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# Neutral effect of nitrogen addition and negative effect of phosphorus addition on topsoil extracellular enzymatic activities in an alpine grassland ecosystem

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ABSTRACT

Soil nitrogen (N) and phosphorus (P) are common limiting nutrients affecting plant primary productivity in alpine ecosystems due to the low decomposition rate, though anthropogenic activities have greatly increased their inputs into ecosystems. Little is known regarding the effects of increasing N and P availabilities on the functioning of belowground microbial communities. To determine how soil microorganisms respond to N and P addition, we measured plant primary productivity, soil microbial biomass, soil mineral N availability, soil respiration, and the activities of soil extracellular enzymes after two years of N- and P-addition in an alpine grassland ecosystem on the Tibetan Plateau. We observed no significant effect of N addition on plant biomass, soil microbial biomass, soil respiration, or the activities of soil extracellular enzymes. In contrast, P addition increased plant biomass but suppressed the activities of most labile-C-cycling enzymes at 0–10 cm of soil depth, although the effects on soil microbial biomass and soil respiration were minor. Moreover, there was no interaction between N and P addition on these variables. Overall, N addition does not appear to exert a significant effect on plant primary productivity and microbial activity, whereas P addition increases plant primary productivity and tends to suppress topsoil microbial activity after two years of nutrient application.



## 1. Introduction

Nutrient availability, e.g., nitrogen (N) and phosphorous (P), frequently limit primary productivity in most terrestrial ecosystems (LeBauer and Treseder, 2008; Vitousek et al., 2010). However, anthropogenic activities have greatly increased the inputs of these nutrients into the biosphere (Falkowski et al., 2000; Vitousek et al., 1997). Increased nutrient availability often has multiple effects on aboveground organisms, including biodiversity loss (Hooper et al., 2012) and their associated ecosystem functioning and services (Isbell et al., 2013; Smith et al., 1999). Meanwhile, nutrient availability can be amplified by climate warming by enhancing microbial decomposition of soil organic matter (Bardgett et al., 2008) which, in turn, stimulates plant growth and mediates ecosystem carbon storage (Koyama et al., 2013; Mack et al., 2004; Waldrop et al., 2004). However, the responses of belowground microorganisms to nutrient addition remains poorly understood (Leff et al., 2015).

Soil microorganisms produce extracellular enzymes to decompose complex organic matter into biologically available nutrients. In turn, these nutrients can mediate microbial metabolism and growth (German et al., 2011). Nutrient availability influences enzymatic activity by regulating microbial allocation to enzyme production (Sinsabaugh and Moorhead, 1994) or by shifting the abundance of specific enzyme-producing microorganisms (Allison et al., 2008; Koyama et al., 2014; Wang et al., 2015). Furthermore, the economic theories of microbial metabolism (resource allocation theory, Allison et al., 2011) predict that enzyme production will increase when simple nutrient is limited (Allison and Vitousek, 2005; Koch, 1985), and decrease when nutrient is available in the soil solution (Chróst, 1991; Pelletier and Sygusch, 1990; Sinsabaugh

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and Moorhead, 1994). For example, soil phosphatase has high activity in P-limiting ecosystems (Turner and Wright, 2014), while P addition tends to suppress phosphatase activity (Olander and Vitousek, 2000). In a similar way, the activities of soil enzymes related to carbon (C)- and N- cycling generally reflect microbial demand for energy and nutrients (Turner and Wright, 2014). Therefore, the activities of soil enzymes could serve as indicators of microbial nutrient demand. However, positive, neutral, and negative effects of nutrient addition on soil enzyme activities have been observed in previous studies (Keeler et al., 2008; Shi, 2011; Tian et al., 2014; Wang et al., 2015), depending on research sites and enzymes assayed (Johnson et al., 1998; Keeler et al., 2008).

The Tibetan Plateau is one of the most sensitive areas to climate change, with substantial soil organic C stocks (Shi et al., 2012; Yang et al., 2008) that may be released by microbial decomposition with ongoing climate change. In addition, this plateau mainly serves as a receptor of environmental pollutants from primarily long-range atmospheric transport (Tao et al., 2011; Wang et al., 2013) due to its unique meteorological and geographical characteristics. For example, Jia et al. (2014) reported that the alpine grasslands receive ~1.0–1.5 g N $m^{-2}$ $yr^{-1}$ atmospheric N deposition. However, soil N and P are still considered as limiting nutrients for plant primary productivity in alpine ecosystems (Jiang et al., 2013) due to the low decomposition rate. Although soil microorganisms are often sensitive to N addition (Leff et al., 2015), little is known regarding the microbial responses to P addition. Therefore, an investigation of the responses of soil microbial communities to N and P addition on the Tibetan Plateau will improve our ability to predict how soil biogeochemical cycles will respond to changes in nutrient availability induced by global change.

The objective of our current study is to assess microbial responses (e.g., soil respiration and enzyme activity as the functional characteristics of microbial communities) to N and P addition. Specifically, we measured plant primary productivity, soil microbial biomass, soil mineral N availability, soil respiration, and the activities of seven soil extracellular enzymes related to C-, N-, and P-cycling (Table 1). We predicted that (1) N addition would increase the activities of both C- and P-cycling enzymes but suppress the activities of N-cycling enzymes, and (2) P addition would increase the activities of both C- and N-cycling enzymes but suppress the activity of P-cycling enzymes according to resource allocation theory (Allison et al., 2008; Keeler et al., 2008).

## 2. Materials and methods

### 2.1. Site description

We collected samples from the Haibei Alpine Grassland Ecosystem Research Station (37°36′ N, 101°19′ E, 3215 m) in 2010. The station is located in the northeast of the Tibetan Plateau in a large valley that is adjacent to the Qilian Mountains (Fig. S1). The seasonal variations of soil temperature at 5 cm of depth, volumetric soil moisture at 5 cm of depth, and daily precipitation in 2010 are shown in Fig. S2. In general, the temperature and precipitation at the study site showed a unimodal pattern with peaks in August. The mean daily temperature varied between 2.5 °C and 18.5 °C during our study period (May to September). The mean daily soil moisture at 5 cm of depth also varied dramatically (5.6–39.6%) with the daily precipitation (0–177 mm), with 83% of the rain falling from May to September. The vegetation is dominated by *Kobresia humilis*, *Stipa aliena*, and *Elymus nutans*. The soil is Gelic Cambisol (WRB, 1998) with an average thickness of 65 cm. Topsoil (0–10 cm) has a pH value of 7.5, and contains 71.4 g $kg^{-1}$ organic C, 7.8 g $kg^{-1}$ total N, and 0.77 g $kg^{-1}$ total P (Huang et al., 2014).

### 2.2. Nutrient addition treatments

We conducted a field experiment of factorial N and P addition with a randomized block design in May 2009. We established six blocks for nutrient application within an area of 110 m × 75 m, and each block contained 3 m × 3 m plots with four nutrient addition treatments. Specifically, the treatments were (1) CK (control); (2) N, nitrogen addition in the form of urea at a rate of 10 g N $m^{-2}$ $year^{-1}$; (3) P, phosphorus addition in the form of triple superphosphate at a rate of 5 g P $m^{-2}$ $year^{-1}$; and (4) NP, a combination of nitrogen and phosphorus addition in the form of urea and triple superphosphate at a rate of 10 g N and 5 g P $m^{-2}$ $year^{-1}$. The background N deposition ranged from 1.0–1.5 g N $m^{-2}$ $year^{-1}$ at our site in the 2000 s (Jia et al., 2014), indicating that the levels of N addition (10 g N $m^{-2}$ $year^{-1}$) in this study were higher than the natural N deposition. These high rates of nutrient addition were chosen because they are typical values applied to alpine grasslands on the Tibetan Plateau (Jiang et al., 2013; Liu et al., 2012; Tian et al., 2014; Zheng et al., 2014). Nutrients were added once per year (July 15th in 2009 and July 5th in 2010) (Yang et al., 2014).

### 2.3. Soil sampling

We removed surface litter and randomly collected three soil cores with a diameter of 3.5 cm from the topsoil (0–10 cm) and subsoil (10–20 cm) on four occasions (May 3, June 14, August 9, and September 16–twice before and twice after the nutrient addition in 2010; Fig. S2). In brief, samples from the same depth in each plot were pooled, packed in polyethylene bags, immediately stored in a portable refrigerator, and transported to the laboratory. The composite samples were passed through a 2-mm sieve and then stored at −20 °C for no more than one week until enzyme analyses could be performed (Jing et al., 2014). We acknowledge that freezing may influence the activities of soil extracellular enzymes (DeForest, 2009), but it should not have differential effects on the activities of soil extracellular enzymes in nutrient-added versus control treatments (Keeler et al., 2008; Stone et al., 2012). Gravimetric water content was determined by drying at 105 °C for 48 h. To minimize the measurement time of the activities of soil

**Table 1**
Extracellular enzymes assayed in our study, and their abbreviations, functions, and corresponding substrates. 4-MUB = 4-methylumvelliferyl; L-DOPA = L-3,4-dihydroxyphenylalanine.

| Enzyme | Abbreviation | Function | Substrate |
|---|---|---|---|
| α-1,4-glucosidase | AG | labile-C cycling | 4-MUB-α-D-glucoside |
| β-1,4-glucosidase | BG | labile-C cycling | 4-MUB-β-D-glucoside |
| β-1,4-xylosidase | BX | labile-C cycling | 4-MUB-β-D-xyloside |
| cellobiohydrolase | CB | labile-C cycling | 4-MUB-β-D-cellobioside |
| β-1,4-N-acetyl-glucosaminnidase | NAG | N cycling | 4-MUB-N-acetyl-β-D-glucosaminide |
| acid phosphatase | AP | P cycling | 4-MUB-phosphate |
| phenol oxidase | POX | recalcitrant-C cycling | L-DOPA |

extracellular enzymes in the laboratory, we only collected soil samples from five out of six blocks.

### 2.4. Plant and microbial biomass

We measured plant aboveground biomass by clipping living plants from September 10–12, 2010. We harvested all living plants from a 0.25 m × 0.25 m quadrat and weighed the resultant dry matter as aboveground biomass after oven-drying the harvested plants for 48 h at 65 °C. Three soil cores (3.5 cm in diameter) were sampled for root biomass analyses in the same quadrat at depths of 0–10 cm, 10–20 cm, 20–30 cm, and 30–40 cm. Root samples were soaked in water and cleaned from soil residuals using a 0.5-mm sieve and then oven-dried at 65 °C for at least 48 h and weighed as belowground biomass.

We measured soil microbial biomass C by using the chloroform fumigation extraction method (Vance et al., 1987) on August 9 and September 16, 2010. Subsamples (10 g) were directly extracted in 0.5 M $K_2SO_4$ for 30 min. Additional subsamples (10 g) were fumigated with ethanol-free chloroform for 24 h and then extracted in 0.5 M $K_2SO_4$ for 30 min. Both non-fumigated and fumigated extracts were filtered and frozen at −20 °C until analysis of dissolved organic C (DOC) was conducted by a TOC analyzer (Multi N/C 3100, Analytik, Germany). To account for incomplete extractability, we used an extraction efficiency factor of 0.45 (Vance et al., 1987) for calculation of soil microbial biomass C. Microbial biomass C was expressed as mg C $kg^{-1}$ dried soil.

### 2.5. Mineral N availability

We measured soil mineral N availability during the growing season (May 3–September 16, 2010) using ion-exchange resin bags (Allison and Treseder, 2008). In brief, each nylon mesh bag was filled with 5 g anion or cation exchange resin, soaked in 0.5 M HCl for 20 min, rinsed with deionized water, and washed with 2 M NaCl. We placed two anion and two cation bags in each plot at a soil depth of 5 cm. We retrieved the bags about six weeks later and replaced them with new bags. Bags were rinsed in deionized water and extracted with 60 ml 2 M KCl on a shaker table for 60 min. We determined $NH_4^+$-N and $NO_3^-$-N concentration by colorimetric analysis with a FIAstar 5000 flow injection analyzer (FOSS Tecator, Höganäs, Sweden). Measurements from two anion and cation bags were averaged to represent the mineral N availability (sum of $NH_4^+$-N and $NO_3^-$-N) in that plot. Mineral N availability was expressed as ng N $g^{-1}$ resin $day^{-1}$.

### 2.6. Soil respiration

We measured soil respiration during the growing season (May 12, June 16, August 6, and September 13) of 2010. In brief, we inserted a PVC collar with a diameter of 20 cm and height of 8 cm into each plot in July 2009 and used an LI-8100 portable soil $CO_2$ flux system (Li-cor, Inc., Lincoln, NE, USA) for measurement of soil respiration rates. Fluxes were expressed as μmol $m^{-2}$ $s^{-1}$. Aboveground plants inside the collars were clipped and removed at least one day before measurements to eliminate aboveground plant respiration. To minimize the measurement time of soil respiration in the field (9:00–11:00 am), we only measured soil respiration from four out of the six blocks.

### 2.7. Soil extracellular enzyme activity

We measured potential activities of six soil hydrolytic enzymes, including four enzymes related to labile-C cycling (α-1,4-glucosidase, β-1,4-glucosidase, β-1,4-xylosidase, and cellobiohydrolase), one related to N cycling (β-1,4-*N*-acetyl-glucosaminnidase), and one related to P cycling (acid phosphatase) (Table 1), following a method modified from Saiya-Cork et al. (2002), Steinweg et al. (2012), and Bell et al. (2013) using fluorometric techniques. In brief, we homogenized 2.75 g soil in 91 ml of 50 mM sodium acetate buffer (pH 5.5) in a Waring blender for 2 min. Slurries of 200 μl were then added to 96-well microplates along with 50 μl of 200 μM fluorometric substrate (saturating concentration) in each well. We used six analytical replicates for each soil sample. The microplates were incubated in the dark at 25 °C for 6 h. The amount of fluorescence was determined using a fluorescence spectrometer

**Table 2**
Summary of the linear mixed-effects model showing the effects of the N and P applications and/or sampling dates on plant total biomass, plant aboveground biomass, plant belowground biomass, microbial biomass C, mineral N availability, and soil respiration. Total plant biomass, aboveground plant biomass, and belowground plant biomass were measured on September 10–12, 2010. df indicates the nominator and denominator degrees of freedom. Bold indicates *P* values < 0.05.

| | | N | P | Date | N × P | N × Date | P × Date | N × P × Date |
|---|---|---|---|---|---|---|---|---|
| Total biomass | df | 1,9 | 1,9 | | 1,9 | | | |
| | F value | 0.00 | 12.61 | | 1.77 | | | |
| | *P value* | 0.952 | **0.006** | | 0.217 | | | |
| Aboveground biomass | df | 1,9 | 1,9 | | 1,9 | | | |
| | F value | 6.04 | 17.04 | | 0.00 | | | |
| | *P value* | **0.036** | **0.003** | | 0.997 | | | |
| Belowground biomass | df | 1,9 | 1,9 | | 1,9 | | | |
| | F value | 0.47 | 6.88 | | 2.11 | | | |
| | *P value* | 0.510 | **0.028** | | 0.180 | | | |
| Microbial biomass C | df | 1,9 | 1,9 | 1,12 | 1,9 | 1,12 | 1,12 | 1,12 |
| | F value | 0.01 | 0.73 | 2.10 | 0.04 | 0.26 | 0.52 | 0.37 |
| | *P value* | 0.921 | 0.414 | 0.173 | 0.849 | 0.620 | 0.487 | 0.557 |
| Mineral N availability | df | 1,12 | 1,12 | 2,32 | 1,12 | 2,32 | 2,32 | 2,32 |
| | F value | 120.65 | 12.65 | 13.00 | 0.08 | 18.50 | 2.04 | 0.88 |
| | *P value* | <**0.001** | **0.004** | <**0.001** | 0.776 | <**0.001** | 0.146 | 0.426 |
| Soil respiration | df | 1,9 | 1,9 | 3,31 | 1,9 | 3,31 | 3,31 | 3,31 |
| | F value | 0.00 | 0.22 | 83.17 | 2.35 | 0.34 | 0.45 | 0.11 |
| | *P value* | 0.988 | 0.648 | <**0.001** | 0.160 | 0.800 | 0.716 | 0.951 |

(Spectramax M2, Molecular Devices, USA) set to 365 nm for excitation and 450 nm for emission. Enzyme activity was expressed as nmol g dry weight$^{-1}$ h$^{-1}$.

We measured one oxidative enzyme related to recalcitrant-C cycling (phenol oxidase) (Table 1) following a modified method described by Saiya-Cork et al. (2002) and German et al. (2011) using L-3,4-dihydroxyphenylalanine (DOPA) as the substrate. We homogenized 1 g of soil in 100 ml of 50 mM sodium acetate buffer (pH 5.5) in a Waring blender for 2 min. The slurries were then added to 96-well microplates along with 50 μl of 5 mM DOPA in each well. The negative controls contained 200 μl of acetate buffer and 50 μl of DOPA in each well. Blanks contained 200 μl sample suspension and 50 μl of acetate buffer in each well. We used six analytical replicates for each soil sample, blank, and control. The microplates were incubated in the dark at 25 °C for 18 h. Activity was quantified using a fluorescence spectrometer (Spectramax M2, Molecular Devices, USA) by measuring the absorbance at 450 nm. Enzyme activity was expressed as μmol g dry weight$^{-1}$ h$^{-1}$.

### 2.8. Statistical analyses

The assay data were analyzed using a linear mixed-effects model (Pinheiro et al., 2011). Treatment, sampling date, and/or soil depth were designated as fixed effects with plots nested in blocks as random effects; plant biomass, microbial biomass C, mineral N availability, soil respiration, and enzyme activity were designated as dependent variables (Table 2 and 3). To make the sampling dates comparable, we considered two dates (sampling dates before and after nutrient application in July 2010) as contrasts in the linear mixed-effects model (Table S1). Because there were no significant interactions between each treatment and the sampling date for enzymes (Table 3) or between each treatment and the contrast sampling date (Table S1), we averaged the activities of each enzyme across the four sampling dates (Fig. S2). To examine the effects of N, P, and NP addition (relative to the control) on plant biomass, microbial biomass C, mineral N availability, soil respiration, and enzyme activity, we used meta-analysis to compare these multiple independent variables. Meta-analysis is a statistical technique for combing the results of multiple independent studies or multiple independent variables within a single study (Koricheva et al., 2013). It has been successfully applied in single studies of responses of extracellular enzymes to N amendment (Saiya-Cork et al., 2002). Specifically, we calculated natural log response ratio (lnRR) for each treatment with the package "metafor" (Viechtbauer, 2010). The effects of N, P, and NP addition (relative to the control) on plant biomass, microbial biomass C, mineral N availability, soil respiration, and enzyme activities (Fig. 1 and 2) were considered significant if the 95% confidence intervals of the lnRR did not overlap with zero. We square root- or log-transformed data where necessary to improve normality and reduce heteroscedasticity. All statistical analyses were performed in R 3.0.0 (R Development Core Team, 2013).

## 3. Results

### 3.1. Plant biomass, microbial biomass C, mineral N availability, and soil respiration rate

Plant biomass, microbial biomass C, mineral N availability, and soil respiration rate all showed additive responses (no significant interaction, $P > 0.10$) to N and P addition (Table 2).

Plant aboveground biomass increased significantly from $476 \pm 70$ (standard deviation) to $630 \pm 111$ g m$^{-2}$ year$^{-1}$ (32% increase) with N addition, to $735 \pm 137$ g m$^{-2}$ year$^{-1}$ (54% increase) with P addition, and to $889 \pm 164$ g m$^{-2}$ year$^{-1}$ (87% increase) with NP addition. Belowground biomass only increased with P addition, from $1716 \pm 405$ to $2550 \pm 579$ g m$^{-2}$ year$^{-1}$ (48% increase), while total biomass increased with both P and NP addition, from $2192 \pm 424$ to $3284 \pm 715$ (50% increase) and $3001 \pm 257$ g m$^{-2}$ year$^{-1}$ (37% increase), respectively (Fig. 1, Table 4).

No significant seasonal variations in soil microbial biomass C were observed (Table 2). Data from the two sampling times were thus pooled, and the meta-analysis showed that soil microbial biomass C was not significantly affected with N, P, or NP addition (Fig. 1). Mean microbial biomass C ranged from $205 \pm 59$ mg C kg$^{-1}$ dried soil in control plots to $260 \pm 113$ mg C kg$^{-1}$ dried soil in P-added plots (Table 4).

Soil mineral N availability (as measured by buried resin bags) varied seasonally, with the lowest concentrations being late in the growing season (August 9–September 16, data not shown). The meta-analysis showed that soil mineral N availability increased significantly from $863 \pm 270$ to $2363 \pm 600$ ng N g$^{-1}$ resin day$^{-1}$ (174% increase) with N addition and to $1925 \pm 349$ ng N g$^{-1}$ resin day$^{-1}$ (123%) with NP addition, while exhibiting a non-significant ($P > 0.10$) decreasing trend with P addition, from $863 \pm 270$ to $689 \pm 138$ ng N g$^{-1}$ resin day$^{-1}$ (20% decrease) (Fig. 1, Table 4).

Soil respiration at the study site followed a unimodal pattern, peaking in August. Soil respiration increased from $2.23 \pm 0.17$ μmol m$^{-2}$ s$^{-1}$ in May to $5.72 \pm 0.17$ μmol m$^{-2}$ s$^{-1}$ in August and then dropped to $3.98 \pm 0.14$ μmol m$^{-2}$ s$^{-1}$ in September. The meta-analysis showed that soil respiration was not significantly affected by N, P, or NP addition (Fig. 1). Mean soil respiration ranged from

**Table 3**
Summary of the linear mixed-effects model for the effects of N and P applications, sampling dates, and soil depths on the activities of soil extracellular enzymes. Abbreviations for each enzyme are shown in Table 1. df indicates nominator and denominator degrees of freedom. Bold indicates $P$ values < 0.05.

| | | AG | | BG | | BX | | CB | | NAG | | AP | | POX | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | F value | *P value* | F value | *P value* | F value | *P value* | F value | *P value* | F value | *P value* | F value | *P value* | F value | *P value* |
| N | 1,12 | 0.7 | 0.413 | 2.7 | 0.125 | 0.0 | 0.859 | 0.8 | 0.390 | 0.6 | 0.472 | 0.1 | 0.798 | 0.8 | 0.391 |
| P | 1,12 | **8.1** | **0.015** | 2.9 | 0.113 | 3.9 | 0.073 | **7.9** | **0.016** | 1.4 | 0.262 | 2.1 | 0.175 | 1.8 | 0.201 |
| date | 3,112 | 1.8 | 0.147 | 1.8 | 0.143 | **5.4** | **0.002** | **9.0** | **<0.001** | 0.1 | 0.952 | **13.5** | **<0.001** | **8.1** | **<0.001** |
| depth | 1,112 | **132.1** | **<0.001** | **351.6** | **<0.001** | **148.3** | **<0.001** | **163.3** | **<0.001** | **415.0** | **<0.001** | **27.7** | **<0.001** | **95.1** | **<0.001** |
| N × P | 1,12 | 2.6 | 0.135 | 0.7 | 0.417 | 0.2 | 0.631 | 0.4 | 0.523 | 0.2 | 0.682 | **5.5** | **0.037** | 0.8 | 0.387 |
| N × date | 3,112 | 0.3 | 0.854 | 0.3 | 0.791 | 0.2 | 0.904 | 0.2 | 0.928 | 0.3 | 0.858 | 0.3 | 0.811 | 0.8 | 0.525 |
| P × date | 3,112 | 1.1 | 0.373 | 2.0 | 0.123 | 1.7 | 0.176 | 1.6 | 0.183 | 0.5 | 0.689 | 2.5 | 0.066 | 1.1 | 0.345 |
| N × depth | 1,112 | 1.1 | 0.302 | 1.3 | 0.257 | 0.5 | 0.472 | 0.5 | 0.500 | 1.6 | 0.214 | 2.2 | 0.139 | **11.2** | **0.001** |
| P × depth | 1,112 | 0.6 | 0.456 | 0.6 | 0.446 | 0.3 | 0.612 | 0.3 | 0.572 | 2.6 | 0.108 | **6.8** | **0.010** | 3.3 | 0.074 |
| date × depth | 3,112 | 1.5 | 0.217 | **4.2** | **0.007** | **5.8** | **0.001** | 2.4 | 0.076 | **6.2** | **0.001** | 1.8 | 0.150 | **5.1** | **0.002** |
| N × P × date | 3,112 | 0.5 | 0.707 | 1.8 | 0.143 | 0.0 | 0.994 | 1.1 | 0.337 | 0.2 | 0.917 | 0.9 | 0.445 | 0.1 | 0.976 |
| N × P × depth | 1,112 | 0.1 | 0.813 | 3.1 | 0.081 | 0.0 | 0.914 | 3.2 | 0.075 | 0.1 | 0.747 | **10.6** | **0.002** | 1.0 | 0.315 |
| N × date × depth | 3,112 | 1.0 | 0.413 | 0.9 | 0.460 | 0.3 | 0.801 | 1.1 | 0.370 | 0.8 | 0.488 | 1.2 | 0.321 | 0.1 | 0.947 |
| P × date × depth | 3,112 | 0.4 | 0.738 | 0.7 | 0.560 | 1.4 | 0.235 | 1.9 | 0.135 | 1.2 | 0.319 | 0.0 | 0.998 | 0.7 | 0.547 |
| N × P × date × depth | 3,112 | 1.5 | 0.216 | 0.9 | 0.424 | 0.8 | 0.490 | 1.2 | 0.314 | 0.2 | 0.874 | 0.7 | 0.530 | 0.4 | 0.742 |

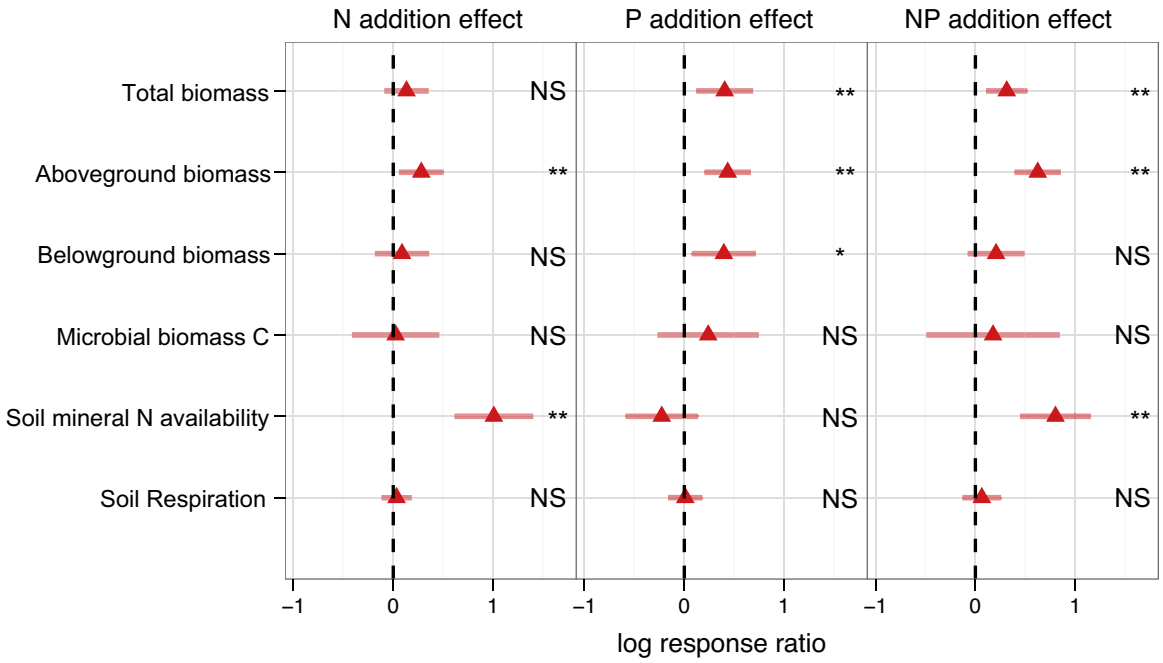


**Fig. 1.** Responses of plant total biomass, plant aboveground biomass, plant belowground biomass, soil microbial biomass, soil mineral N availability, and soil respiration to N, P, and NP addition. Log response ratio = $\ln(X_T/X_C)$, $X_T$ is the means of nutrient application group, $X_C$ is the means of control group. Values less than one indicate nutrient addition has a negative effect; values greater than one indicate nutrient addition has a positive effect. Error bars are the 95% confidence intervals for the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, NS = non-significant ($P > 0.1$) difference between the log response ratio and zero.

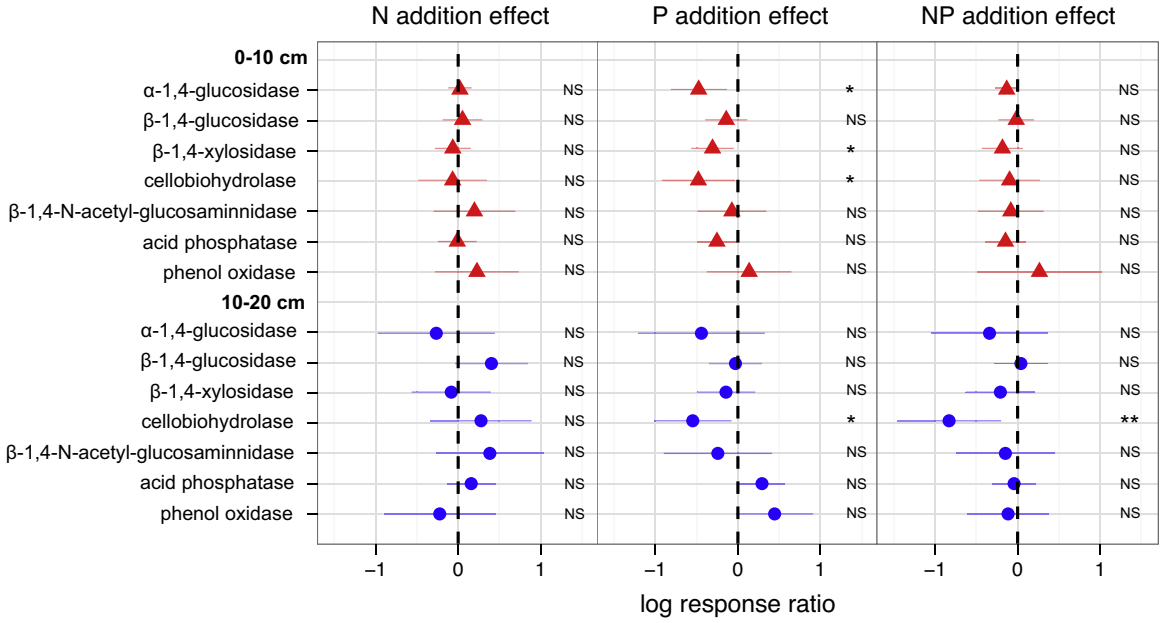


**Fig. 2.** Responses of soil extracellular enzymes to N, P, and NP addition at 0–10 cm (triangles) and 10–20 cm (circles) of soil depth. Log response ratio = $\ln(X_T/X_C)$, $X_T$ is the means of nutrient application group, $X_C$ is the means of control group. Values less than one indicate nutrient addition has a negative effect; values greater than one indicate nutrient addition has a positive effect. Error bars are the 95% confidence intervals for the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, NS = non-significant ($P > 0.1$) difference between the log response ratio and zero.

$4.00 \pm 0.60$ μmol $m^{-2}$ $s^{-1}$ in control plots to $4.27 \pm 0.55$ μmol $m^{-2}$ $s^{-1}$ in NP-addition plots (Table 4).

### 3.2. Extracellular enzyme activity

N addition and NP addition had no detectable effects ($P > 0.10$) on the hydrolytic and oxidative enzymes at either depth (0–10 cm and 10–20 cm). The exceptions to this were that N addition marginally ($P < 0.10$) increased β-1,4-glucosidase activity from $5.76 \pm 1.92$ to $8.62 \pm 3.25$ nmol g dry weight$^{-1}$ $h^{-1}$ at 10–20 cm of depth, and that NP addition significantly ($P < 0.01$) decreased cellobiohydrolase activity from $0.55 \pm 0.21$ to $0.24 \pm 0.14$ nmol g dry weight$^{-1}$ $h^{-1}$ at 10–20 cm of depth (Fig. 2, Table 4). In contrast, P addition tended to suppress the activity of most labile-C-cycling enzymes at 0–10 cm of depth ($P < 0.05$), but had no detectable effect at 10–20 cm ($P > 0.10$, Fig. 2). Specifically, P addition

**Table 4**
Plant biomass, microbial biomass C, mineral N availability, soil respiration rate, and enzyme activities of the studied site. Data shown are the mean and standard deviation for each variable for *n* replicates of N and P treatment.

| Variables | *n* | CK | N | P | NP |
|---|---|---|---|---|---|
| Total biomass ($g\,m^{-2}\,year^{-1}$) | 4 | 2192.3 ± 423.8 | 2503.5 ± 299.0 | 3284.3 ± 714.6 | 3000.8 ± 257.5 |
| Aboveground biomass ($g\,m^{-2}\,year^{-1}$) | 4 | 476.0 ± 70.3 | 630.3 ± 110.5 | 735.0 ± 136.7 | 888.8 ± 164.4 |
| Belowground biomass ($g\,m^{-2}\,year^{-1}$) | 4 | 1716.3 ± 404.6 | 1873.3 ± 273.0 | 2549.5 ±579.3 | 2112.0 ± 362.6 |
| Soil microbial biomass C ($mg\,C\,kg^{-1}$ dried soil) | 4 | 204.9 ± 58.5 | 209.8 ± 72.2 | 260.3 ± 112.6 | 244.5 ± 151.6 |
| Soil mineral N availability ($ng\,N\,g^{-1}$ resin $day^{-1}$) | 4 | 863.0 ± 269.7 | 2362.5 ± 599.8 | 688.6 ± 137.6 | 1925.1 ± 348.9 |
| Soil respiration ($\mu mol\,m^{-2}\,s^{-1}$) | 4 | 4.00 ± 0.60 | 4.14 ± 0.16 | 4.04 ± 0.37 | 4.27 ± 0.55 |
| 0–10 cm[a] | | | | | |
| α-1,4-glucosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 2.67[b] ± 0.25 | 2.73 ± 0.36 | 1.66 ± 0.62 | 2.34 ± 0.28 |
| β-1,4-glucosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 18.73 ± 4.14 | 19.74 ± 3.17 | 16.27 ± 3.07 | 18.41 ± 1.98 |
| β-1,4-xylosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 3.36 ± 0.72 | 3.15 ± 0.40 | 2.47 ± 0.50 | 2.80 ± 0.52 |
| cellobiohydrolase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 2.65 ± 1.07 | 2.48 ± 0.61 | 1.64 ± 0.48 | 2.40 ± 0.28 |
| β-1,4-N-acetyl-glucosaminnidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 11.15 ± 4.31 | 13.57 ± 5.58 | 10.36 ± 2.87 | 10.26 ± 2.43 |
| acid phosphatase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 40.12 ± 9.14 | 39.71 ± 5.09 | 31.13 ± 4.16 | 34.64 ± 5.32 |
| phenol oxidase (μmol g dry $weight^{-1}\,h^{-1}$) | 5 | 3.21 ± 1.68 | 4.03 ± 0.99 | 3.68 ± 0.95 | 4.18 ± 2.82 |
| 10–20 cm[a] | | | | | |
| α-1,4-glucosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 0.76 ± 0.47 | 0.58 ± 0.30 | 0.49 ± 0.30 | 0.54 ± 0.28 |
| β-1,4-glucosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 5.76 ± 1.92 | 8.62 ± 3.25 | 5.60 ± 0.84 | 6.00 ± 0.97 |
| β-1,4-xylosidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 1.07 ± 0.37 | 0.99 ± 0.42 | 0.93 ± 0.19 | 0.87 ± 0.28 |
| cellobiohydrolase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 0.55 ± 0.21 | 0.72 ± 0.42 | 0.32 ± 0.12 | 0.24 ± 0.14 |
| β-1,4-*N*-acetyl-glucosaminnidase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 2.63 ± 1.40 | 3.86 ± 2.02 | 2.06 ± 1.08 | 2.27 ± 0.96 |
| acid phosphatase (nmol g dry $weight^{-1}\,h^{-1}$) | 5 | 26.16 ± 6.75 | 30.64 ± 6.40 | 35.03 ± 6.37 | 25.07 ± 3.70 |
| phenol oxidase (μmol g dry $weight^{-1}\,h^{-1}$) | 5 | 10.04 ± 4.44 | 8.03 ± 5.05 | 15.67 ± 4.64 | 8.95 ± 3.10 |

[a] Enzyme activities at 0–10 cm and 10–20 cm of soil depth.
[b] Averaged across four sampling dates.

significantly decreased α-1,4-glucosidase activity from 2.67 ± 0.25 to 1.66 ± 0.62 nmol g dry $weight^{-1}\,h^{-1}$, β-1,4-xylosidase activity from 3.36 ± 0.72 to 2.47 ± 0.50 nmol g dry $weight^{-1}\,h^{-1}$, and cellobiohydrolase activity from 2.65 ± 1.07 to 1.64 ± 0.48 nmol g dry $weight^{-1}\,h^{-1}$ at 0–10 cm of soil depth (Fig. 2, Table 4). In addition, P addition significantly decreased cellobiohydrolase activity, from 0.55 ± 0.21 to 0.32 ± 0.12 nmol g dry $weight^{-1}\,h^{-1}$ at 10–20 cm of soil depth (Fig. 2, Table 4).

Moreover, soil enzyme activities primarily varied with soil depth and sampling date ($P < 0.05$, Table 3). For example, the activities of the six hydrolytic enzymes tended to be higher in the topsoil (0–10 cm), whereas the activity of phenol oxidase was higher in the subsurface soil (10–20 cm; Table 4). In brief, acid phosphatase activity exhibited the lowest decrease with soil depth, from 40.12 ± 9.14 to 26.16 ± 6.75 nmol g dry $weight^{-1}\,h^{-1}$ (35% decrease) in the control plots, and cellobiohydrolase activity showed the highest decrease with soil depth, from 2.65 ± 1.07 to 0.55 ± 0.21 nmol g dry $weight^{-1}\,h^{-1}$ (79% decrease) in the control plots. In contrast, phenol oxidase activity increased with soil depth from 3.21 ± 1.68 to 10.04 ± 4.44 μmol g dry $weight^{-1}\,h^{-1}$ in the control plots. Additionally, four and six out of the seven measured enzymes at 0–10 cm and 10–20 cm of soil depth, respectively, showed significant seasonal variations, with the highest activity being in the growing season (June–August; Table S2). For the control plots, cellobiohydrolase activity showed the highest seasonal variation, increasing from 1.88 ± 0.59 nmol g dry $weight^{-1}\,h^{-1}$ on May 3 to 4.67 ± 0.74 nmol g dry $weight^{-1}\,h^{-1}$ (61% increase) on June 14 in the topsoil (Table S2).

## 4. Discussion

### 4.1. Neutral effect of N addition on soil enzymes

In this study, we found no detectable effect of N addition on soil enzyme activities after two years of N addition in an alpine grassland ecosystem. This result did not support the resource allocation theory (Allison et al., 2008; Keeler et al., 2008), which predicted that N addition would increase the activities of C- and P-cycling enzymes, but suppress the activity of N-cycling enzymes. The lack of a N addition effect on soil enzymes in this study was unexpected, given that the amount of N we added was at the high end of N-addition experiments and that a positive effect of N addition on C- and P-acquiring enzymes has often been observed in other ecosystems (e.g., Allison et al., 2008; Koyama et al., 2013; Saiya-Cork et al., 2002; Wang et al., 2008). However, Bell et al. (2010) also found that soil enzyme activity was unresponsive to N addition across all seasons in a temperate old field. Here we provide two potential explanations for the neutral effect of N addition on soil enzyme activity observed in this study.

One potential explanation is that the short treatment period might prevent us from detecting statistically significant changes in soil enzymes after two years of N addition. The time scale over which we examined the effect of N addition on soil enzymes is important (Allison et al., 2010; Wang et al., 2015). For example, Fauci and Dick (1994) found that short-term (~165 days) N addition has limited effects on the activity of soil enzymes, whereas long-term (~306 days) N addition has a large effect on soil biological activity in a greenhouse experiment. In addition, Olander and Vitousek (2000) reported that phosphatase and chitinase activities in tropical montane rainforest soils did not respond to one month of N and P fertilization, but showed negative responses to four years of N and P fertilization. Therefore, soil enzyme activities may start to show significant responses to N addition in following years. Indeed, both soil respiration and heterotrophic respiration started to show negative responses after four years of N addition in this ecosystem (Ren et al., unpublished data). Further measurements in the following years may reveal the long-term impact of N addition on soil enzyme activities in this alpine grassland ecosystem. In addition, sampling time may also be responsible for the lack of enzymatic responses to N addition. As the added compound (urea) could provide readily available N for microbes, a stronger response of soil enzyme activity may be expected right after N addition. The first two samplings (May 3 and June 14) were before the second N application in July 5 2010 (with the first N application in July 2009), and the last two samplings (August 9 and September 16) were about 1 and 2 months after the

N application. These sampling dates were chosen to represent seasonal dynamics, but might fail to detect a significant pulse response of microbial enzyme activity to N addition in this alpine grassland ecosystem.

Another non-exclusive explanation may be related to the N status of this ecosystem. The accumulation of available N in the soil in the N and NP addition plots compared to the control plots (Fig. 1) suggests that the amount of added N ($10\,g\,N\,m^{-2}\,yr^{-1}$) exceeds the capacity of N uptake by plants and microbes (Aber et al., 1989; Bell et al., 2010). Likely, the background of N deposition ($1.0–1.5\,g\,N\,m^{-2}\,yr^{-1}$) at this site over the past decades (Jia et al., 2014) had provided enough available N for these alpine plants to grow. The lack of a significant effect of N addition on plant total and belowground biomass, soil microbial biomass C, and soil respiration in this study (Fig. 2) provides further evidence that this alpine grassland ecosystem may not be N-limited.

### 4.2. Negative effect of P addition on soil enzymes

Compared to the effect of N addition, the effect of P addition on soil enzymes is less explored (Marklein and Houlton, 2012). In this study, P addition generally suppressed the activity of most labile-C-cycling enzymes at 0–10 cm of depth but had insignificant or weak effects at 10–20 cm of depth (Fig. 2). Specifically, acid phosphatase activity at 0–10 cm of depth was marginally suppressed by P addition (Fig. 2), which is consistent with previous studies (Clarholm, 1993; Olander and Vitousek, 2000; Treseder and Vitousek, 2001; Wang et al., 2008), likely because microbes reduce the production cost of N-rich phosphatase enzymes in soils with high available P (Allison et al., 2011; Treseder and Vitousek, 2001). Moreover, two enzymes (β-1,4-*N*-acetyl-glucosaminnidase and phenol oxidase) were unresponsive to P addition, whereas three C-cycling related enzymes (α-1,4-glucosidase, β-1,4-xylosidase, and cellobiohydrolase) at depths of 0–10 cm all showed negative responses to P addition. Although this finding is not consistent with our hypothesis that P addition stimulates C- and N-cycling enzyme activities, it has been observed in a few other studies. For example, Turner and Wright (2014) showed that 10 years of P addition had no effect on β-1,4-glucosidase activity, yet suppressed β-1,4-*N*-acetyl-glucosaminnidase activity in a lowland tropical forest.

Additionally, P addition increased plant growth in terms of both aboveground and belowground biomass (Fig. 1), suggesting that plant primary productivity is limited by available P in this alpine grassland ecosystem. However, microbial biomass C and soil respiration rate were unresponsive to P addition, and mineral N availability tended to decline (by 20%) with P addition in this study (Fig. 1). Based on the evidence above, we propose that P addition may stimulate plant growth and the uptake of mineral N from the soil, reducing the amount of N available in the soil and limiting microbes to produce enzymes to decompose soil organic matter (Wang et al., 2008). Therefore, the hypothesis of secondary N-limitation due to P addition (Vitousek and Howarth, 1991; Vitousek et al., 2010) may help explain our finding that soil labile-C-cycling enzyme activities were suppressed by P addition but not by NP addition. It is likely that P addition causes secondary N-limitation for microbial activity, while combined N and P addition relieves this N-limitation by providing additional available N (Fig. 2). This working hypothesis is based on indirect evidence and awaits further test in future studies.

### 4.3. Implications for soil carbon storage in this alpine grassland ecosystem

In this study, we measured potential activities of seven hydrolytic and oxidative soil extracellular enzymes in response to a factorial N and P addition experiment in a Tibetan alpine grassland ecosystem. In contrast to our original hypotheses, we found that N and NP addition generally had no detectable effect on soil enzymes, while P addition tended to suppress most labile-C-cycling enzyme activities in the topsoil. This result is consistent across the four sampling dates during the growing season after two years of N and P addition, and is supported by the result from a recent sampling after five years of nutrient addition in a nearby grassland which had a similar experimental design (Jing et al.,

**Table 5**
Summary of nitrogen and phosphorus addition effects on plant productivity, soil microbial biomass, soil nutrient availability, soil respiration, and soil enzymes, based on results in this study. "+" indicates a positive effect, "-" indicates a negative effect, "ns" indicates no effect.

| | N addition effect | P addition effect | N + P addition effect |
|---|---|---|---|
| Plant productivity | | | |
| Total biomass | ns | + | + |
| Aboveground biomass | + | + | + |
| Belowground biomass | ns | + | ns |
| Soil microbial biomass | | | |
| Soil microbial biomass C | ns | ns | ns |
| Soil nutrient availability | | | |
| Soil mineral N availability | + | ns | + |
| Soil respiration | | | |
| Total soil respiration | ns | ns | ns |
| Root respiration[a] | ns | + | ns |
| Heterotrophic respiration[a] | ns | – | ns |
| Soil enzymes | | | |
| Labile-C cycling enzymes | ns | –[b] | ns |
| N cycling enzyme | ns | ns | ns |
| P cycling enzyme | ns | ns | ns |
| Recalcitrant-C cycling enzyme | ns | ns | ns |

[a] Data were not directly measured in this study.
[b] Most of the labile-C cycling enzymes were depressed in the topsoil.

unpublished data). It is worthy to note that the effects of nutrient addition on soil enzymes in this study are combination of direct effects (through changes in nutrient availability, Allison and Vitousek, 2005) and indirect effect (through changes in root biomass and substrate inputs, Stone et al., 2013). A few recent studies that included both rooted and root-free soils (Phillips and Fahey, 2008; Zhu et al., 2015) suggest that the present of living roots do not remarkably affect the responses of soil enzyme activities to nutrient addition. Nevertheless, future studies may include a root-free reference soil to separate the direct and indirect effects of nutrient addition on soil enzyme activities.

Taking measurements on all variables (plant biomass, soil respiration, microbial biomass, and enzyme activity) together could shed light on soil carbon cycling and storage in response to N and P addition in this alpine grassland ecosystem (Table 5). Although we did not directly measure heterotrophic respiration, we could infer it from the difference between soil respiration (directly measured) and root respiration (assumed to be positively correlated with root biomass, Geng et al., 2012; Vicca et al., 2010). In this study, N and NP addition had no effect on root biomass (Fig. 1) and root respiration presumably. As soil respiration was unresponsive to N and NP addition, heterotrophic respiration should also be unresponsive to N and NP addition. This reasoning was supported by the undetectable response of microbial biomass and enzyme activity to N and NP addition (Fig. 1 and 2). Additionally, P addition stimulated root biomass (Fig. 1) and root respiration presumably. Because soil respiration was not affected by P addition, heterotrophic respiration should be suppressed by P addition. Such prediction is consistent with the comparable microbial biomass and lower enzyme activities in P-added soils than in control soils (Fig. 1 and 2). Taken together, these results suggest that P addition may have a more positive effect on soil carbon storage compared to N and NP addition because of the higher carbon input from root biomass and lower carbon output from heterotrophic respiration. Overall, our findings suggest that soil enzyme activities of this alpine grassland ecosystem may be more sensitive to increased P availability than to increased N availability. Note that this finding is based on results after two years of N and P addition. Further monitoring of plant primary productivity, microbial activity, and soil carbon fluxes in the following years should be helpful for understanding the long-term response of soil carbon storage to nutrient addition in this alpine grassland ecosystem.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.06.004.

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