxidase activity to added N were strongly dependent on soil C/N ratio: responses were negative in low C/N ratio soil and positive in high C/N ratio soil. In the present study soil, the C/N ratio was located within the 95% confidence intervals of the break-point threshold between the negative and positive response of oxidase activity to N addition; in contrast, the CLPP analysis, which showed N addition improved microbial utilization of recalcitrant C sources, such as phenolic acids, and functional diversity of microbial metabolism (Fig. 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*) was lower in N-fertilized than unfertilized plots in a Mediterranean forest; however, Zhang *et al*. (2008) reported that AWCD, *H*, and *S* increased at low rates of N, but decreased at >160 kg N ha^{-1} yr^{-1}. Hence, low rates of N, along with relatively high C/N ratios may have been less likely to suppress oxidative enzyme activity, but rather increase in the activities of enzymes such as phenol oxidase, and accelerate the SOC losses in the present studied N-limited grassland.

In a wetland bog under N enrichment, Bragazza *et al*. (2012) suggested that increased activity of phenol oxidase was attributed to amelioration of peat chemistry, that decreased the ratio of lignin to lignin+cellulose. Accordingly, it is likely that N amendment reduced lignin in litter (Zhu *et al*., 2016) that enhanced the activity of phenol oxidase (Fig. 4b). Based on measurements from 25 grasslands worldwide, Leff *et al*. (2015) found that N addition promoted growth of dominant fungi Ascomycota. Thus, it is possible that there was proliferation of Ascomycota rather than Basidiomycota in the present studied grassland soil, as has been observed in forests, that led to an increase in oxidase activity.

Resource allocation theory suggests that N enrichment decreases activity of N-acquisition enzymes (Allison and Vitousek, 2005). In contrast, the activity of NAG, which is involved in the degradation of the organic compounds chitin, increased with N addition (Fig. 4c), as was soil available N. We also found a positive correlation between NAG activity and fungal biomass (r^2 = 0.291, p = 0.031), which supports 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 *et al*., 2013) that caused 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. 4d), indicating that a large

amount of available N in soil could have substantially relieved N limitations for microbes and caused more conservative production of N-acquisition enzymes (Sinsabaugh *et al*., 2008). Considering 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 reduction of SOC through mineralization of organic N following N addition in the present study.

CONCLUSIONS

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

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TABLE I

Effects of different rates of N amendment on soil properties

Treatment	pH	SOC (g C kg^{-1})	TN (g N kg^{-1})	C/N	DOC (mg C kg^{-1})	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})	DON (mg N kg^{-1})	AP (mg P kg^{-1})	MBC (mg C kg^{-1})	MBN (mg N kg^{-1})
CK	7.21 ± 0.10 a	66.7 ± 1.0 a	6.7 ± 0.1 a	9.9 ± 0.1 a	88.7 ± 1.1 a	3.7 ± 0.1 b	22.3 ± 1.1 b	33.8 ± 0.6 c	7.0 ± 0.4 a	1113 ± 83 c	124 ± 8 b
N25	7.45 ± 0.02 a	63.4 ± 0.8 b	6.5 ± 0.1 ab	9.8 ± 0.1 a	86.1 ± 7.8 a	3.7 ± 0.1 b	31.5 ± 1.8 ab	35.4 ± 2.9 bc	6.7 ± 0.3 a	1194 ± 83 bc	134 ± 8 b
N50	7.31 ± 0.01 a	63.6 ± 0.5 b	6.4 ± 0.1 b	9.9 ± 0.1 a	80.8 ± 1.3 a	4.1 ± 0.1 a	33.9 ± 2.8 a	40.6 ± 1.7 ab	6.9 ± 0.5 a	1377 ± 50 ab	162 ± 1 a
N100	7.11 ± 0.18 a	59.9 ± 0.9 c	6.1 ± 0.3 c	9.8 ± 0.1 a	96.7 ± 8.4 a	3.5 ± 0.1 b	40.5 ± 5.1 a	44.6 ± 1.5 a	6.6 ± 0.5 a	1502 ± 82 a	181 ± 7 a

Means ± SE ($n = 4$). Different letters within the same column indicate significant differences among treatments at $p < 0.05$. SOC, soil organic carbon; TN, total nitrogen; C/N, ratio of SOC to TN; NH_4^+, ammonium; NO_3^-, nitrate; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; AP, available phosphorus; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen.

Figure legends

Fig. 1 Relationships between soil microbial biomass C (MBC), microbial biomass N (MBN) and aboveground plant biomass (AGB; a and c) or soil dissolved organic nitrogen concentration (DON; b and d). Shaded sections indicate the 95% confidence intervals of the regression models.

Fig. 2 Non-metric multidimensional scaling (NMDS) plot of soil microbial community composition based on individual PLFAs concentrations across different treatments.

Fig. 3 Effects of N addition on abundance or ratios of soil microbial groups as indicated by phospholipid fatty acids (PLFAs). Error bars are SEMs (n = 4), and different letters indicate significant differences among treatments at $p < 0.05$.

Fig. 4 Activities of β-1,4-glucosidase (BG, a), phenol oxidase (POX, b), β-1,4-N-acetyl-glucosaminidase (NAG, c) and leucine aminopeptidase (LAP, d) in the different treatments. Error bars are SEMs ($n = 4$), and different letters indicate significant differences among treatments at $p < 0.05$.

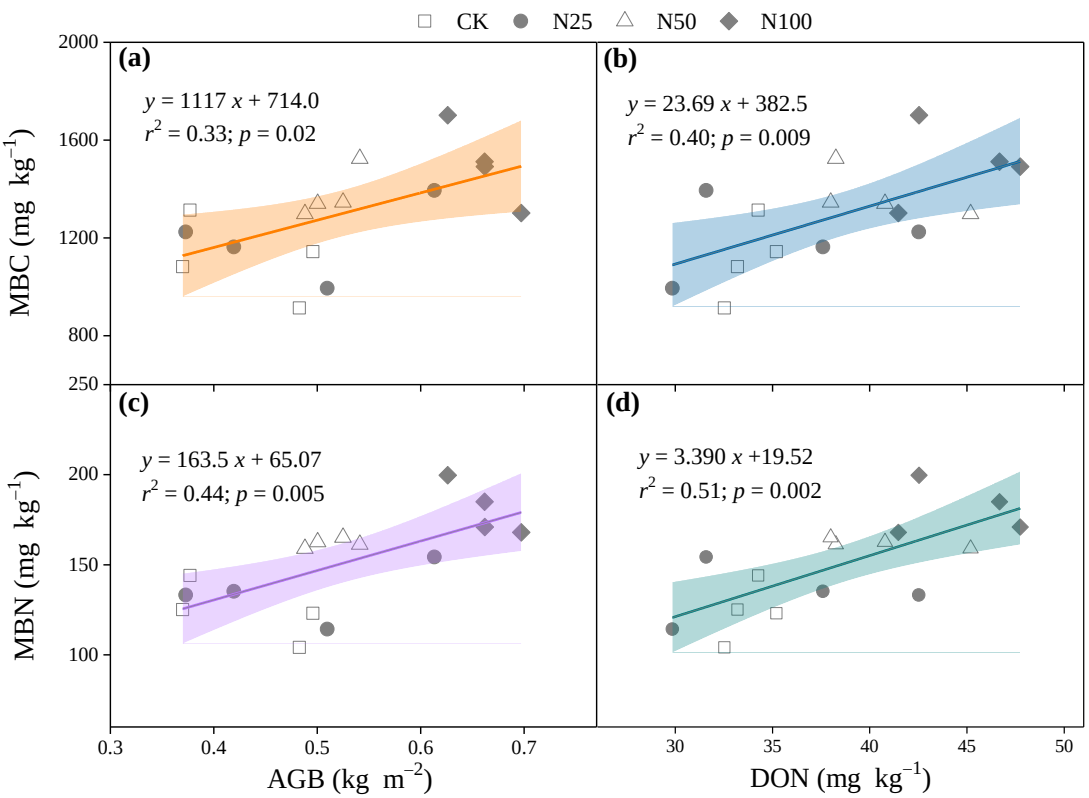
Fig. 5 Redundancy analysis (RDA) between enzyme activities and soil properties or plant biomass. BG, β-1,4-glucosidase; POX, phenol oxidase; NAG, β-1,4-N-acetyl-glucosaminidase; LAP, leucine aminopeptidase; SOC, soil organic carbon; TN, total nitrogen; NO_3^-, nitrate; DON, dissolved organic nitrogen; AGB, aboveground plant biomass; Grasses, grasses biomass; Forbs, forbs biomass.

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

Fig. 7 Changes in average well-color development (AWCD), Shannon-Wiener index (*H*), McIntosh index (*U*), and Richness index (*S*) along the N addition gradient. Error bars are SEMs ($n = 4$), and different letters indicate significant differences among treatments at $p < 0.05$.

Fig. 1

Fig. 2



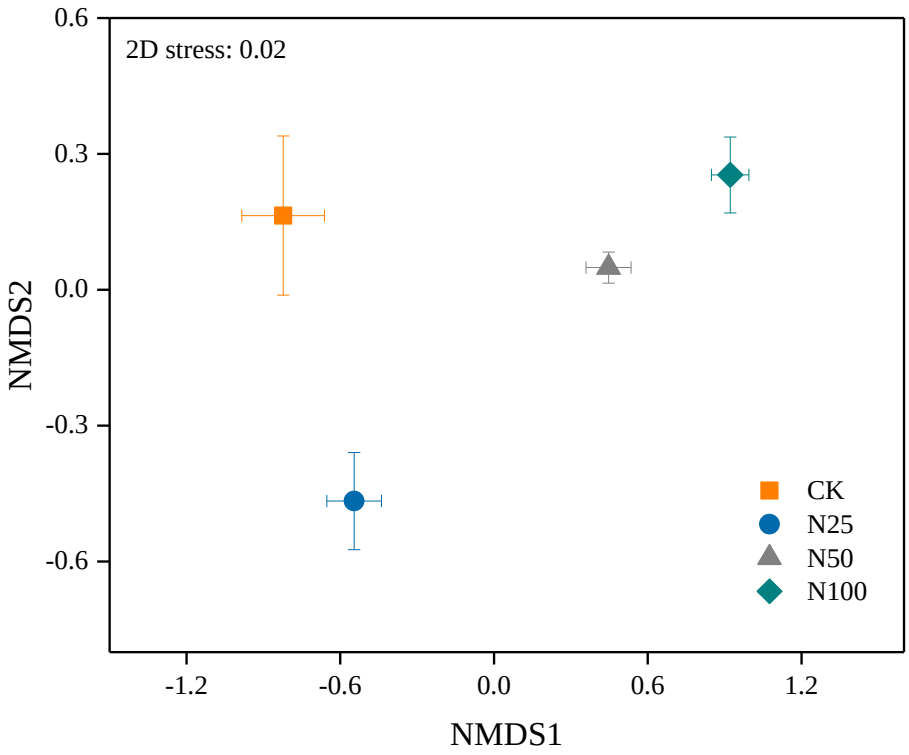

2D stress: 0.02
0.6
0.3
0.0
-0.3
-0.6
NMDS2
-1.2
-0.6
0.0
0.6
1.2
NMDS1
CK
N25
N50
N100

Fig. 3

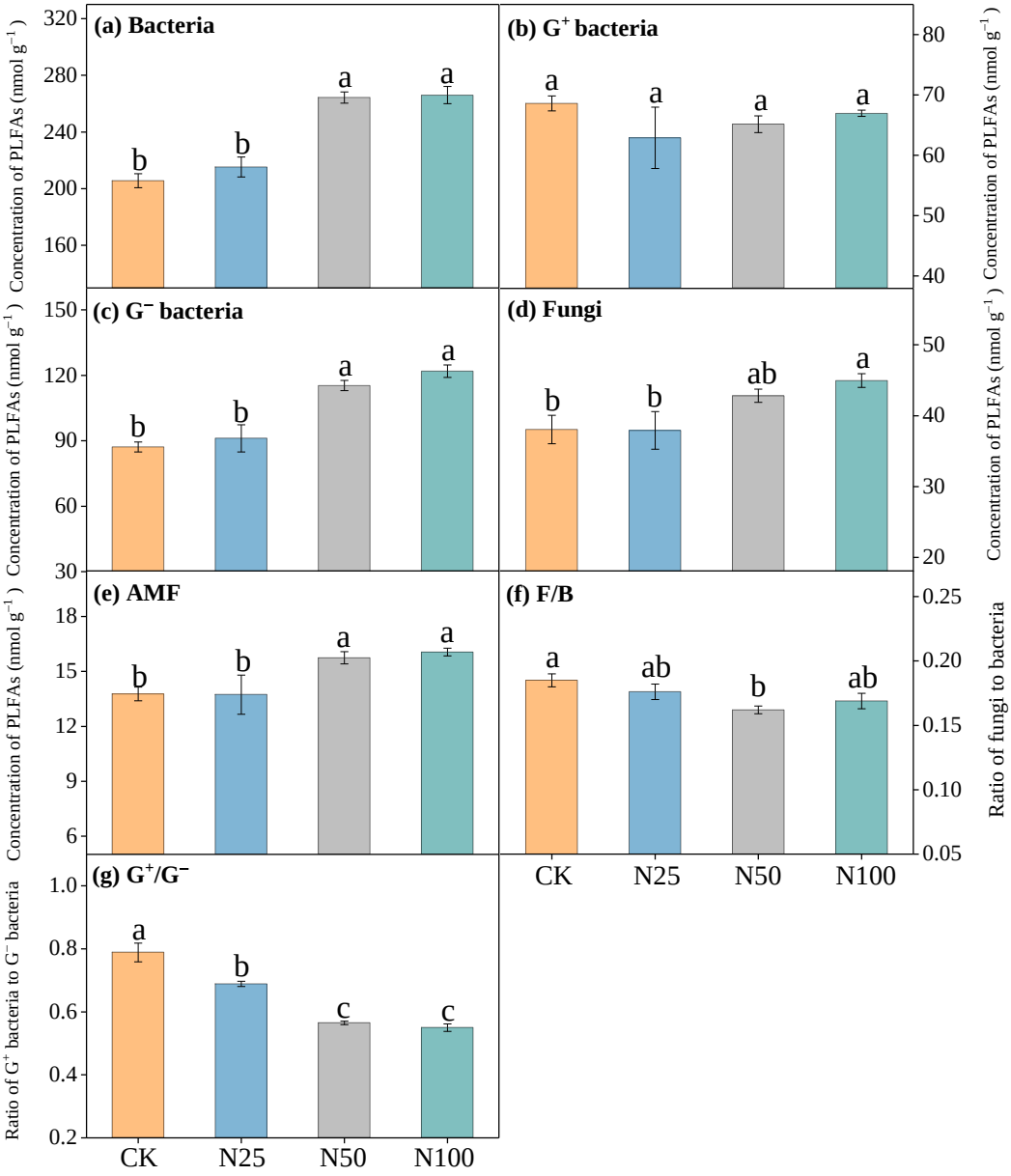

(a) Bacteria
(b) G+ bacteria
(c) G− bacteria
(d) Fungi
(e) AMF
(f) F/B
(g) G+/G−
Concentration of PLFAs (nmol g−1)
Ratio of G+ bacteria to G− bacteria
Ratio of fungi to bacteria
CK
N25
N50
N100

Fig. 4

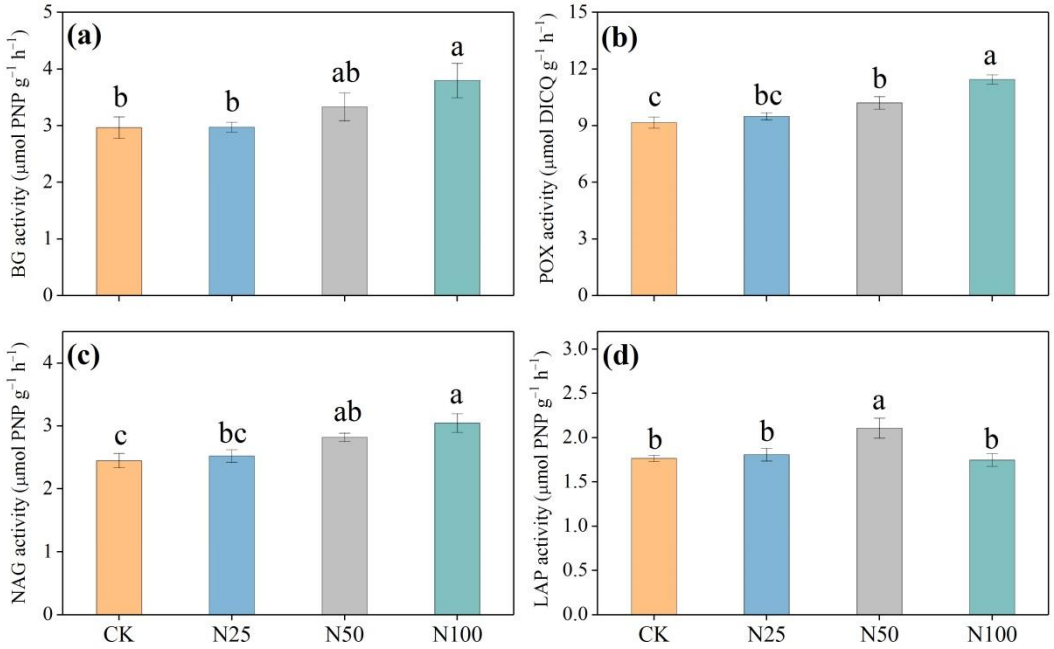

(a)
BG activity (μmol PNP g⁻¹ h⁻¹)
b
b
ab
a
(b)
POX activity (μmol DICQ g⁻¹ h⁻¹)
c
bc
b
a
(c)
NAG activity (μmol PNP g⁻¹ h⁻¹)
c
bc
ab
a
(d)
LAP activity (μmol PNP g⁻¹ h⁻¹)
b
b
a
b
CK
N25
N50
N100

Fig. 5

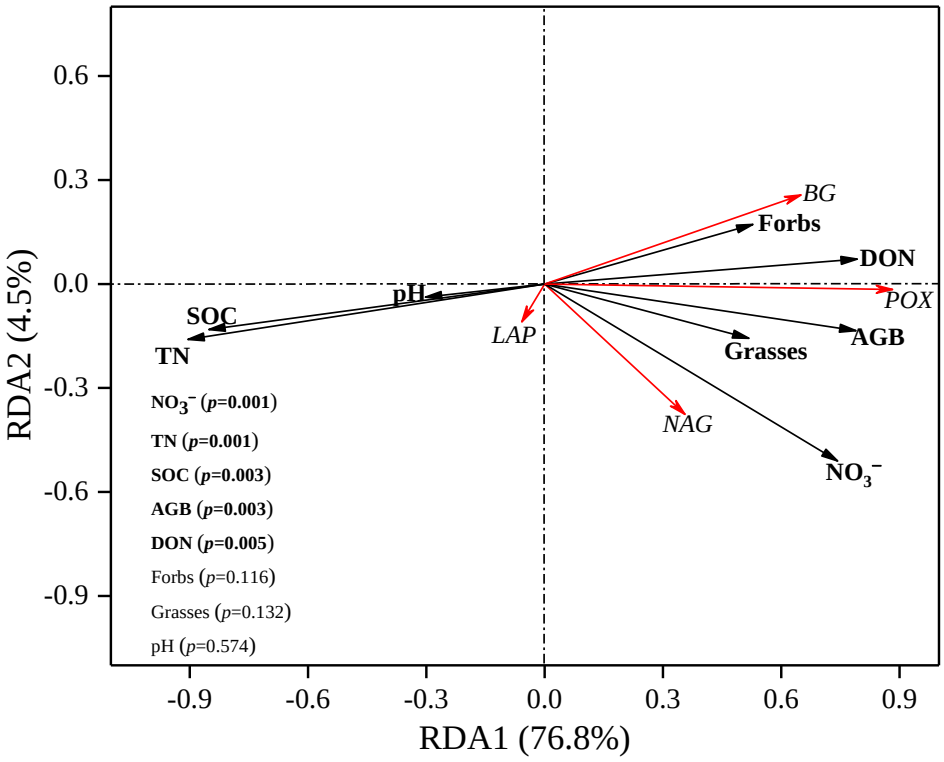

RDA2 (4.5%)
RDA1 (76.8%)
BG
Forbs
DON
POX
AGB
Grasses
NAG
NO3−
LAP
pH
SOC
TN
NO3− (p=0.001)
TN (p=0.001)
SOC (p=0.003)
AGB (p=0.003)
DON (p=0.005)
Forbs (p=0.116)
Grasses (p=0.132)
pH (p=0.574)

Fig. 6

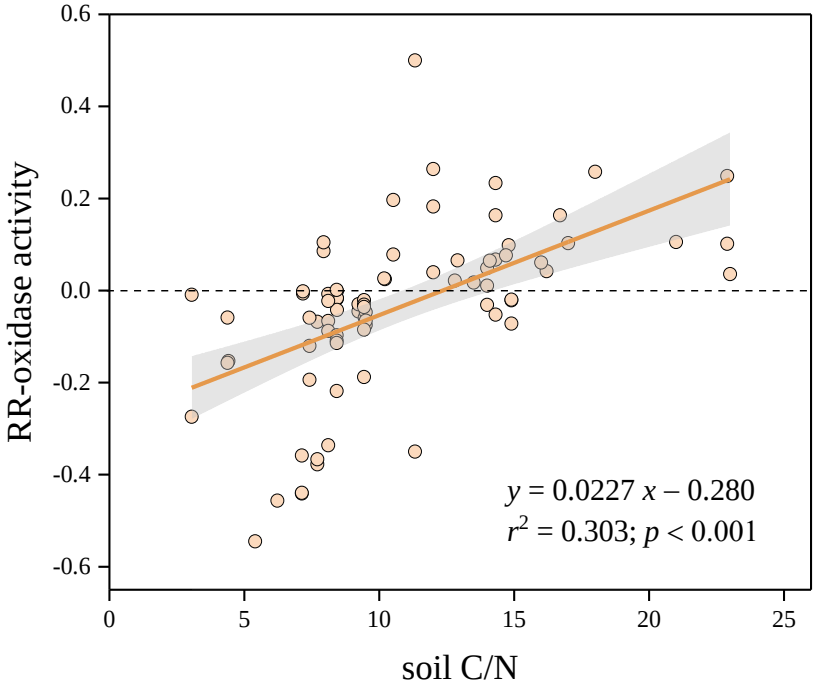
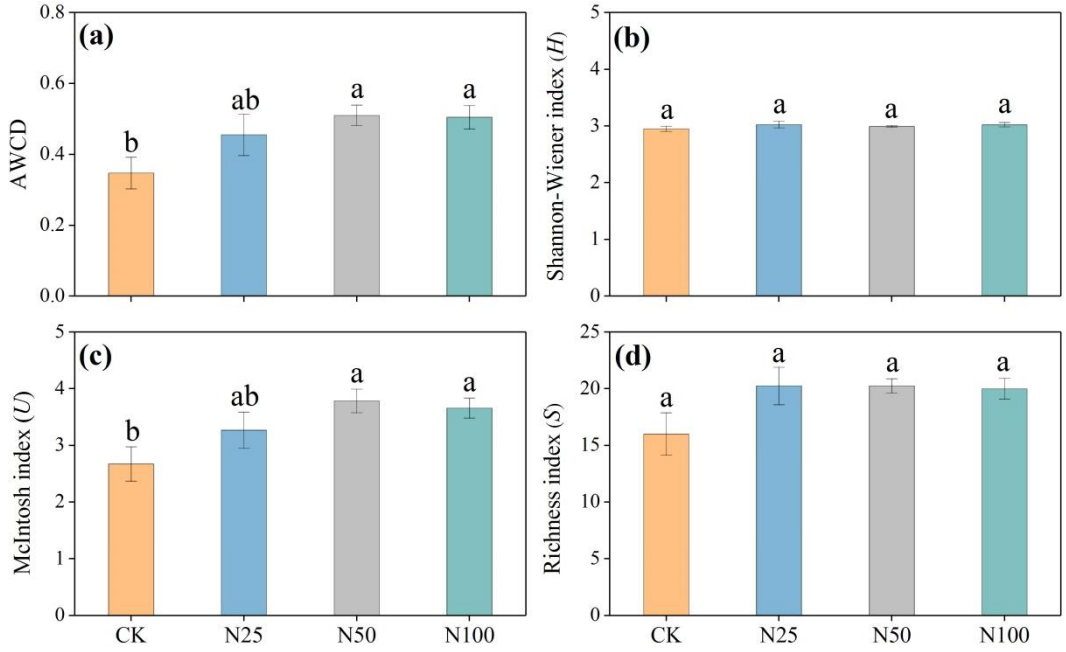

RR-oxidase activity
soil C/N
y = 0.0227 x – 0.280
r2 = 0.303; p < 0.001

Fig. 7


(a)
AWCD
(b)
Shannon-Wiener index (H)
(c)
McIntosh index (U)
(d)
Richness index (S)
CK
N25
N50
N100

Supplementary Material

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

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TABLE SI

Summary of published cellulase or oxidase activity and soil organic carbon (SOC) content from different sites worldwide in the control and N-added treatments, and relevant data including N addition rates, total nitrogen (TN) content, and C/N ratio

Site	N addition rates	SOC	TN	C/N	RR-SOC	Cellulase activity		Oxidase activity		Reference
	kg N ha^{-1} yr^{-1}	g C kg^{-1}	g N kg^{-1}			μmol g^{-1} h^{-1}	RR	μmol g^{-1} h^{-1}	RR	
Minnesota, USA	0	5.65	0.47	12.0		0.0251		0.6479		Chung et al., 2007
	40	6.07			0.0713	0.0260	0.0354	0.6741	0.0395	
	0	5.65	0.47	12.0		0.0244		0.4693		
	40	6.07			0.0713	0.0360	0.3866	0.6112	0.2640	
	0	5.65	0.47	12.0		0.0244		0.4954		
	40	6.07			0.0713	0.0391	0.3911	0.5945	0.1823	
Sanming, China	0	14.30	1.40	10.2		0.0911		2.74		Gao et al., 2017
	80	13.60			−0.0502	0.0739	−0.2093	2.81	0.0246	
Lenoir County, USA	0	98.00	7.00	14.0		21.86		38.47		Iyyemperumal et al., 2008
	200	112.00			0.1335	32.23	0.3883	40.39	0.0487	
	400	99.00			0.0102	31.81	0.3752	37.28	−0.0312	
	600	118.00			0.1857	40.28	0.6113	38.87	0.0105	
	0	112.00	7.52	14.9		24.44		47.51		
	200	114.00			0.0177	37.12	0.4180	46.51	−0.0213	
	400	111.00			−0.0090	39.12	0.4704	44.22	−0.0719	
	600	127.00			0.1257	41.03	0.5183	46.58	−0.0199	
Wuyishan, China	0	0.05	0.003	14.3		0.0970		7.60		Jing et al., 2017
	50	0.05			0.0887	0.0940	−0.0313	9.60	0.2333	
	100	0.04			−0.0639	0.0837	−0.1471	8.95	0.1634	
Wuying, China	0	0.22	0.015	14.3		0.2708		53.60		
	50	0.21			−0.0476	0.2794	0.0310	57.32	0.0671	

	100	0.23			0.0567	0.2948	0.0849	50.86	−0.0525	
Genhe, China	0	0.22	0.009	22.9		0.7324		12.38		
	50	0.21			−0.0476	0.9182	0.2262	15.88	0.2488	
	100	0.23			0.0567	0.6943	−0.0534	13.71	0.1015	
Minnesota, USA	0	16.13	0.95	17.0		0.1320		1.20		Keeler et al., 2009
	100	16.08			−0.0031	0.1361	0.0306	1.33	0.1029	
	0	6.44	0.46	14.1		0.1047		1.50		
	100	5.53			−0.1523	0.0925	−0.1239	1.60	0.0645	
	0	14.38	0.89	16.2		0.1751		2.10		
	100	14.99			0.0415	0.1959	0.1122	2.19	0.0420	
	0	12.24	0.83	14.8		0.1734		2.42		
	100	13.38			0.0891	0.1646	−0.0521	2.67	0.0983	
	0	10.76	0.67	16.0		0.1431		1.92		
	100	10.69			−0.0065	0.1595	0.1085	2.04	0.0606	
	0	8.21	0.49	16.7		0.1051		1.86		
	100	8.51			0.0359	0.1348	0.2489	2.19	0.1633	
Seoul, South Korea	0	250.00	82.00	3.0		0.0380		0.18		Kim et al., 2011
	100	250.00			0.0000	0.0398	0.0447	0.18	−0.0092	
	0	250.00	82.00	3.0		0.0380		0.18		
	100	300.00			0.1823	0.0389	0.0223	0.14	−0.2743	
	0	120.00	13.00	9.2		0.1254		0.10		
	167	120.00			0.0000	0.1523	0.1937	0.09	−0.0452	
	0	120.00	13.00	9.2		0.1254		0.10		
	167	130.00			0.0800	0.1592	0.2383	0.09	−0.0296	
Huitong, China	0	16.55	3.78	4.4		8.25		3.23		Li et al., 2016
	200	14.01			−0.1666	7.73	−0.0656	3.04	−0.0589	

	0	16.90	3.83	4.4		8.25		3.22	
	200	17.23			0.0193	6.16	−0.2922	2.76	−0.1532
	0	16.55	3.78	4.4		8.25		3.23	
	200	17.23			0.0403	6.16	−0.2922	2.76	−0.1572
Taiyueshan, China	0	42.80	6.00	7.1		18.87		8.57		Liu et al., 2015
	50	53.50			0.2231	16.80	−0.1162	5.99	−0.3585
	100	53.90			0.2306	17.64	−0.0675	5.51	−0.4409
	150	48.30			0.1209	15.36	−0.2062	5.52	−0.4396
	0	44.70	5.80	7.7		15.73		8.69	
	50	49.20			0.0959	13.48	−0.1544	8.12	−0.0681
	100	53.20			0.1741	13.33	−0.1659	5.96	−0.3775
	150	56.50			0.2343	14.78	−0.0629	6.03	−0.3664
Hong Kong, China	0	26.30	2.50	10.5		3.62		4.35		Luo et al., 2017
	10	26.40			0.0038	2.90	−0.2218	4.70	0.0782
	39	25.40			−0.0348	2.90	−0.2202	5.29	0.1967
	0	12.70	1.60	7.9		0.47		2.18	
	10				0.0156	0.48	0.0084	2.38	0.0854
	39				0.0310	0.41	−0.1342	2.42	0.1048
Kyunggi, South Korea	0	4.60	0.62	7.4		0.28		0.15		Min et al., 2011
	45					0.21	−0.2790	0.13	−0.1940
	0	4.60	0.62	7.4		0.28		0.15	
	45					0.21	−0.2650	0.15	−0.0590
	0	4.60	0.62	7.4		0.28		0.15	
	45					0.23	−0.1984	0.14	−0.1202
	0	11.10	0.98	11.3		1.55		0.12	
	45					1.22	−0.2423	0.09	−0.3498

	0	11.10	0.98	11.3		1.55		0.12		
	45					0.98	−0.4574	0.20	0.4998	
Michigan, USA	0	34.90	1.52	23.0		5.97		24.90		Sinsabaugh et al., 2005
	80	28.52			−0.2018	5.72	−0.0428	25.80	0.0355	
New Mexico, USA	0	5.17	0.38	13.5		0.0799		0.0858		Stursova et al., 2006
	10	5.51			0.0637	0.0863	0.0773	0.0873	0.0173	
	0	5.13	0.35	14.7		0.2010		0.0808		
	10	5.54			0.0769	0.1913	−0.0495	0.0872	0.0762	
Michigan, USA	0	17.50	0.83	21.0		3.76		304.00		Waldrop et al., 2004
	80	20.10			0.1385	4.40	0.1571	337.78	0.1054	
	0	29.60	1.64	18.0		5.94		239.38		
	80	21.50			−0.3197	6.24	0.0491	309.79	0.2578	
Heilongjiang, China	0	47.38	5.01	9.5		2.57		0.50		Yang et al., 2018
	100	47.11			−0.0057	2.89	0.1178	0.47	−0.0590	
New Mexico, USA	0	0.17	0.01	12.8		2.55		458.65		Zeglin et al., 2007
	100	0.17			0.0000	3.57	0.3378	468.65	0.0216	
Kansas, USA	0	0.82	0.06	12.9		3.89		3.63		
	100	0.82			0.0000	4.33	0.1066	3.88	0.0653	
Hubei, China	0	17.67	1.74	10.2		0.43		9.45		Zhang et al., 2016
	150	18.46			0.0437	0.48	0.1101	9.70	0.0261	
Haidian, China	0	17.36	2.42	7.2		2.75		9.59		Zhang et al., 2017
	50	18.03			0.0379	2.39	−0.1427	9.53	−0.0065	
	150	18.30			0.0527	6.83	0.9098	9.49	−0.0018	
Sichuan, China	0	26.70	4.94	5.4				4.76		Zhao et al., 2014
	250	30.60			0.1363			2.76	−0.5450	
	0	32.80	5.27	6.2				5.02		

	250	31.20			−0.0500			3.18	−0.4565	
Hebei, China	0	7.90	0.83	9.5		0.0811		7.29		Zhao et al., 2016
	360	9.50			0.1844	0.1336	0.4984	6.76	−0.0750	
	0	7.90	0.83	9.5		0.0560		7.29		
	360	10.20			0.2555	0.0941	0.5191	6.95	−0.0472	
	0	7.90	0.83	9.5		0.0560		7.29		
	360	11.10			0.3401	0.1072	0.6489	6.82	−0.0655	
Urumqi, China	0	1.00	0.11	9.4				1.49		Zhou et al., 2012
	5	1.10			0.0953			1.46	−0.0210	
	10	1.10			0.0953			1.44	−0.0309	
	30	1.10			0.0953			1.44	−0.0358	
	60	1.00			0.0000			1.37	−0.0851	
	240	1.00			0.0000			1.23	−0.1878	
	0	0.70	0.08	8.4				1.62		
	5	0.60			−0.1542			1.59	−0.0178	
	10	0.70			0.0000			1.62	−0.0015	
	30	0.60			−0.1542			1.59	−0.0163	
	60	0.70			0.0000			1.59	−0.0163	
	240	0.60			−0.1542			1.55	−0.0420	
	0	1.20	0.15	8.1				1.75		
	5	1.20			0.0000			1.73	−0.0074	
	10	1.40			0.1542			1.71	−0.0229	
	30	1.40			0.1542			1.63	−0.0662	
	60	1.50			0.2231			1.60	−0.0877	
	240	1.90			0.4595			1.25	−0.3363	
	0	0.80	0.10	8.4				1.72		

5	0.70	−0.1335	1.73	0.0014	
10	0.80	0.0000	1.56	−0.0973	
30	0.60	−0.2877	1.55	−0.1089	
60	0.60	−0.2877	1.54	−0.1138	
240	0.60	−0.2877	1.39	−0.2183	

TABLE SII

Pearson correlations between microbial community structure and soil properties or plant biomass

Variable	Bacteria	G^+	G^-	Fungi	AMF	F/B	G^+/G^-
pH	−0.261	−0.199	−0.193	−0.093	−0.041	0.417	0.046
SOC	−0.490	0.154	**−0.547***	−0.361	−0.252	0.411	**0.647****
TN	**−0.543***	0.116	**−0.594***	−0.400	−0.288	0.439	**0.668****
C/N	−0.135	0.186	−0.160	−0.116	−0.043	0.146	0.284
NH_4^+	0.019	−0.043	0.067	−0.058	0.033	−0.119	−0.096
NO_3^-	**0.640****	−0.029	**0.698****	**0.510***	0.445	−0.438	**−0.724****
DOC	0.228	0.089	0.184	0.196	0.116	−0.154	−0.102
DON	**0.791*****	0.309	**0.783*****	**0.648****	**0.602***	**−0.501***	**−0.644****
AP	−0.084	0.021	−0.118	−0.294	−0.229	−0.451	0.175
AGB	0.467	−0.246	0.521	0.251	0.136	**−0.539***	**−0.619***
BGB	0.240	0.006	0.316	0.415	0.459	0.260	−0.376
Grasses	0.149	**−0.505***	0.237	0.002	−0.058	−0.331	−0.464

Sedges	0.263	−0.071	0.358	0.188	0.224	−0.239	−0.432
Legumes	−0.141	−0.020	−0.189	−0.069	−0.043	0.160	0.206
Forbs	0.484	0.446	0.397	0.380	0.262	−0.300	−0.163

SOC, soil organic carbon; TN, total nitrogen; C/N, ratio of SOC to TN; NH_4^+, ammonium; NO_3^-, nitrate; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; AP, available phosphorus; AGB, aboveground plant biomass; BGB, belowground plant biomass; Grasses, grasses biomass; Sedges, sedges biomass; Legumes, legumes biomass; Forbs, forbs biomass; G^+, gram-positive bacteria; G^-, gram-negative bacteria; AMF, arbuscular mycorrhizal fungi; F/B, ratio of fungi to bacteria. Values in bold indicate significant correlations at * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

TABLE SIII

Pearson correlations between enzyme activities and soil properties or plant biomass

	pH	SOC	TN	C/N	NH_4^+	NO_3^-	DOC	DON	AP	AGB	BGB	Grasses	Sedges	Legumes	Forbs
BG	−0.255	**−0.649****	**−0.685****	−0.232	−0.102	0.306	−0.150	**0.576***	−0.392	**0.578***	−0.184	0.435	0.070	−0.187	0.296
POX	−0.282	**−0.802*****	**−0.834*****	−0.396	−0.018	**0.725****	0.287	**0.757****	−0.043	**0.733****	0.183	0.460	0.067	−0.197	**0.529***
NAG	−0.200	−0.488	**−0.519***	−0.242	0.024	**0.735****	0.392	0.417	0.195	**0.670****	0.317	**0.581***	0.220	0.094	0.092
LAP	−0.028	0.192	0.161	0.402	0.459	−0.100	−0.301	0.162	−0.004	−0.111	0.070	−0.090	0.458	−0.265	−0.222

SOC, soil organic carbon; TN, total nitrogen; C/N, ratio of SOC to TN; NH_4^+, ammonium; NO_3^-, nitrate; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; AP, available phosphorus; AGB, aboveground plant biomass; BGB, belowground plant biomass; Grasses, grasses biomass; Sedges, sedges biomass; Legumes, legumes biomass; Forbs, forbs biomass; BG, β-1,4-glucosidase; POX, phenol oxidase; NAG, β-1,4-N-acetyl-glucosaminidase; LAP, leucine aminopeptidase. Values in bold indicate significant correlations at * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

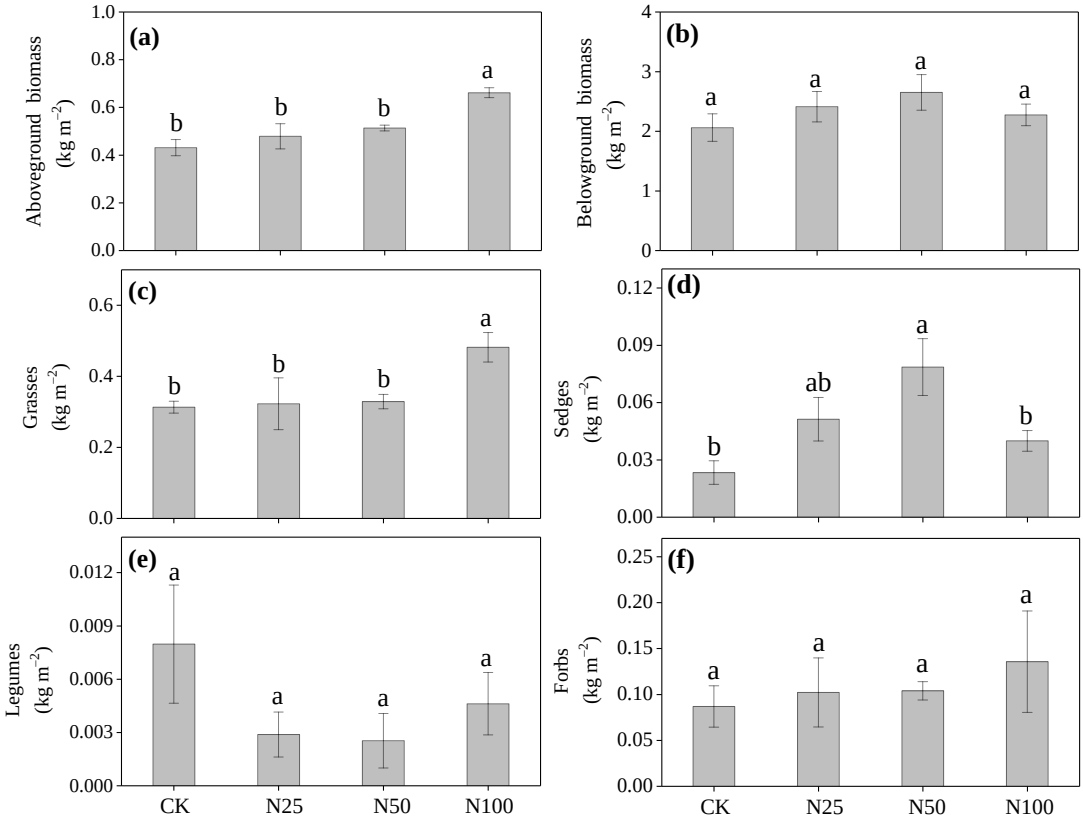

Fig. S1 Effects of N addition on above- (a) and belowground (b) biomass of the plant community and aboveground biomass of the four plant functional groups (c-f). Error bars are SEMs (n = 4), and different letters indicate significant differences among treatments at $p < 0.05$.

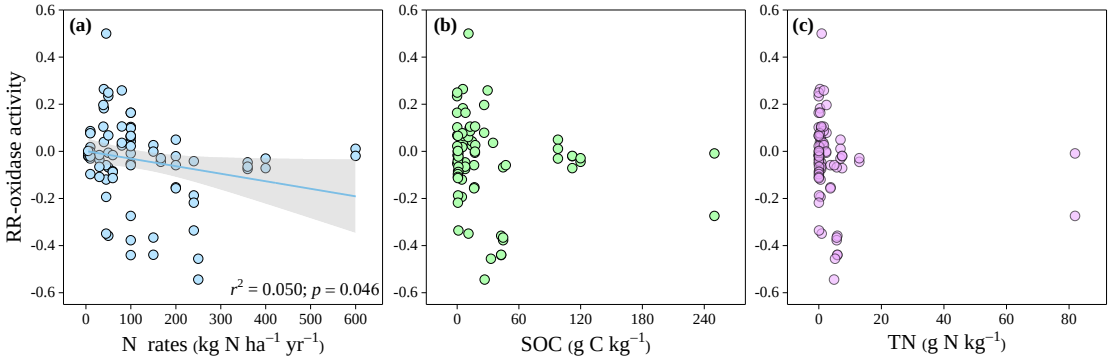

Fig. S2 Correlation between response ratio (RR) of oxidase activity and (a) rate of N addition, (b) SOC and (c) TN based on collected literature data. Shaded sections indicate the 95% confidence intervals of the regression models.

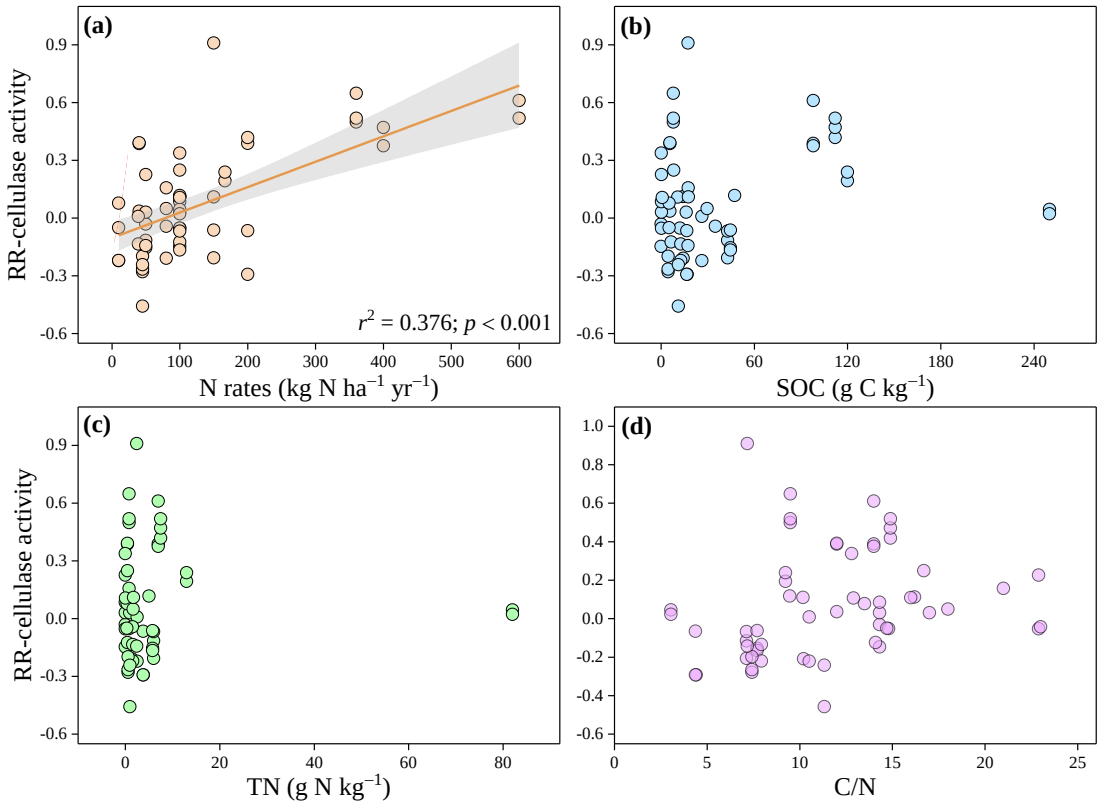

Fig. S3 Correlation between response ratio (RR) of cellulase activity and (a) rate of N addition, (b) SOC, (c) TN, and (d) C/N based on collected literature data. Shaded sections indicate the 95% confidence intervals of the regression models.

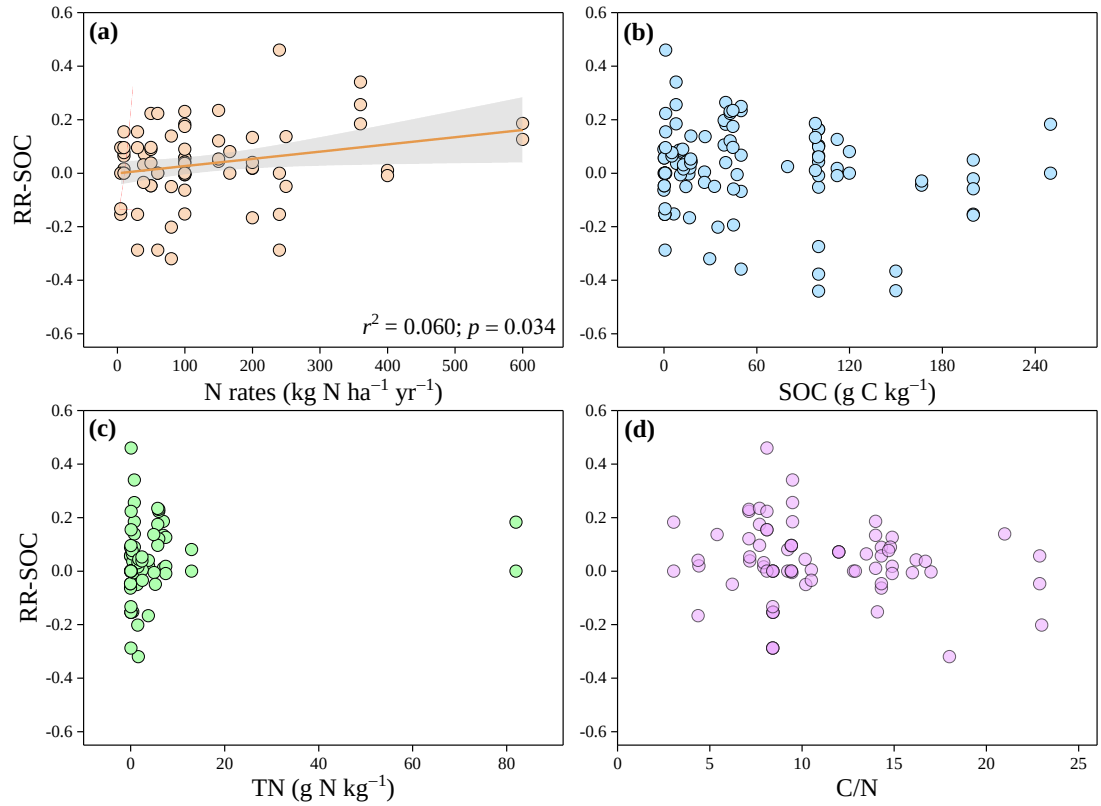

Fig. S4 Correlation between response ratio (RR) of SOC content and (a) rate of N

addition, (b) SOC, (c) TN, and (d) C/N based on collected literature data. Shaded sections indicate the 95% confidence intervals of the regression models.

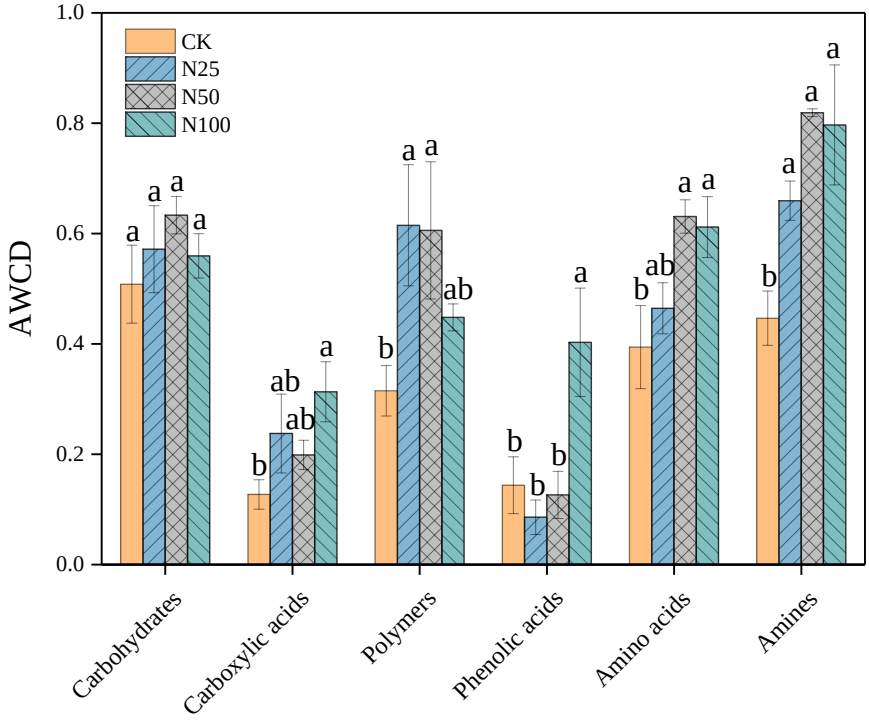

Fig. S5 Effects of N addition on microbial metabolism of different types of C substrates, based on changes in average well-color development (AWCD). Error bars are SEMs (n = 4), and different letters indicate significant differences among treatments at $p < 0.05$.

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