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REGULAR ARTICLE

# Minor responses of soil microbial biomass, community structure and enzyme activities to nitrogen and phosphorus addition in three grassland ecosystems

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**Abstract**

*Background and aims* Human activities have significantly increased nitrogen (N) and phosphorous (P) inputs to terrestrial ecosystems. However, the impact of N and P enrichment on soil microbial community structure and functioning in temperate and alpine grassland ecosystems remains unclear.

*Methods* In this study, we investigated the responses of soil microbial communities to nutrient (N and P) additions in two temperate and one alpine grassland ecosystems in China. We measured soil chemical properties, microbial community composition (indicated by the phospholipid fatty acids, PLFA) and potential enzyme activities related to carbon (C), N, and P cycling in the peak growing season after 4 years of nutrient addition.

*Results* We found that N addition reduced soil pH and increased soil total N content at two meadow sites, P addition increased soil total P content at all three sites, but both N and P additions had minimal effects on soil organic C content. Bacteria and total microbial abundances did not change after N and P additions, while fungi and arbuscular mycorrhizal fungi (AMF) abundances were suppressed by N addition. Moreover, the activity of soil extracellular enzymes involved in C, N and P cycling and their stoichiometric ratios were not responsive to N and P additions, except for inhibition of acid phosphatase by P addition at the temperate meadow site.

*Conclusions* Despite significant changes in soil chemistry (e.g., pH and available nutrients), soil microbial biomass (except fungi and AMF abundances), community structure, and enzyme activities (except phosphatase) were generally resistant to 4 years of N and P addition in the three temperate and alpine grassland ecosystems in China.

**Keywords** Grassland · Phospholipid fatty acids · Arbuscular mycorrhizal fungi · Soil extracellular enzymes · Nitrogen addition · Phosphorus addition

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## Introduction

Grassland is one of the most widely distributed terrestrial ecosystems in the world. It covers 26% of the land and also present distinctive formats of C cycling (Schimel 1995). Soil microbes drive belowground biogeochemical processes in terrestrial ecosystems (Falkowski et al. 2008). They also regulate aboveground primary productivity and mediate the response of multiple ecosystem functions (Jing et al. 2015). Therefore, soil microbes play a crucial role in mediating the responses of biodiversity and biogeochemistry to global changes in grassland ecosystems (van der Heijden et al. 2008; Leff et al. 2015).

Human activities have greatly enhanced nitrogen (N) and phosphorus (P) deposition to terrestrial ecosystems in the past decades (Galloway et al. 2004; Peñuelas et al. 2013). The mean rate of inorganic N deposition is comparably high in heavily populated industrial countries. For example, China's atmospheric deposition of N is 21 kg N $ha^{-1}$ $yr.^{-1}$ in the 2000s and will reach 50 kg N $ha^{-1}$ $yr.^{-1}$ by 2050 in hotspot areas (Liu et al. 2013). Moreover, the deposition of P has also been increasing in some areas recently, though to a lesser degree than N deposition (Peñuelas et al. 2013). This human-induced N and P inputs have dramatically altered plant productivity, microbial community composition, and the biogeochemistry of terrestrial ecosystems across the globe (Bobbink et al. 2010; Peñuelas et al. 2013; Luo et al. 2019).

Nitrogen enrichment increases soil N availability, reduces soil pH (acidification), and alters soil C and N cycling, as indicated by recent meta-analyses (e.g., Treseder 2008; Liu and Greaver 2010; Tian and Niu 2015). A large number of studies have reported on the impact of N addition on soil microbes, including biomass, community composition, and enzyme activities (e.g., Nemergut et al. 2008; Cusack et al. 2011; Leff et al. 2015; Sinsabaugh et al. 2015). For example, in a global network of nutrient-addition experiments in 25 grassland sites, N additions had a consistent effect on soil microbial community composition, modified relative abundance of mycorrhizal fungi and oligotrophic bacteria, and changed the relative abundance of functional genes (Leff et al. 2015). Moreover, in a temperate grassland in Inner Mongolia, China, N addition enhanced above- and belowground plant biomass, but suppressed soil bacterial and fungal biomass, largely due to N-induced soil acidification (Chen et al. 2015). Such shifts in microbial biomass and community composition following N addition may further lead to shifts in soil extracellular enzyme activity, which drives soil organic matter decomposition and nutrient cycling (Schimel and Weintraub 2003; Burns et al. 2013). Based on the resource allocation theory of enzyme production (Sinsabaugh and Moorhead 1994; Allison and Vitousek 2005), N addition may suppress the activity of N-cycling and oxidative enzymes targeting soil organic nitrogen and recalcitrant organic matter, respectively, but may enhance the activity of hydrolytic enzymes involved in C and P cycling. This theory has been experimentally supported in some (Olander and Vitousek 2000; Marklein and Houlton 2012), but not all studies (Keeler et al. 2009; Jing et al. 2017; Xiao et al. 2018).

Terrestrial ecosystems may shift from nitrogen limitation to phosphorus limitation due to anthropogenic nitrogen (N) inputs and N-based fertilizers (Peñuelas et al. 2013). Although the intensity of atmospheric deposition of P has been increasing and the impact of P deposition on carbon cycling of terrestrial ecosystems has been increasingly recognized (Peñuelas et al. 2013; Wieder et al. 2015), relatively fewer field manipulation studies have been conducted to investigate the impact of atmospheric deposition of P or fertilization on grassland ecosystems. Generally, tropical ecosystems are considered more P-limited, and temperate and boreal ecosystems (including forests and grasslands) are likely more N-limited (Vitousek et al. 2010). However, whether grassland ecosystems are sensitive to atmospheric deposition of P remains poorly understood. For example, Massey et al. (2016) reported that soil microbial communities were insensitive to long-term P fertilization in two grassland ecosystems in the UK, while Ling et al. (2017) showed that soil microbial community structure shifted with P addition in a temperate grassland in China. In the global-scale study of 25 grassland sites, P addition consistently shifted bacterial and fungal community composition and the relative abundance of microbial functional genes (Leff et al. 2015).

In this study, we investigated the responses of soil microbial communities to N and P addition in three contrasting grassland ecosystems. The three sites include an alpine meadow on the Tibetan Plateau, and a temperate meadow and a temperate steppe on the Inner Mongolian Plateau in China. The objectives of this study were to (1) evaluate how N and P addition affected soil physicochemical and microbial properties; and (2)

explore the underlying mechanisms of these properties in response to N and P addition in each grassland ecosystem. We hypothesized that nutrient addition would lead to consistent changes in soil pH and nutrient availability, and further shifts in microbial biomass and community structure in the three grassland sites. Specifically, N addition would cause soil acidification, suppress microbial biomass, and change microbial community structure (Treseder 2008; Leff et al. 2015; Ling et al. 2017); while P addition would have a weaker impact on these variables because grassland ecosystems are generally not P-limited (Vitousek et al. 2010). Moreover, we hypothesized that both N and P addition would significantly affect soil enzyme activity, consistent with the resource allocation theory (Sinsabaugh and Moorhead 1994; Allison and Vitousek 2005). Specifically, N addition would suppress N-acquisition and oxidative enzymes and stimulate C and P acquisition enzymes, while P addition would suppress P-acquisition enzyme and stimulate other enzymes.

## Materials and methods

### Site description

Three grassland sites assayed in this study are located in northern China (Fig. S1). The first site is an alpine meadow in Haibei (37°36′N, 101°19′E) on the Tibetan Plateau, with a typical and continental monsoon climate. The mean annual temperature (MAT) is −1.7 °C and mean annual precipitation (MAP) is 500 mm (Table 1). The plant community is dominated by *Kobresia humilis* and *Elymus nutans*, and the soil is Cambisols. The second site is a temperate meadow in Hailaer (49°21′ N, 120°07′E) on the Mongolian Plateau, which has a temperate continental monsoon climate. The MAT is −2.0 °C and MAP is 350 mm (Table 1). The plant community is dominated by *Leymus chinensis* and *Stipa baicalensis* and the soil is Chernozem. The third site is a temperate steppe in Maodeng (44°10′N, 116°28′E) on the Mongolian Plateau, with *Stipa krylovii* and *Leymus chinensis* as dominant plants and Kastanozem as the dominant soil. The climate here is semi-arid continental climate with MAT of 1.5 °C and MAP of 275 mm (Table 1). More detailed site information can be found in Table 1.

Since May 2011, bifactorial N and P addition experiments were established in these three sites. The two experiments in Hailaer and Maodeng were randomized block designs with five field replicates. The two factors were N addition (10 g N $m^{-2}$ $yr.^{-1}$) and P addition (10 g P $m^{-2}$ $yr.^{-1}$). The same experiment design was used in Haibei with six field replicates (samples were taken from five replicates). The two factors were N addition (10 g N $m^{-2}$ $yr.^{-1}$) and P addition (5 g P $m^{-2}$ $yr.^{-1}$). These rates of addition were considerably higher than the natural deposition rates of N and P at these sites (Zhu et al. 2016), but were comparable to the rates of addition (10 g N or P $m^{-2}$ $yr.^{-1}$) in a global-scale nutrient-addition experimental network in grassland ecosystems (NutNet, Borer et al. 2014). Nitrogen fertilizers were selected as urea ($CO(NH_2)_2$), and phosphorus fertilizers were selected as triple superphosphate ($Ca(H_2SO_4)_2{\cdot}H_2O$). These fertilizers were dissolved in deionized water and evenly applied on the ground using a backpack sprayer at the beginning of each month during the growing season (from May to August). The unfertilized plots received the same amount of water.

### Soil sampling and measurement

In August 2015 (peak month for standing plant biomass), three soil cores (5 cm in diameter) at 0–10 cm depth were randomly taken from each plot (6 × 6 m) and then mixed to a composite sample. A total of 60 soil samples (3 sites × 4 treatments × 5 replicates) were taken and transported to the lab refrigerated by a cooler with ice bags. Once in the lab, the samples were passed through a 2-mm sieve and the remaining roots were picked out. After homogenizing, soil water content (SWC) was measured immediately by drying at 105 °C for 24 h and weighted. The remaining soil was divided into two parts: one was stored at −20 °C for measuring extracellular enzyme activities (EEA) and microbial phospholipid fatty acids (PLFA); one was air-dried to measure soil pH, soil organic carbon (SOC), total nitrogen (TN) and total phosphorus (TP). Soil pH was measured using a pH meter (S210 SevenCompact™, Mettler-Toledo, Switzerland) after shaking a soil water suspension (soil: water ratio 1:2) for 30 min. After removing inorganic carbon by acid fumigation, SOC and TN content were determined by an elemental analyzer (Vario EL III, Elementar, Germany). Soil TP content was measured by a molybdate/ascorbic acid method after $H_2SO_4$-$HCLO_4$ digestion.

In early September 2015, we measured potential activities of seven extracellular enzymes, including five

**Table 1** The basic information about the three grassland sites

| Site | Location | MAT (°C) | (mm) | Vegetation type | Dominant species | Soil type | pH | SOC (mg $g^{-1}$) | TN (mg $g^{-1}$) | TP (mg $g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Haibei | 101°19′N, 37°36′E | −1.7 | 500 | Alpine meadow | *Kobresia humilis, Festuca ovina* | Cambisols | 7.50(0.01) | 68.58(1.20) | 5.59(0.21) | 0.06(0.07) |
| Hailaer | 49°21′N, 120°07′E | −2.0 | 350 | Temperate meadow | *Stipa baicalensis, Leymus chinensis* | Chernozem | 6.86(0.09) | 46.29(3.16) | 4.28(0.28) | 0.64(0.64) |
| Maodeng | 44°10′N, 116°28′E | 1.5 | 275 | Temperate steppe | *Stipa krylovii, Leymus chinensis* | Kastanozem | 8.55(0.02) | 11.45(0.49) | 1.43(0.04) | 0.42(0.40) |

MAT: mean annual temperature (°C); MAP: mean annual precipitation (mm $yr.^{-1}$); SOC: soil organic C content (mg $g^{-1}$, 0–10 cm); TN: soil total N content (mg $g^{-1}$); TP: soil total P content (mg $g^{-1}$). Values in the bracket indicate standard error (SE, $n = 5$). Soil type is based on the FAO soil taxonomy system

hydrolytic enzymes (BG: β-1,4-glucosidase, CB: β-D-cellobiohydrolase, involved in C cycling; NAG: β-1,4-N-acetyl-glucosaminidase, LAP: Leucine aminopeptidase, involved in N cycling; AP: Acid phosphatase, involved in P cycling) and two oxidative enzymes (POX: phenol oxidase and PER: peroxidase, involved in C cycling) (Table S2). As the mean pH of our soil samples is about 7.6 (Fig. 1), we performed enzyme assays in soil slurries made with Tris buffer (adjust pH with HCl to 8.0) using 96-well micro-plates (German et al. 2011). All plates were incubated in the dark at 25 °C before analysis. The incubation time is 2.5 h for hydrolytic enzymes and 4 h for oxidative enzymes. We used a micro-plate reader (SynergyH1M, Biotek, USA) to measure the quantity of fluorescence for hydrolytic enzymes or absorbance for oxidative enzymes (Chen et al. 2018).

We measured soil microbial community structure indicated by PLFA (Bossio and Scow 1998; Chen et al. 2018) in late September 2015. We extracted total lipids from freeze-dried soil samples (equivalent to 8 g fresh soil) using a chloroform-methanol extraction modified to incorporate a 0.05 M phosphate buffer. The PLFAs were purified from the lipids extracts, quantified and identified using a Gas Chromatograph (6890, Hewlett Packard, USA) fitted with a 25-m Ultra 2 (5%-phenyl)-methylpolysiloxane column. Individual PLFAs were identified using microbial fatty acid standards and Microbial ID peak identification software. All fatty acids accounting for >0.5% of total fatty acids were used in further analyses (Wang et al. 2010; Chen et al. 2018). The fatty acids were quantified by comparing the individual PLFA peak areas with that of the internal standard 19:00. PLFAs were expressed in nmol PLFA $g^{-1}$ dry soil. The 48 markers (except 19:00) were divided into three groups (Table S1): 40 markers for bacteria, two markers for fungi including one for arbuscular mycorrhizal fungi (AMF) and six markers for actinomycetes.

## Statistical analysis

All statistical analyses were performed with the statistical software R 3.3.3 (R Development Core Team 2016). We used linear mixed-effects model to test for the effects of N addition, P addition, and their interaction on soil properties, microbial biomass, community composition and enzyme activities. In the model, block was treated as a random factor, N addition, P addition, and their

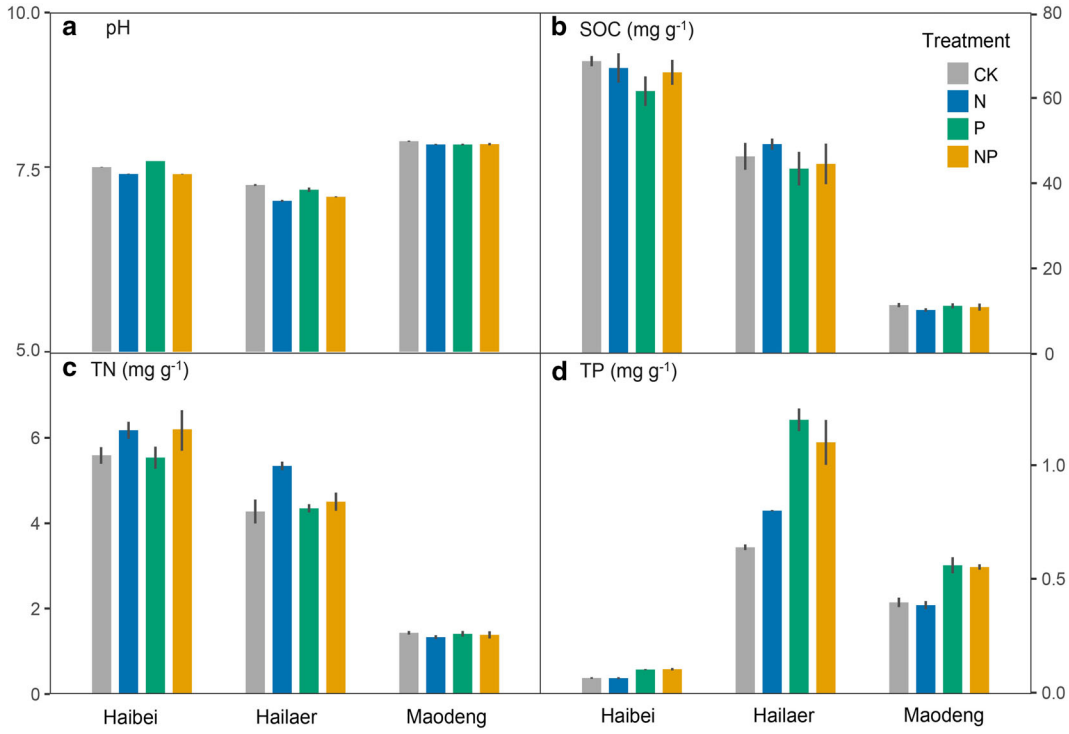



**Fig. 1** The effects of nutrient addition on soil properties in the three grassland sites. **a** Soil pH; (**b**) SOC: soil organic carbon (mg $g^{-1}$); (**c**) TN: total nitrogen (mg $g^{-1}$); (**d**) TP: total phosphorus (mg $g^{-1}$). Values are means ± SE ($n = 5$)

interaction were treated as fixed factors at each site (Table 2), and N addition, P addition, site and their interactions were treated as fixed factors with all data combined (Table S3). The models were fitted with the *lmer* function in the *lme4* package (Bates et al. 2015).

Principal components analysis (PCA) were conducted with the *rda* function in the *vegan* package (Oksanen et al. 2019) in R to analyze the data of all the PLFA markers and the seven extracellular enzymes for differentiating sampling sites and nutrient treatments with data at all three sites (Fig. 4) as well as at each site (Fig. S2). We conducted correlation tests by using *cor* function in *psych* package (Revelle 2018) to examine the bivariate correlation between soil variables (pH, SOC, TN, TP) and microbial variables (total PLFA, actinomycetes, bacteria, fungi, AMF, fungi:bacteria, C-acquisition enzyme, N-acquisition enzyme, P-acquisition enzyme and oxidase) in each site respectively (Fig. 5) and together with data from all three sites (Fig. S3).

As the relative abundance of fungi and AMF had been altered by nutrient additions (Table 2, Fig. 2), we fitted a structural equation model (SEM) based on the known and potential relationships among different variables to determine the effect of N and P addition on soil properties (pH, SOC, TN, TP) and eventually on fungi or AMF abundance (Fig. 6) in each site. Natural-log transformations were selected in SEM to improve the normality of the data. We chose the $\chi^2$ test and the root mean square error (RMSE) of approximation to evaluate the fitness of the model. We also sequentially optimized SEMs until we attained the final model. SEMs were conducted in R with *lavaan* package (Rosseel 2012).

## Results

### Soil chemical properties

Soil pH was lower in the two relatively wet meadow sites (Haibei and Hailaer) than the relatively dry steppe site (Maodeng, Fig. 1). Notably, N addition significantly reduced soil pH at the two meadow sites but not at the steppe site, while P addition only increased soil pH at the alpine meadow (Haibei) (Table 2; Fig. 1).

**Table 2** Summary of the linear mixed-effects models showing the effects of nutrient addition on soil properties, microbial biomass, microbial community composition and enzyme activity in three research sites

| | | Haibei | | | Hailaer | | | Maodeng | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | N | P | N*P | N | P | N*P | N | P | N*P |
| Soil properties | pH | 720.60*** | 63.58*** | 70.09*** | 92.82*** | 0.08 | 13.54** | 2.98 | 3.19 | 4.82 |
| | SOC | 1.55 | 0.19 | 0.52 | 0.34 | 1.19 | 0.07 | 1.55 | 0.19 | 0.52 |
| | TN | 9.56** | 0.01 | 0.04 | 10.44** | 4.08 | 5.80* | 1.18 | 0.07 | 0.45 |
| | TP | 0.02 | 92.03*** | 0.08 | 0.32 | 60.15*** | 5.51* | 0.21 | 56.33*** | 0.01 |
| Microbial biomass and community composition | MBC | 0.76 | 2.45 | 1.35 | 2.88 | 0.19 | 0.34 | 4.00 | 2.74 | 7.88* |
| | Total PLFA | 0.03 | 0.85 | 2.48 | 3.35 | 0.16 | 2.13 | 3.35 | 0.16 | 2.13 |
| | Actinomycetes | 0.06 | 1.82 | 0.80 | 1.28 | 3.04 | 0.77 | 2.56 | 0.04 | 0.60 |
| | Bacteria | 0.05 | 0.67 | 2.78 | 2.97 | 3.13 | 0.39 | 3.06 | 0.30 | 2.29 |
| | Fungi | 1.75 | 0.84 | 6.78* | 7.72* | 4.14 | 1.04 | 8.18* | 0.29 | 3.28 |
| | AMF | 7.21* | 3.23 | 1.33 | 6.00* | 4.40 | 1.34 | 5.80* | 1.66 | 2.55 |
| | Fungi: Bacteria | 2.53 | 3.00 | 0.06 | 8.33* | 2.93 | 0.69 | 1.99 | 3.15 | 0.00 |
| | G+ | 0.05 | 2.34 | 4.65 | 3.69 | 0.49 | 0.00 | 3.98 | 1.19 | 2.21 |
| | G- | 0.04 | 0.45 | 0.22 | 1.40 | 3.15 | 0.11 | 0.36 | 0.14 | 0.40 |
| | G+: G- | 0.00 | 3.76 | 1.87 | 0.00 | 1.41 | 0.00 | 3.65 | 1.80 | 1.80 |
| Enzyme activities | C enzyme | 0.20 | 2.41 | 0.14 | 0.77 | 0.13 | 3.27 | 0.32 | 0.49 | 1.35 |
| | N enzyme | 0.02 | 0.64 | 0.79 | 0.42 | 0.45 | 0.54 | 1.25 | 0.60 | 1.38 |
| | P enzyme | 0.76 | 2.14 | 1.64 | 0.49 | 7.03* | 2.22 | 0.07 | 0.43 | 0.07 |
| | Oxidase | 0.35 | 1.67 | 0.47 | 2.80 | 0.08 | 2.49 | 2.44 | 10.78** | 1.66 |
| | Enzyme C: N | 0.17 | 4.67 | 0.28 | 0.01 | 0.40 | 3.91 | 0.01 | 3.54 | 0.08 |
| | Enzyme C: P | 0.38 | 1.11 | 0.16 | 0.27 | 2.76 | 1.50 | 0.61 | 2.71 | 3.32 |
| | Enzyme N: P | 0.35 | 0.00 | 0.03 | 0.25 | 5.38* | 0.00 | 1.42 | 0.13 | 4.09 |

The F values are shown in the table. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. SOC, soil organic carbon (mg $g^{-1}$); TN, total nitrogen (mg $g^{-1}$); TP, total phosphorus (mg $g^{-1}$); MBC, microbial biomass carbon (mg $g^{-1}$); PLFA, phospholipid fatty acid; microbial community compositions include total PLFA (nmol $g^{-1}$), actinomycetes (nmol $g^{-1}$), bacteria (nmol $g^{-1}$), fungi (nmol $g^{-1}$), AMF, arbuscular mycorrhizal fungi (nmol $g^{-1}$), the ratio of fungi to bacteria (unitless). G+, Gram-positive bacteria (nmol $g^{-1}$); G-, Gram-negative bacteria (nmol $g^{-1}$); C-enzyme, BG + CB (nmol $g^{-1}$ $h^{-1}$), N-enzyme, NAG+LAP (nmol $g^{-1}$ $h^{-1}$); P-enzyme, AP (nmol $g^{-1}$ $h^{-1}$); Oxidase, PER+POX (μmol $g^{-1}$ $h^{-1}$). Ratios of ln(BG+CB): ln(NAG+LAP), ln(BG + CB): ln(AP) and ln(NAG+LAP): ln(AP) were calculated to represent enzyme C: N, C: P and N: P ratio

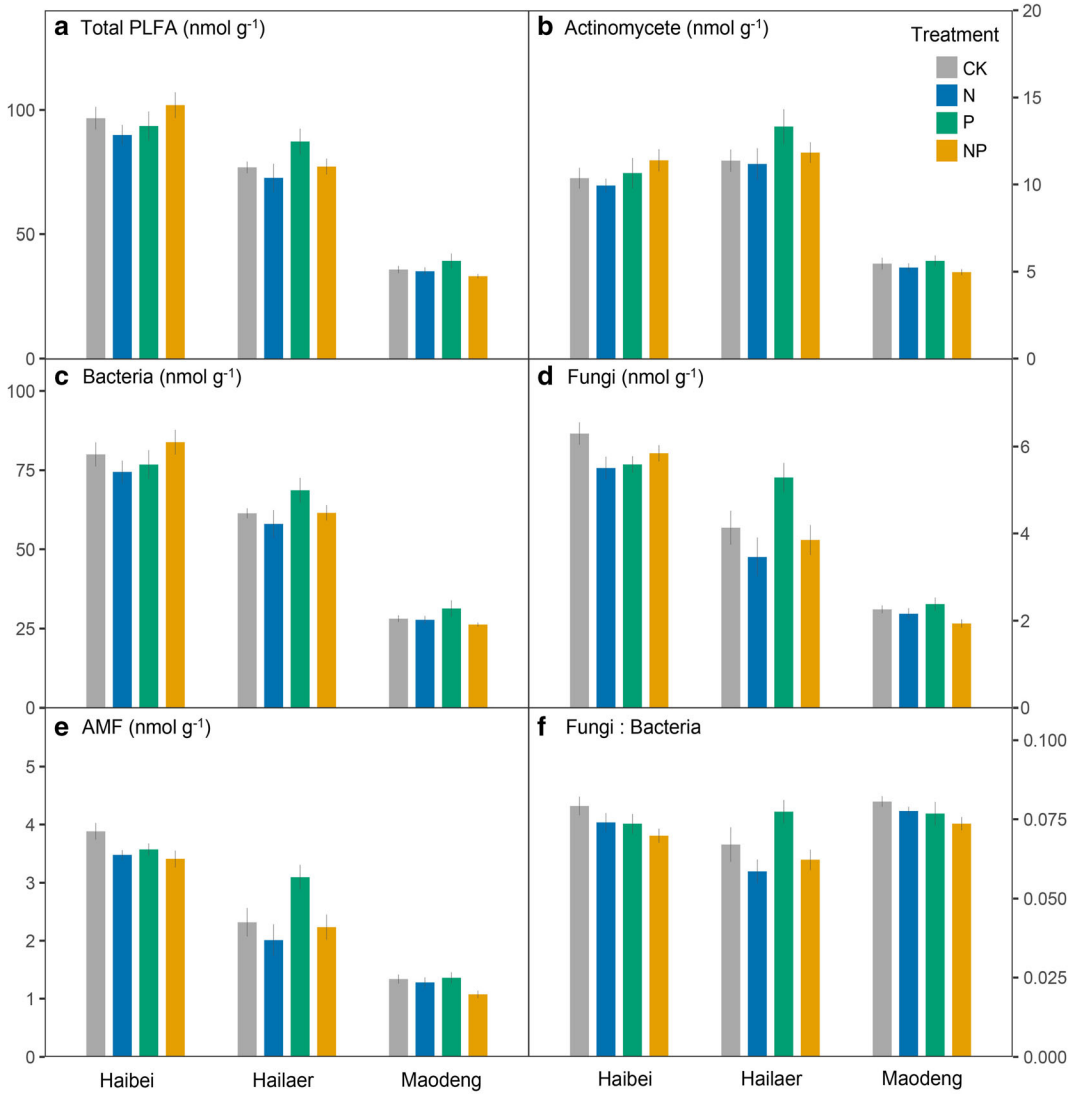



**Fig. 2** The effects of nutrient addition on soil microbial biomass and the major soil microbial community groups in the three grassland sites. (**a**) Total PLFA (nmol $g^{-1}$); (**b**) Actinomycete (nmol $g^{-1}$); (**c**) Bacteria (nmol $g^{-1}$); (**d**) Fungi (nmol $g^{-1}$); (**e**) AMF: arbuscular mycorrhizal fungi (nmol $g^{-1}$) and (**f**) the ratio of fungi and bacteria. Values are means ± SE ($n = 5$)

Soil organic carbon (SOC) was higher in the two meadow sites than the steppe site and was not responsive to N or P addition at all three sites (Fig. 1, $P > 0.05$). Soil total nitrogen (TN) was increased by N addition at the two meadow sites but was not sensitive to N or P addition at the steppe site (Table 2; Fig. 1). Different from SOC and TN, soil total phosphorus (TP) was highest at the temperate meadow (Hailaer), intermediate at the temperate steppe (Maodeng), and lowest at the alpine meadow (Haibei). Notably, P addition consistently increased soil TP content at all three sites (Fig. 1, $P < 0.001$).

## Soil microbial groups and enzyme activities

Total PLFA and all microbial groups tended to be higher at the two meadow sites (Haibei and Hailaer) than the steppe site (Maodeng) (Fig. 2). Generally, nutrient addition had little effect on the total PLFA and different microbial groups at each site (Table 2, Fig. 2, $P > 0.05$), except that fungi, AMF and F:B ratio were suppressed by N addition, particularly at Hailaer (Table 2, Fig. 2, $P < 0.05$).

The EEA showed similar variation across the sites as SOC and most microbial groups, being highest at Haibei

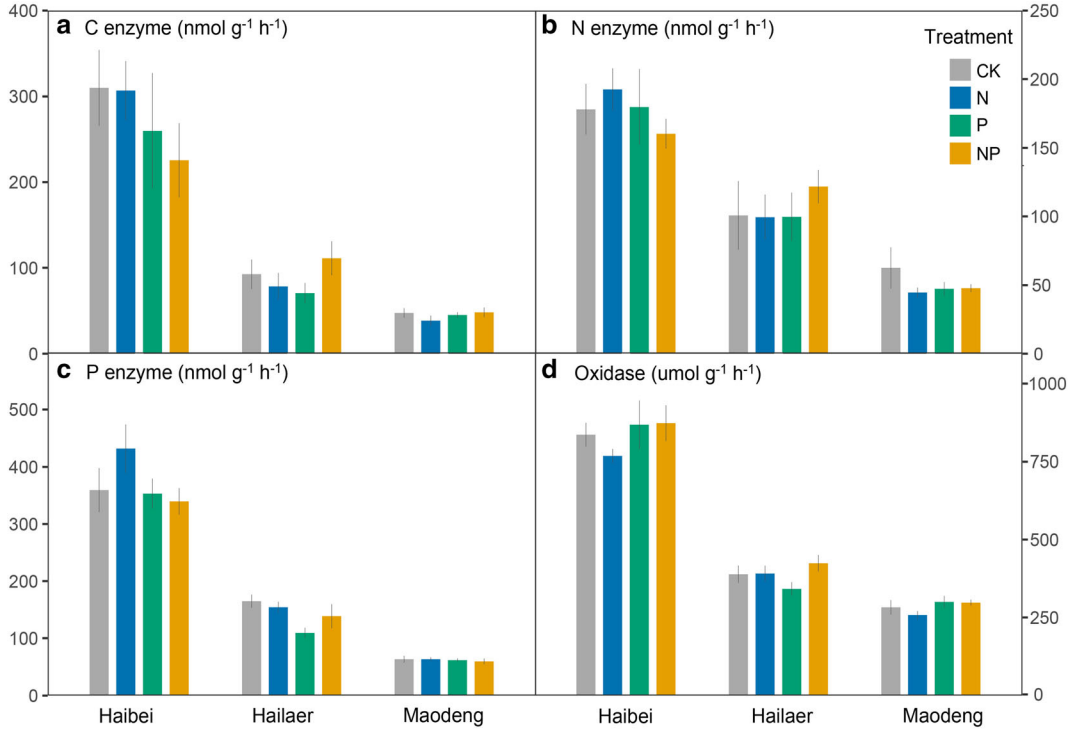



**Fig. 3** The effects of nutrient addition on soil enzyme activities in the three grassland sites. (**a**) C enzyme: BG+CB (nmol $h^{-1}$ $g^{-1}$); (**b**) N enzyme: NAG+LAP (nmol $h^{-1}$ $g^{-1}$); (**c**) P enzyme: AP (nmol $h^{-1}$ $g^{-1}$); (**d**) Oxidase: POX+PER (μmol $h^{-1}$ $g^{-1}$). Values are means ± SE ($n = 5$)

and lowest at Maodeng (Fig. 3). Both C- and N-acquisition hydrolytic and oxidative enzymes were responsive to nutrient addition at none of the three sites (Table 2, Fig. 3, $P > 0.05$). In contrast, the P-acquisition enzyme (acid phosphatase) was suppressed by P addition (33.7%) at Hailaer (Table 2, Fig. 3, $P < 0.05$) where had the highest soil TP content (Fig. 1), but was not sensitive to nutrient addition at the other two sites (Table 2, Fig. 3, $P > 0.05$).

Overall, a consistent pattern across sampling sites and nutrient treatments emerges between microbial groups and enzyme activities. The PCA results (Fig. 4) clearly showed that microbial community composition and enzyme activities differed significantly among the three grassland sites ($P < 0.001$), but were insensitive to nutrient addition treatments ($P > 0.05$). The overall insensitivity of PLFA and enzyme to nutrient addition (with notable exceptions for fungi, AMF and phosphatase as mentioned earlier) was evident when data from all three sites were analyzed together (Fig. 4) or separately (Fig. S2). This pattern was also reflected in the result of the mixed-effect model (Table 2, S3).

## Relationships between soil properties and microbial variables

We then used correlation analyses to address the relationships between microbial variables and soil physicochemical properties in the three grasslands respectively (Fig. 5), and we only detected significant negative correlations between soil TP and P-acquisition enzyme in Hailaer. However, when we analyzed the whole data across the three sites and the four treatments (Fig. S3), soil pH and TP had negative correlations ($r < 0$) while SOC and TN had positive correlations ($r > 0$) with most of the microbial groups and enzyme activities.

Among the microbial variables (PLFA and EEA), only fungi and AMF responded significantly to nutrient additions (Table 2, Figs. 2 and 3). Therefore, we quantified the various pathways of nutrient addition on soil properties (pH, SOC, TN, and TP) and further on fungi and AMF at the three sites by constructing a structural equation model (SEM, Fig. 6). In the two meadow sites (Haibei and Hailaer, Fig. 6a and b), N addition acidified soils and increased N content, which had a negative effect

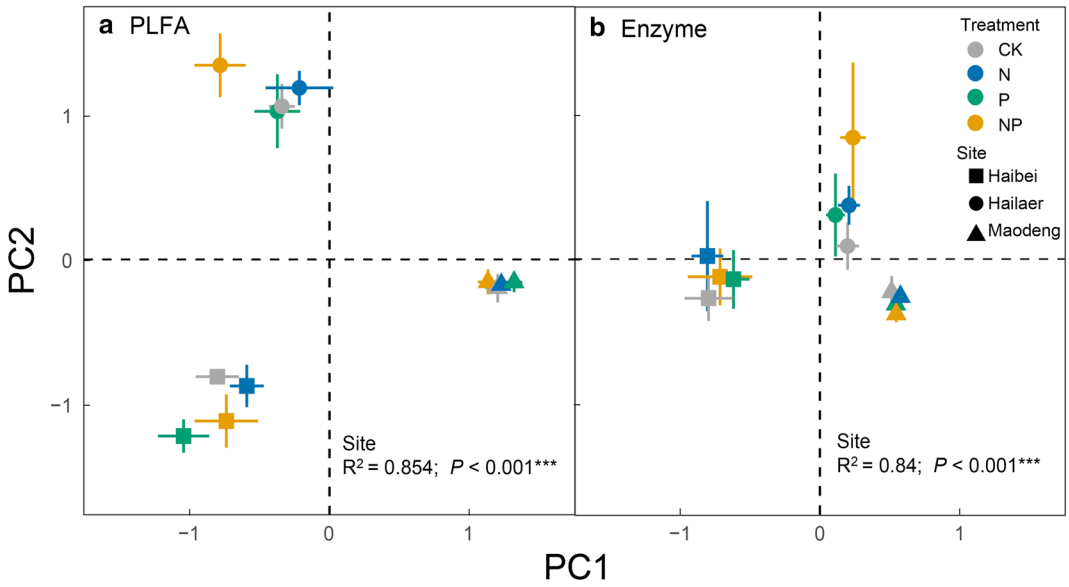



**Fig. 4** Principal component analysis (PCA) for (**a**) all the PLFA markers, in which PC1 explains 59.1% and PC2 explains 8.9%; (**b**) seven extracellular enzymes (BG, CB, NAG, LAP, AP, POX, PER), in which PC1 explains 85.0% and PC2 explains 5.1%. Average of PC values with standard error are shown in (A) and (B). PERMANOVA statistics refer to significant site effects

on fungi and AMF abundance. Such negative effect of N addition on fungi and AMF abundance was less significant at the steppe site (Maodeng, Fig. 6c). In addition, P addition had consistent positive effect on soil total P content across all three sites, and tended to stimulate fungi and AMF abundance only at Hailaer (Fig. 6b) but not at Haibei and Maodeng (Fig. 6a and c). These results support earlier analyses (Table 2, Fig. 2).

## Discussion

We originally hypothesized N addition would cause soil acidification, suppress microbial biomass, and change microbial community structure (Treseder 2008; Leff et al. 2015), while P addition would have a weaker impact on these variables. Our results only partly supported this hypothesis because 4 years of both N and P addition had minimal effects on microbial biomass and community structure (indicated by PLFA). Moreover, we hypothesized that both N and P addition would significantly affect soil enzyme activity, consistent with the resource allocation theory (Sinsabaugh and Moorhead 1994; Allison and Vitousek 2005). However, our results did not support this hypothesis as the hydrolytic and oxidative enzymes and their stoichiometric ratios were mostly not responsive to N or P addition. Collectively, these results suggest that despite significant changes in soil chemistry (e.g., pH and available nutrients), soil microbial biomass (except fungi and AMF abundances), community structure (indicated by PLFA), and enzyme activities (except phosphatase) were generally resistant to 4 years of N and P addition in the three temperate and alpine grassland ecosystems in China.

### Effects of nutrient addition on soil chemical properties

After 4 years of N and P addition, we detected significant changes in soil pH at the two meadow sites, but not at the steppe site. The soil acidification by N addition (10 g urea-N $m^{-2}$ $yr.^{-1}$) at the two meadow sites is not surprising, because it was frequently observed in grassland ecosystems (Chen et al. 2015; Tian and Niu 2015). The weaker effect at Maodeng was likely associated with its lower precipitation, initial SOC and TN content, and higher initial soil pH. A synthesis study (Tian and Niu 2015) showed that the effect size of N addition on soil pH was positively related to precipitation, initial SOC and TN content, but negatively related to initial soil pH. Compared to N addition, the effect of P addition

on soil pH was much weaker, being only significant at Haibei. This pattern is consistent with the literature. For example, a long-term nutrient addition experiment at three alpine meadows in the Rocky Mountain also showed strong soil acidification by N addition, but no effect of P addition on soil pH (Yuan et al. 2016).

The total SOC content of bulk soil did not respond to the 4-year addition of nutrients at any of the three sites in this study (Fig. 1). This result was consistent with case studies and meta-analyses in non-agricultural (particularly grassland) ecosystems. For example, an earlier meta-analysis by Lu et al. (2011) found no change in soil carbon by N addition in grassland ecosystems, which was confirmed by recent work (e.g., Riggs et al. 2015; Ling et al. 2017; Chen et al. 2019; Crowther et al. 2019). Two mechanisms may explain the lack of significant response of SOC to nutrient addition in grassland ecosystems. Firstly, the enhanced plant input to soil (through both aboveground and belowground biomass) by nutrient addition may have been cancelled by stimulated decomposition of litter and soil organic matter at the three sites (Wang 2018). Secondly, the slow turnover and high spatial heterogeneity of SOC in the cold or dry grassland ecosystems of this study may require a long observation period to detect a statistically significant change in SOC of bulk soil with nutrient treatments (Hungate et al. 1996; Schrumpf et al. 2011).

Unlike SOC, soil TN or TP content were sensitive to N or P addition. Soil TN was higher in the plots with urea addition in the two meadow sites, but not in the steppe site (Fig. 1). The weaker effect at the dry steppe site (Maodeng) was likely related to its high soil pH and presumably higher ammonia vitalization (Zhang et al. 2014). In contrast, soil TP content was elevated by P addition at all three sites, particularly at the two temperate sites which received a higher dose of P addition (10 g $m^{-2}$ per year), which was also found in similar P-addition studies in temperate grasslands (He et al. 2013). The shifts in soil organic matter stoichiometry (C:N and particularly C:P) may have implications for microbial activity and biogeochemical cycling (Cleveland and Liptzin 2007; Sinsabaugh et al. 2008), which merits further in-depth investigation.

## Effects of nutrient addition on soil microbial groups

We used a PLFA approach to quantify the abundance of different microbial groups (i.e. fungi, bacteria, actinomycetes) and microbial community composition. Total PLFAs were significantly positively correlated with microbial biomass carbon (Fig. S5), but did not respond to N or P additions at any of the three sites (Fig. 2). The same pattern was also observed for actinomycetes and bacteria, but not for fungi and AMF (Fig. 2). The lack of response of total PLFAs to N addition was also reported by a recent meta-analysis (Zhou et al. 2017). Possibly, the positive effect on microbial growth through enhanced litter input and nitrogen availability was cancelled by the negative effect of soil acidification and metal toxicity (Treseder 2008; Tian and Niu 2015), resulting in overall no effect on total PLFAs. Notably, the effect of N addition on microbial biomass (indicated by PLFA) was variable across sites in the literature. For example, N addition (up to 15 g N $m^{-2}$ $yr.^{-1}$) reduced PLFA in a temperate grassland in Inner Mongolia (Yang et al. 2017), but it first stimulated then suppressed PLFA (with peak at 10 g N $m^{-2}$ $yr.^{-1}$) in another temperate grassland in Inner Mongolia (Liu et al. 2017). Two recent meta-analyses also reported either negative (Zhang et al. 2018) or neutral (Zhou et al. 2017) effect of N addition on soil PLFA, likely due to the different numbers of observations (111 vs. 140) in these syntheses.

Similar to total PLFA, bacteria and actinomycetes were unresponsive to N or P additions. In the literature, the two microbial groups showed various responses to nutrient addition. For example, the meta-analysis of Zhou et al. (2017) reported an increase in bacteria PLFA with N addition, while that of Zhang et al. (2018) found a decrease in bacteria PLFA with N addition. These two synthesis studies differed in the number of observations in the database, indicating large variations in the effect size of N addition on bacteria among individual studies. Likewise, actinomycetes showed negative (Zhou et al. 2017) or neutral (Zhang et al. 2018) response to N addition in these two synthesis studies. Differences in plant and soil variables (e.g., forest vs. grassland, acidic vs. neural/alkaline soil) and experimental approaches (e.g., type, amount and duration of N addition) may explain the site-specific responses of microbial abundance to N addition in the literature.

Given the large variances in the responses of microbial groups to nutrient (particularly N) addition in the literature (recently reviewed by Zhou et al. 2017 and Zhang et al. 2018), it was unsurprising that bacteria (both Gram-positive and Gram-negative bacteria, as well as their ratio, Fig. S6), actinomycetes and total PLFA did not respond to N and P addition in this study.

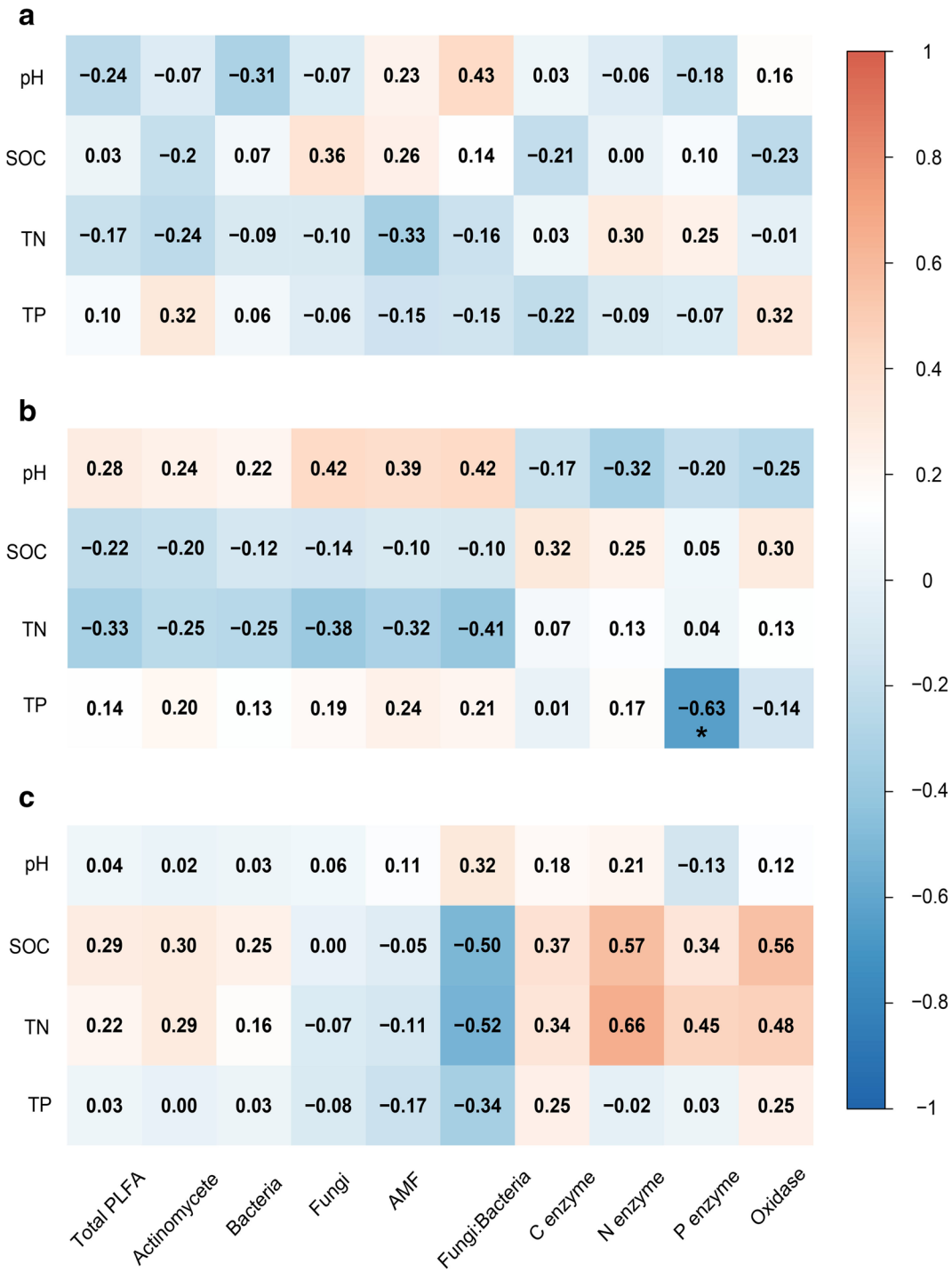
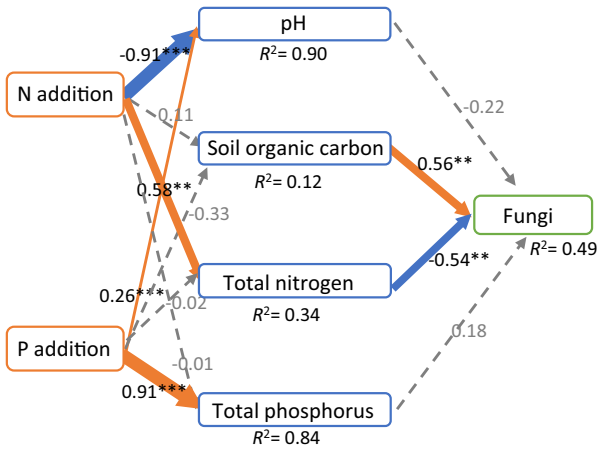
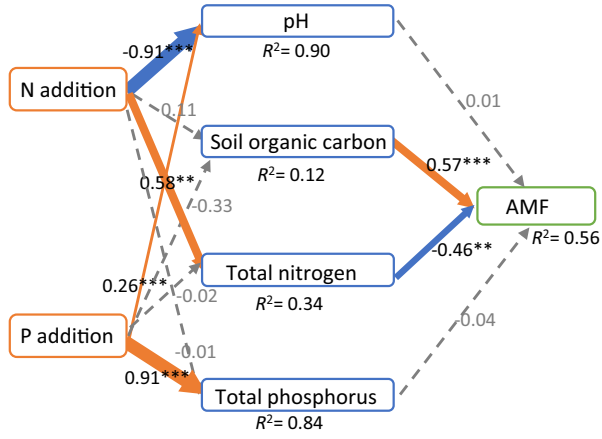


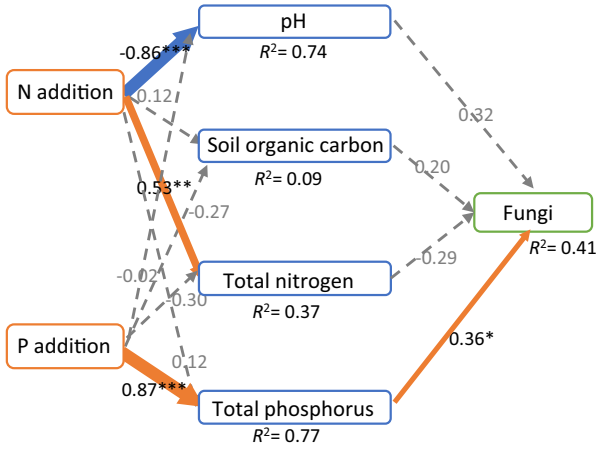

**Fig. 5** The correlation between soil properties and microbial groups, enzyme activities in (**a**) Haibei, (**b**) Hailaer and (**c**) Maodeng. SOC, soil organic carbon (mg $g^{-1}$); TN, total nitrogen (mg $g^{-1}$); TP, total phosphorus (mg $g^{-1}$); PLFA, phospholipid fatty acid; microbial community compositions include total PLFA (nmol $g^{-1}$), actinomycetes (nmol $g^{-1}$), bacteria (nmol $g^{-1}$), fungi (nmol $g^{-1}$), AMF, arbuscular mycorrhizal fungi (nmol $g^{-1}$), the ratio of fungi to bacteria (unitless). C-enzyme, BG+CB (nmol $g^{-1}$ $h^{-1}$), N-enzyme, NAG+LAP (nmol $g^{-1}$ $h^{-1}$); P-enzyme, AP (nmol $g^{-1}$ $h^{-1}$); Oxidase, PER+POX (μmol $g^{-1}$ $h^{-1}$). All correlation analysis use spearman rank correlation. Correlation coefficient r value is shown in the figure, * $P < 0.05$

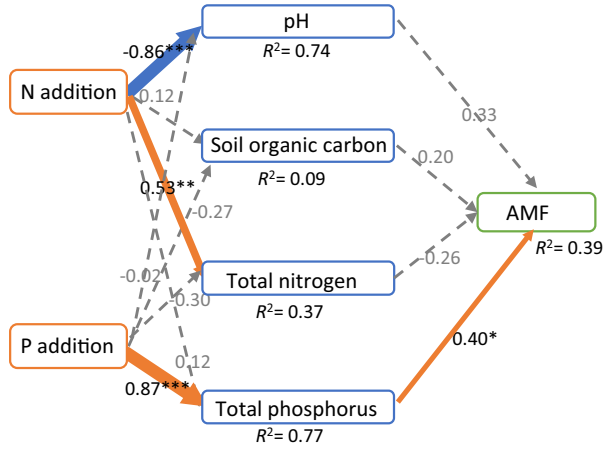
**a** Haibei



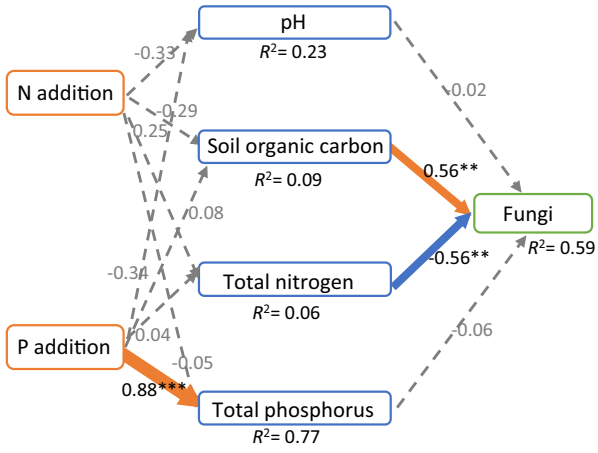



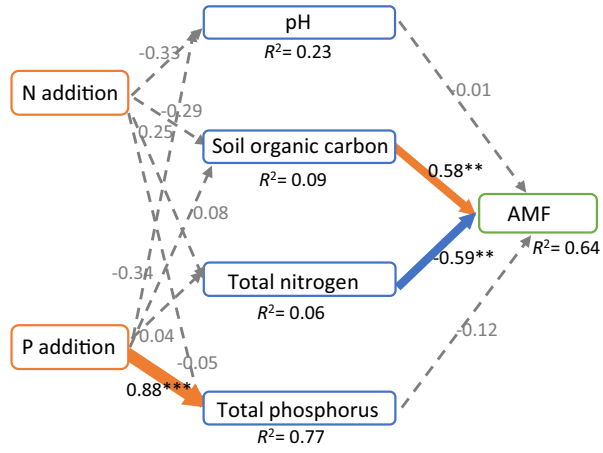

**b** Hailaer




**c** Maodeng



◀ **Fig. 6** Structure equation model (SEM) investigate the multivariate effects on AMF and Fungi in the three sites: (**a**) Haibei (**b**) Hailaer (**c**) Maodeng. Arrows indicate the hypothesized direction of causation. Orange arrows indicate positive relationships and blue arrows indicate negative relationships. Width of arrows represents the strength of the relationship. The numbers next to arrows are standardized path coefficients. The solid represent significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and the dashed represent non-significant ($P > 0.05$). The proportion of variance explained ($R^2$) appears alongside each response variables in the model. Goodness-of-fit statistics for the model: Fungi in Haibei: $\chi^2 = 28.42$, df = 8, $P < 0.01$, root mean square error of approximation (RMSEA) = 0.36, $P < 0.01$. AMF in Haibei: $\chi^2 = 23.79$, df = 8, $P < 0.01$, root mean square error of approximation (RMSEA) = 0.31, $P < 0.01$. Fungi in Hailaer: $\chi^2 = 32.15$, df = 8, $P < 0.01$, root mean square error of approximation (RMSEA) = 0.39, $P < 0.01$. AMF in Hailaer: $\chi^2 = 30.33$, df = 8, $P < 0.01$, root mean square error of approximation (RMSEA) = 0.37, $P < 0.01$. Fungi in Maodeng: $\chi^2 = 59.44$, df = 8, $P < 0.01$, root mean square error of approximation (RMSEA) = 0.57, $P < 0.01$. AMF in Maodeng: $\chi^2 = 59.49$, df = 8, $P < 0.01$ root mean square error of approximation (RMSEA) = 0.57, $P < 0.01$

Two possible mechanisms may explain this result. Firstly, although aboveground biomass was slightly enhanced by both N and P addition (Yang et al. 2014; He et al. 2015), root biomass (Wang 2018) and SOC content (Fig. 1) showed a minor response, suggesting that belowground resources for soil microbes did not shift with nutrient addition. Secondly, although N addition led to soil acidification in the two meadow sites, soil pH remained near neutral (between 6.1 and 7.7). Such small shift in soil pH (by up to 0.6 units, Fig. 1) within the neutral range may not change the physiology of bacteria, which was more sensitive to pH change at acidic range (pH < 6.0, Lauber et al. 2009).

In contrast to bacteria, actinomycetes, and total PLFA, fungi and AMF were sensitive to either N or P addition, and the responses were consistent among the three sites (Fig. 2, Table 2). Specifically, N addition inhibited fungi mainly through its direct effect (likely through increasing soil $NO_3^-$, Fig. S8) and an indirect effect through soil acidification (Fig. 6). Such negative effect of N addition on fungi may be due to two mechanisms. First, fungi have a lower demand for nitrogen compared to bacteria (Zechmeister-Boltenstern et al. 2015), and thus the higher soil nitrogen availability after N addition would favor bacteria over fungi (Zhou et al. 2017). Second, N-induced soil acidification may cause physiological stress for fungi and thus result in lower fungal abundance in soil (Rousk et al. 2009). Likewise, N addition inhibited AMF all three sites (Fig. 2). Soil acidification seems to play a minor role in causing the decline in AMF (Fig. 6), while increases in soil nitrogen availability and other factors (e.g., loss of AMF host plants, particularly forbs, Jansa et al. 2002; Wang 2018) may mainly drive the decline in AMF at the three sites in this study (Treseder 2008; Li et al. 2018).

The significant response of fungi and AMF to N addition in this study are in line with most studies in the literature. In particular, two recent syntheses (Zhou et al. 2017; Zhang et al. 2018) showed significant inhibition of N addition on fungi and AMF, despite that they showed somewhat divergent results on bacteria and actinomycetes. A notable study across 25 grassland sites globally also found that fungi, particularly AMF, was inhibited by N addition (Leff et al. 2015). It also indicated that changes in soil fungal community were associated with changes in the plant community, which may also apply to this study because N addition reduced both plant species richness (Wang 2018) and fungal abundance in the three sites. Taken together, it appears that N addition in grasslands had a more consistent effect on soil fungi and AMF (mostly negative) than bacteria and actinomycetes (variable and site-specific).

Compared to N addition, P addition had a relatively minor effect on soil microbial groups (Table 2, Table S3). Although P addition increased soil total and available phosphorus content (Fig. 1, S8), it had no significant effect on root biomass and plant species richness (Wang 2018), which may explain its minor effect on soil microbial groups. Notably, this result is not consistent with earlier studies which reported a negative effect of P addition on fungi (He et al. 2016) and AMF (Liu et al. 2012; Leff et al. 2015) in grasslands. Future studies may use DNA-based sequencing technology (Leff et al. 2015) to increase the taxonomic and functional resolution of the microbial community and to provide a mechanistic understanding of the response of AMF to nutrient addition in the three grasslands.

## Effects of nutrient addition on soil enzyme activities

We did not find significant effects of N or P addition on potential activities (Fig. 3) and stoichiometric ratios (Fig. S7) of the main hydrolytic and oxidative enzymes involved in soil C and N cycling in this study. This result supports a recent meta-analysis (Xiao et al. 2018) which also showed no significant effects of N addition on soil C- and N-acquisition enzyme activities in grasslands

globally. This is in sharp contrast to the significant effects in forests, where N addition generally stimulates hydrolytic enzymes but inhibits oxidative enzymes (Waldrop et al. 2004; Cusack et al. 2011; Xiao et al. 2018). The mechanisms for the difference in enzyme response to N addition between forest and grassland ecosystems remain unclear. One possible factor is related to the difference in soil microbial (particularly fungal) community composition between the two ecosystems. Specifically, forest soils (particularly temperate and boreal forests where most N-addition experiments occurred) were dominated by Basidiomycota, which had a higher capacity to produce enzymes and were sensitive to nitrogen availability (Sinsabaugh 2010). However, grassland soils were dominated by Glomeromycotina and Ascomycota (Leff et al. 2015), which contributed little to enzyme production. This hypothesis should be further tested with more observations from grassland ecosystems (e.g., Keeler et al. 2009; Cenini et al. 2015; Riggs and Hobbie 2016; Jing et al. 2018) which received far less attention on soil enzymes compared to forest ecosystems (Xiao et al. 2018).

Particularly for this study, all three grassland sites showed no response of enzyme activities and ratios with N and P addition. The changes in soil chemical properties, such as pH, total and available N and P may not be large enough to drive changes in soil enzyme activities with N and P addition. Also, microbial biomass, as measured by both PLFA (Fig. 2) and chloroform fumigation and extraction (Fig. S5) were not significantly affected by nutrient addition, which may explain the minor response of soil enzymes to nutrient addition in this study. Moreover, soil heterotrophic respiration, as measured in root-free soil columns in deep (0–65 cm) PVC collars, did not respond strongly to N or P addition (Wang 2018), consistent with the data on microbial biomass and enzyme activities. Taken together, multiple lines of evidence suggest that belowground soil carbon cycling showed a minimal response to 4 years of N and P addition at the three grassland sites. This is in contrast to the shifts in plant community composition and increases in aboveground plant biomass with N and P addition at these grassland sites (Wang 2018). Together, these results may suggest that belowground carbon cycling is less sensitive to N and P addition than plant diversity and aboveground carbon cycling (Wang 2018) in the three grassland ecosystems.

Although most C- and N-acquisition enzymes were not responsive to N and P addition, the P-acquisition enzyme (acid phosphatase) was sensitive to P addition. It was significantly inhibited by P addition at Hailaer (Fig. 3c) and showed a negative relationship with soil TP in Hailaer (Fig. 5) as well as across all three sites and four treatments (Fig. S3). These results suggest that P-acquisition enzyme activity was down-regulated with increasing soil P availability (Margalef et al. 2017), consistent with the resource-allocation theory for microbial enzyme production (Allison and Vitousek 2005). This finding was also confirmed by a recent meta-analysis (Xiao et al. 2018) which reported that P addition suppressed P-acquisition enzyme activity by 20%.

The enzyme stoichiometric ratios were mostly not responsive to N and P addition (Fig. S7), consistent with the results of individual enzymes. This result indicates that the nutrient demand and resource allocation of the microbial decomposer communities showed a minor response to N and P addition at the three grassland ecosystems, perhaps driven by the minor change in the microbial biomass and community structure (Fig. 4, S2).

## Limitations

Our study has two main limitations. First, the rates of N and P addition in this study (5 and 10 g $m^{-2}$ $yr.^{-1}$) were considerably higher than the natural deposition rates at these sites (Zhu et al. 2016). Thus, the results of this study cannot directly infer the responses of these grassland ecosystems to natural deposition of N and P. Given the fact that most field experiments use comparably high rates of fertilizers (e.g. 10 g $m^{-2}$ $yr.^{-1}$ in the grassland Nutrient Network globally, Borer et al. 2014; Leff et al. 2015), our study is comparable to those studies addressing responses of soil microbial communities to nutrient additions in grassland ecosystems. Second, this study was based on one-time sampling at the peak growing season after 4 years of nutrient addition which may miss the season- or year-specific responses, and microbial biomass and community structure were measured by the PLFA technique which may not detect a potential shift in the bacterial and fungal community by the DNA-based sequencing technology. Further studies based on more experimental sites, more frequent samplings, and cutting-edge molecular methods will help further elucidate the responses and underlying mechanisms of soil microbial community to nutrient addition in grassland ecosystems.

## Conclusion

In summary, based on a 4-year nutrient fertilization experiment at three contrasting grasslands, this study showed that N addition reduced soil pH and increased soil total N content at two meadow sites, P enrichment increased soil total P content at all three sites, but N and P addition had minimal effect on soil organic C content across all sites. Bacteria and total microbial abundances (as indicated by PLFA) did not change after N and P addition, while fungi and AMF abundances generally showed negative responses to N and P addition. Moreover, the activity soil extracellular enzymes involved in C and N cycling and their stoichiometric ratios were not responsive to N and P addition, except for suppression of acid phosphatase by P addition at one meadow site. Despite limitations associated with the high rates of nutrient addition and the techniques used in this study as discussed above, the mostly consistent results at three contrasting grassland ecosystems suggest that 4 years of N and P addition may have minimal effect on soil microbial biomass, community composition and enzyme activities in these grassland ecosystems.

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