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Exogenous P compounds differentially interacted with N availability to regulate enzymatic activities in a meadow steppe

Ruzhen Wang\textsuperscript{a,1,*}, Yanzhuo Cao\textsuperscript{a,b,1}, Hongyi Wang\textsuperscript{a,c}, Feike A. Dijkstra\textsuperscript{d}, Jinglun Jiang\textsuperscript{a}, Ruonan Zhao\textsuperscript{a}, Wang Ma\textsuperscript{a}, Tianpeng Li\textsuperscript{a}, Maxim Dorodnikov\textsuperscript{e}, Zhengwen Wang\textsuperscript{a}, Jordi Sardans\textsuperscript{f,g}, Josep Peñuelas\textsuperscript{f,g}

\textsuperscript{a}Erguna Forest-Steppe Ecotone Ecosystem Research Station, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China

\textsuperscript{b}Key Laboratory of Regional Environment and Eco-Remediation, College of Environment, Shenyang University, Shenyang 110044, China

\textsuperscript{c}Heilongjiang Bayi Agricultural University, Daqing 163319, China

\textsuperscript{d}Sydney Institute of Agriculture, School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia

\textsuperscript{e}Department of Soil Science of Temperate Ecosystems, Georg-August University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany

\textsuperscript{f, g}CSIC, Global Ecology Unit CCREAF-CSIC-UAB, 08913 Bellaterra, Catalonia, Spain

\textsuperscript{f, g}CREAF, 08913 Cerdanyola del Vallès, Catalonia, Spain

\textsuperscript{1}Authors contributed equally to this work.

\textsuperscript{*}Corresponding author: Ruzhen Wang, Tel: +86 24 83970603; Fax: +86 24 83970300

E-mail address: ruzhenwang@iae.ac.cn
Increased inputs of ecosystem nitrogen (N) and phosphorus (P) may affect the activity of soil enzymes that play essential roles in the metabolism of carbon (C), N and P for microbial growth. However, the associations between soil enzymatic activities and N and P availability remain poorly understood. We conducted a study in a meadow steppe to elucidate the effects of the addition of N, as ammonium nitrate (NH₄NO₃), and two forms of P with contrasting solubility, comprising monopotassium phosphate (KH₂PO₄) that is more soluble than triple superphosphate (Ca(H₂PO₄)₂, on activity of β-glucosidase (BG), N-acetyl-glucosaminidase (NAG) and acid and alkaline phosphomonoesterases (PMEs). In general, there was a positive effect of N on BG, NAG and alkaline PME activity as a result of enhanced soil N availability, plant-microbe nutrient competition and plant P uptake. Addition of KH₂PO₄ increased activity of BG, NAG and alkaline PME, but had no impact on acid PME activity. Addition of Ca(H₂PO₄)₂ increased NAG activity, but only increased activity of BG and alkaline PME with the addition of N. Concentration of soil available P and microbial biomass P increased with added P, particularly KH₂PO₄. These results provide the first evidence for the N- and P-mediated stimulation of microbial activity depending on the chemical form of added P in this ecosystem. Relationships between activity of BG and NAG, and between that of NAG and PME were allometric, indicating disproportionate changes in activity of these soil enzymes. This further suggests shifts in microbial acquisition of C, N and P along with increases in availability of N and P that may potentially affect plant productivity. We conclude that scenarios of global environmental change, in which...
ecosystem availability of N and P are affected, may result in variable activity responses among soil enzymes, while the chemical form of P input should be considered as an important factor influencing meadow steppe grassland ecosystem function.

**Keywords** enzymatic stoichiometry, extracellular enzymatic activity, microbial biomass phosphorus, nitrogen availability, phosphorus fertilization

**Highlights**

- Chemical N and P increased enzyme activity but effects varied with P form and rate.
- N addition promoted soil enzyme activity through enhanced plant-microbe interactions.
- P and N availability resulted in variable activity responses among soil enzymes.
- Enzymatic stoichiometry showed varying microbial responses in C-, N- and P-acquisition.
Introduction

Current inputs of nitrogen (N) to ecosystems are 2- to 3-fold greater than levels prior to the green revolution and are >4-fold greater than those of phosphorus (P) (Peñuelas et al., 2013; Wang et al., 2018). Although global anthropogenic N inputs have steadily increased from 120-150 Mt $y^{-1}$ in the 1980s to 165-250 Mt $y^{-1}$ in the 2000s (Peñuelas et al., 2012) and are largely derived from crops that fix $N_2$, industrial fertilizers and emissions from fossil fuels, global anthropogenic inputs of P, which mostly stem from fertilizer use, have remained relatively stable. Thus, anthropogenic inputs of N and P have become increasingly unbalanced, with N:P ratios that are often much greater than those for terrestrial plants (Peñuelas et al., 2012, 2013). Changes in N and P cycles influence ecosystem stability and functions, such as primary productivity, plant-litter decomposition, nutrient release and C balance, particularly in temperate and boreal (limited by N) and tropical (limited by P) regions (Peñuelas et al., 2013; Fernández-Martínez et al., 2014; Jing et al., 2016; Niu et al., 2016; Chen et al., 2017).

Soil enzymes play a key role in the decomposition of soil organic matter and recycling of soil nutrients for plant and microbial growth (Shukla & Varma 2011; Trivedi et al., 2016). For example, β-glucosidase (BG) enzymes, which hydrolyze cellulose and other β-linked glucans into glucose, and N-acetyl-glucosaminidase (NAG) enzymes, which hydrolyze chitin and other β-linked aminopolysaccharides into glucosamine, are commonly used indicators of microbial C and N acquisition, respectively (Carreiro et al., 2000; Sinsabaugh et al., 2014). Acid and alkaline phosphomonoesterases (PMEs), required to hydrolyze phosphate from phospholipids
and phosphosaccharides are used as indicators of microbial P acquisition (Sinsabaugh et al., 2014; Jian et al., 2016). β-glucosidase, NAG and acid/alkaline PME catalyze terminal and rate-limiting reactions to produce C, N and P products that are assimilable by microbes, so their activities represent microbial C, N and P demand (Tabatabai, 1994; Sinsabaugh et al., 2014). As a result, these four enzymes have been used in studies to improve understanding of C, N and P cycling in soils (Sinsabaugh et al., 2009, 2014; Waring et al., 2014; Cenini et al., 2015). Soil C-cycling enzymes regulate the activity of N- and P-cycling enzymes via influencing microbial C availability and consequentially enzymatic activity, so activities of these enzymes are often tightly coupled with stoichiometric relationships (Waring et al., 2014). Activities of soil enzymes have been shown to be positively correlated with plant productivity (Margalef et al., 2017; Sterkenburg et al., 2018), plant nutrient demand (Sardans et al., 2007) and soil C:N:P stoichiometry of an ecosystem (Sinsabaugh et al., 2009), and may be affected by anthropogenic mediated changes in ecosystem availability of N and P. However, the magnitude and direction of single and combined effects of N and P inputs remain uncertain.

Effects of N addition on soil enzymatic activities in grassland ecosystems have been widely studied (summarized in Wang et al. 2014). and have been shown to vary. For example, positive, negative and neutral effects of soil N availability on C- and P-cycling enzyme activities have been reported (refer to Wang et al., 2014), indicating that enzyme activity may be mediated by other soil physicochemical properties, such as soil pH, moisture and P availability (Sardans et al., 2007; Sinsabaugh et al., 2008;
Indeed, N-induced soil acidification has been found to decrease BG and PME activities by inhibiting microbial growth, but higher levels of N availability led to reduced limitation of microbial N (Yang et al., 2017).

Effects of P addition, particularly those related to chemical form of P, on enzymatic activities (Table 1) and potential ecosystem responses in grasslands are less clear than those of N. For example, the addition of the relatively soluble monosodium phosphate (NaH$_2$PO$_4$) increased plant productivity on average by 22% in 98 grassland soils across North America (Craine & Jackson, 2010), while the less soluble triple superphosphate (Ca(H$_2$PO$_4$)$_2$) elicited positive (+59%) and neutral effects in South African grasslands (Craine et al., 2008), and—And additions of NaH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$ decreased (Phoenix et al., 2003) and increased (Tian et al., 2016) PME activity in natural calcareous grasslands, respectively.

These contrasting effects of different chemical forms of P on enzyme activity may be due to differences in soil pH, P-absorption capacity, time since application and their varying use efficiencies by plants and soil microorganisms related to P compound chemistry (Mori et al., 2018). For example, KH$_2$PO$_4$ addition was found to increase BG, NAG and acid PME activities in both a savannah and a semi-natural grassland (Mganga et al., 2015), while superphosphate application had no impact on the activity of the three enzymes in an alpine grassland (Jing et al., 2016). However, no comparison has been made for the differential effects of soluble and less soluble P forms on enzyme activities in the same grassland ecosystem with the same environmental conditions and fertilization history. Pre-input levels of ecosystem N and P are essential factors that
influence microbial activity in response to P inputs (Waring et al., 2014; Tian et al., 2016; Margalef et al., 2017), as demonstrated by increased PME activity in a P-poor steppe in response to P addition, but decreased activity in a relatively P-rich old-field grassland (Tian et al., 2016). Although little is known about the combined effects of inputs of N and chemical forms of P on C-, N- and P-cycling enzyme activities, microbial economic theory suggests that higher levels of P availability suppress P-cycling enzyme activity, but promote C- and N-cycling enzyme activities (Allison et al., 2010), where these effects would be stronger with more soluble forms of P.

The meadow steppe of northeastern China represents one of the dominant types of grassland in Eurasia and plays a vital role in supporting the regional economy, floral diversity and environmental health (Wang & Ba, 2008). This cold meadow steppe is highly sensitive to global climate change and is within the vulnerable ecotone between forest and steppe that receives 1-2 g N m$^{-2}$ y$^{-1}$ (Xu et al., 2015). Given increased productivity of the grassland is required to support a growing human population, more efficient P fertilization is urgently needed to prevent the gradual depletion of natural P reserves (Sattari et al., 2016). Therefore, we investigated the effects of combined additions of N and chemical forms of P on ecosystem processes in a meadow steppe field experiment, to test the hypotheses that (1) combined P and N addition would alleviate the decrease of NAG and increase of PME activities but stimulate the increase of BG activity as caused by N addition alone due to increasing microbial C and N requirements with enhanced P inputs; and (2) Ca(H$_2$PO$_4$)$_2$ would have less impact on enzyme activities than KH$_2$PO$_4$ where increases in BG and NAG activities and
decreases in PME activities are more pronounced under the more soluble form of P.

**Materials and methods**

**Study site and experimental design**

The field experiment was conducted at the Erguna Forest-Steppe Ecotone Research Station, Chinese Academy of Sciences, located at the southern boundary of the Eurasian permafrost region in Inner Mongolia. The climate of this area belongs to the transition zone between cold- and mid-temperate climates, with mean annual temperature and precipitation of -3 °C and 375 mm, respectively. The meadow steppe ecosystem is dominated by *Leymus chinensis* (Trin.) Tzvel, *Stipa baicalensis* Roshev and *Carex duriuscula* C.A.Mey, and soils are classified as Chernozem (IUSS Working Group WRB 2014). The relatively low background inputs of N and P render this an ideal location for the study of ecosystem responses to global environmental change and nutrient management. The bulk soil pH was 6.8 ± 0.07. Elemental analysis showed the bulk soil to have 24.0 ± 0.57 g kg⁻¹ C, 1.8 ± 0.06 g kg⁻¹ N and 0.5 ± 0.02 g kg⁻¹ P.

The experiment started in May 2014, and annual applications of fertilizers in May comprised KH₂PO₄ or Ca(H₂PO₄)₂ applied at 0, 2, 4, 6, 8 and 10 g P m⁻² y⁻¹, with (elevated) and without (ambient) N, applied as NH₄NO₃ at 0 and 10 g N m⁻² y⁻¹, arranged as three factors in a randomized block design, with five replicates; blocks were separated by 2-m buffer strips, and the 24 plots (8 × 8 m) within a block were separated by 1-m buffer strips (Figure S1). Fertilizers were applied to the soil surface as pellets. We chose 10 g N m⁻² y⁻¹ as it has been suggested to be the upper threshold for affecting
aboveground productivity, species richness and composition of plant functional groups in Inner Mongolian grasslands (Bai et al., 2010). However, an upper threshold for P has not been clearly established. In this study, we chose two commonly used P fertilizers of KH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$ to compare their effects on soil enzyme activities. We used KCl in the KH$_2$PO$_4$ plots to ensure similar levels of potassium (K) that were equal to those in the highest P treatment (13.2 g K m$^{-2}$); CaCl$_2$ was used to maintain a constant annual chloride (Cl) input (12.1 g Cl m$^{-2}$) with KCl addition. Calcium was not compensated, because of its high natural abundance in the calcareous soils.

Field sampling

Aboveground biomass of all plant material, including litterfall, was harvested from a 1 × 1-m quadrat that was placed randomly in each plot in August 2016. Plants were sorted to species and oven-dried with the litterfall biomass at 65 °C for 48 h, and then weighed. Five samples of soil, which were collected from the 0-10 cm layer using a 5-cm diameter auger in August 2016, were combined to form a single composite sample per plot and then stored in insulated cans at 4 °C during transport to the laboratory. The soil samples were sieved through a 2-mm screen to remove stones and visible roots; samples were subdivided, where one subsample was stored at 4 °C until analysis of microbial parameters, and another was air-dried.

Soil pH was determined in a 1:5 (w/v) soil-water suspension using a PHS-3G digital pH meter (Precision and Scientific Instrument Co. Ltd., Shanghai, China). A subsample
of the air-dried soil was ground using a ball mill (Retsch M400, Haan, Germany) for analysis of soil organic C (SOC), total N (TN) and total P (TP). SOC and TN concentrations were determined using K$_2$Cr$_2$O$_7$ oxidation (Nelson & Sommers, 1982) and the Kjeldahl method (Bremner, 1996), respectively, and soil TP concentration was determined using molybdenum-blue colorimetry following acid digestion of 0.1 g of soil (HNO$_3$, HClO$_4$ and HF). Total P concentration in the three dominant plant species (L. chinensis, S. Baicalensis and C. duriuscula) was determined using molybdenum-blue colorimetry following acid digestion of 0.3 g of plant material (HNO$_3$ and HClO$_4$) (Murphy & Riley, 1962) and P uptake in the species was determined as the product of its biomass and P concentration. Total inorganic N (TIN) concentration was calculated as the sum of NO$_3^-$-N and NH$_4^+$-N concentrations that were determined using a continuous-flow analyzer (AutoAnalyzer III, Bran & Luebbe, Norderstedt, Germany), following extraction from fresh soil using 2 M KCl.

Microbial biomass C (MBC) was determined using fumigation-extraction (Vance et al., 1987), where a 15-g subsample of fresh soil was fumigated with chloroform for 24 h; another 15-g subsample was not fumigated. Following extraction with 0.5 M K$_2$SO$_4$, the concentration of C was determined using a TOC analyzer (Multi N/C 3100, Analytik Jena, Jena, Germany), and MBC concentration was calculated as the difference between the fumigated and unfumigated samples. To correct for incomplete extraction, an efficiency factor of 0.38, which has previously been used for this grassland, was used to calculate actual MBC concentration (Wang et al., 2015). Concentration of microbial biomass P (MBP) was determined as for MBC, except we used 0.5 M NaHCO$_3$ as the
extractant (Brookes et al., 1982). Phosphate concentration in the extracts was measured using molybdenum-blue colorimetry with a UV-visible spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) (Murphy & Riley, 1962). The efficiency factor for MBP (0.39) was determined according to the equation proposed by Bilyera et al. (2018), using SOC (24.5 g kg\(^{-1}\)), total P (0.53 g kg\(^{-1}\)) and clay content (23.6%). Extractable C in the unfumigated samples was classed as dissolved organic C (DOC), and available (Olsen) P was extracted from 2.5 g of air-dried soil with 50 ml of 0.5 M NaHCO\(_3\) (pH 8.5) (Olsen et al., 1954) and then filtered; Olsen P was measured using molybdenum-blue colorimetry. Biomass of arbuscular mycorrhizal fungi (AMF) was estimated from analysis of phospholipid fatty acids that were extracted, fractionated and quantified (after Bossio & Scow, 1998) from frozen soil samples; the extraction mixture comprised CHCl\(_3\), methanol and citrate buffer (1:2:0.6). The extracted phospholipids were separated from neutral lipids and glycolipids and then methylated into fatty acid methyl esters via a mild alkaline methanolysis; the fatty acid methyl esters were then analyzed using a gas chromatograph (Agilent 7890A, Wilmington, USA) and identified using a microbial identification system (Microbial ID. Inc., Newark, DE, USA). We used fatty acid 16:1\(\omega5\) as a biomarker for AMF (Zhang et al., 2015).

**Soil enzymatic activity**

Maximum potential activities of BG, NAG and acid/alkaline PME required to catalyze specific reactions were determined at their respective optimal pHs and temperatures from fresh soil samples to allow comparison with other studies. The incubation
temperature was not adjusted to the mean annual temperature (-3 °C) that may have been a rate-limiting factor and obscured any treatment effects (Nannipieri et al., 2018).

For BG activity, 1 g of soil was mixed with 0.25 ml of toluene, 4 ml of modified universal buffer (comprising 0.1 M tris(hydroxymethyl)aminomethane, 0.067 M citric acid monohydrate and 0.1 M boric acid; pH 6.0) and 1 ml of 0.5 M p-nitrophenyl-β-D-glucopyranoside (CAS:2492-87-7) substrate; the mixture was incubated at 37 °C for 1 h, and the reaction was stopped by adding 1 ml of 0.5 M CaCl$_2$ and 4 ml of 0.1 M tris(hydroxymethyl)aminomethane (pH 12.0). Then, the mixture was filtered and production of p-nitrophenol (PNP) was measured colorimetrically using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 410 nm. Activities of NAG and acid/alkaline PME were measured as for BG activity, except we used a different substrate and pH for the reaction system: we used p-nitrophenyl-N-acetyl-β-D-glucosaminidine, p-nitrophenyl phosphate and p-nitrophenyl phosphate as substrates buffered at pHs of 5.5 (Parham & Deng, 2000), 6.5 and 11.0 (Tabatabai et al., 1994), respectively. Activities were expressed as the rate of PNP production (mg PNP kg soil$^{-1}$ h$^{-1}$). We acknowledge that our enzyme “activity” measurements only provide an indication of enzyme concentrations and do not represent actual soil enzyme activities (Wallenstein and Weintraub 2008).

**Statistical analyses**

Data were tested for normality using the Kolmogorov-Smirnov test, and homogeneity of variance was determined using Levene’s test; data were log-transformed for analysis.
of variance (ANOVA) as appropriate. Three-way ANOVA, with N addition, rate of P addition and chemical form of P fertilizer as factors, was used to test for treatment differences in soil physicochemical properties and enzymatic activities. Associations between enzymatic activities and physicochemical properties were tested using Pearson correlation analysis. Statistical analyses were performed in SPSS 19.0 for Windows (SPSS Inc., Chicago, USA). Redundancy analysis (RDA) (Canoco for Windows 5.0, Plant Research International, Wageningen, The Netherlands) was used to determine the relationships between soil physicochemical properties and enzymatic activities; prior to analysis, enzymatic activity data were \( \log_{10}(x+1) \)-transformed to correct for positive skewing, and the soil physicochemical properties were zero-centered for data standardization. We used a Monte Carlo test (499 permutations) (Canoco for Windows 5.0, Plant Research International, Wageningen, The Netherlands) to determine the relative contribution of soil parameters to variation in enzymatic activities, and the relationship between BG, NAG and acid PME enzymatic activities was tested using standardized major-axis (SMA) regression in R software (http://www.r-project.org, last accessed: February 2018) and compared with regression slopes of unity to identify isometric (not different from unity) or allometric (different from unity) relationships at \( P < 0.05 \).

**Results**

**Effects of N and P on soil physicochemical properties**

Soil pH decreased with addition of Ca(H$_2$PO$_4$)$_2$ under ambient and elevated levels of N
input ($P < 0.01$; Tables S1 and S2) due to release of H$^+$ during hydrolysis of Ca(H$_2$PO$_4$)$_2$.

There was a tendency towards a decrease in SOC concentration with increasing rate of KH$_2$PO$_4$, irrespective of N treatment ($P = 0.06$). Addition of N increased TIN concentration, regardless of form of P ($P < 0.01$; Table S2). TP concentration was greater with increasing rate of KH$_2$PO$_4$, irrespective of addition of N, and with addition of Ca(H$_2$PO$_4$)$_2$, without N (Tables S1 and S2). Olsen-P concentration was greater with increasing rate of P, irrespective of form of P and addition of N ($P < 0.01$; Table S2).

We found that MBP concentration was greater with increasing rate of added P, regardless of form of P and addition of N ($P < 0.01$; Figures 1a and b, Table S1) and it was greater under KH$_2$PO$_4$ than Ca(H$_2$PO$_4$)$_2$ ($P < 0.01$; Table S3, Figure 1). In general, there was a negative effect of N on AMF biomass (Figure S2a).

**Effects of N and P on enzymatic activities**

In general, addition of N positively affected BG, NAG and alkaline PME activities, while there was an interaction between the effects of N and rate of P on acid PME activity (Figure 2, Table S3). β-glucosidase activity increased with higher rates of KH$_2$PO$_4$ under ambient N ($P < 0.01$; Figure 2a) and at 4, 8 and 10 g m$^{-2}$ y$^{-1}$ KH$_2$PO$_4$ and 6 g m$^{-2}$ y$^{-1}$ Ca(H$_2$PO$_4$)$_2$ under elevated N (Figures 2a and b). The higher rates of P (6-10 g m$^{-2}$ y$^{-1}$) increased NAG activity, regardless of N and form of P ($P < 0.01$; Figures 2c and d). Acid PME activity was not affected by the addition of KH$_2$PO$_4$, irrespective of N (Fig. 2e), and increased with addition of Ca(H$_2$PO$_4$)$_2$ with elevated N (Figure 2f). Alkaline PME activity increased with increasing rate of KH$_2$PO$_4$, irrespective of N.
Effects of N and P on plant and soil function

Aboveground plant biomass increased with elevated N and, when averaged across rates of P and N treatment, was greater with addition of KH$_2$PO$_4$ than Ca(H$_2$PO$_4$)$_2$ ($P = 0.036$; Figure S2b). Phosphorus uptake in the three plant species was greater with addition of KH$_2$PO$_4$ than Ca(H$_2$PO$_4$)$_2$ ($P = 0.02$; Figure S2c), with increasing rate of added P ($P < 0.01$) and with elevated N ($P < 0.01$). Addition of N and P resulted in greater levels of aboveground litterfall biomass (Figures 4a and b).

$\beta$-glucosidase activity was positively correlated with litterfall biomass, regardless of form of P and addition of N (Figures 4c and d), while under addition of KH$_2$PO$_4$, it was positively correlated with TP (Figure S3a) and Olsen P (Figure S3b) concentrations and negatively associated with total C:P ($P = 0.01$; Figure S3c) and total N:P ($P = 0.01$; Figure S3d) ratios.

$N$-acetyl-glucosaminidase activity was positively correlated with TP ($P < 0.01$; Figure S3e) and Olsen P ($P < 0.05$; Figure S3f) and negatively correlated with...
total C:P ($P < 0.01$; Figure S3g) and total N:P ($P < 0.05$; Figure S3h) ratios, under both forms of P type, across N treatments. Acid PME activity was correlated negatively with Olsen P concentration with addition of KH$_2$PO$_4$ and positively correlated in the Ca(H$_2$PO$_4$)$_2$ treatment ($P < 0.05$; Figure S3i). Alkaline PME activity was positively correlated with Olsen P concentration with addition of KH$_2$PO$_4$ ($P = 0.001$; Figure S3j).

Under addition of KH$_2$PO$_4$, activities of BG, NAG and alkaline PME were positively associated with the first axis of the RDA, together with plant P uptake, plant biomass and TP, Olsen P and TIN concentrations; activity of acid PME was negatively associated, together with SOC concentration (Figure 5a). Activities of BG, NAG and acid PME under addition of Ca(H$_2$PO$_4$)$_2$ were correlated with plant P uptake and Olsen P and TP concentrations, whereas activity of alkaline PME was correlated with pH (Figure 5b). Overall variation in enzymatic activities under the addition of KH$_2$PO$_4$ tended to be driven by plant P uptake, plant biomass and concentrations of Olsen-P, TP, SOC and TIN that, together, explained 42.2% of the total variation (Fig. 5a). In contrast, soil pH, plant P uptake and Olsen-P and TP concentrations explained 25.5% of the variation in enzymatic activities under the addition of Ca(H$_2$PO$_4$)$_2$ (Figure 5b).

**Discussion**

Microbial biomass PMBP concentration increased under the two chemical forms of P, irrespective of addition of N, indicating immobilization of P in microbial biomass and limitation of P in this typical meadow steppe ecosystem. It is likely that alleviation of microbial P limitation would trigger the activity of extracellular enzymes, because our
multivariate analyses showed that P stocks and availability and plant P uptake were key drivers of the increases in enzymatic activities.

Effects of P on enzymatic activities

Our study is one of few that have reported increases in BG activity in response to greater availability of P, and thus far the only study in the cold to mid-temperate transitional climatic zone (Table 1). We detected a positive effect of P on BG activity, indicating soil-C cycling in this meadow steppe may be constrained by P availability; this finding supported our first hypothesis. However, the effect of P depended on its chemical form, because BG activity was greater with increasing rate of KH$_2$PO$_4$ but unaffected by Ca(H$_2$PO$_4$)$_2$. This finding was supported by the positive correlations of BG activity with soil TP and with Olsen P concentrations when KH$_2$PO$_4$ was added, and the lack of such associations under addition of Ca(H$_2$PO$_4$)$_2$ (Figure S3a and b). It is also possible that optimal BG activity decreased with the lower pH levels recorded under the addition of Ca(H$_2$PO$_4$)$_2$. The addition of the less soluble Ca(H$_2$PO$_4$)$_2$ may have reduced decomposition rates, because microbial BG activity was lower than in soils treated with the more soluble KH$_2$PO$_4$.

Previous studies in wetland and alpine meadow soils found that BG activity was unaffected by P loading availability, but positively correlated with DOC concentration (Wright & Reddy, 2001; Jing et al., 2016). In our study, litterfall biomass, but not DOC concentration, positively affected BG activity, regardless of form of P, indicating that plant litter played a more important role than DOC concentration in the regulation of
BG activity. Higher increased levels of soil N and P may improve substrate quality, such as reduced litterfall C:N and C:P, and increase quantity (as litterfall biomass) (Hobbie, 2005; Li et al., 2017). The negative correlations between soil C:P and N:P with BG activity (Figures S3c and d) support the premise that substrate quality plays an important role in the regulation of enzymatic activities (Wallenstein et al., 2009; Phillips et al., 2014). Indeed, litter contains abundant cellulose and hemicellulose that then serve as substrates and induce BG activity (Allison et al., 2013; Sinsabaugh et al., 2008); however, dissolved organic matter (including DOC) is a enzymatic product of litter decomposition that may inhibit BG activity (Tian et al., 2010). We found that BG activity was stimulated by the increased N and P inputs, likely due to the direct positive roles of P and N availability in the synthesis of proteins and soil enzymes (Sinsabaugh et al., 2014; Tian et al., 2016).

Addition of P led to an increase in microbial N demand, as indicated by the greater levels of NAG activity (regardless of form of P), which support our first hypothesis. Microbial NAG activity may eventually be subjected to soil C limitation in this meadow steppe, because we found that increased application of the more soluble KH$_2$PO$_4$ decreased the concentration of SOC that was possibly linked to an increase in decomposition. Indeed, addition of P also increased loss of soil C by increasing SOC mineralization in Swedish meta-replicated long-term field experiments (Poeplau et al., 2016a, b).

The positive effects of soil P (TP and Olsen P) levels on BG and NAG activities contrasted with the lack of effects reported from a meta-analysis of 17 studies of tropical...
ecosystems (Waring et al., 2014). Paradoxically, microbial enzyme activities may be
constrained by P in relatively fertile chernozems, but not in highly weathered and P-
limited tropical soils, and it is possible these contrasting results may be due to
differences in data synthesis from large-scale ecosystems and small-scale field-
manipulative experiments (Niu et al., 2016). Contrasting correlations between Olsen P
concentration and acid PME activity under addition of KH$_2$PO$_4$ (negative) and
Ca(H$_2$PO$_4$)$_2$ (positive) may have been due to the greater levels of plant P demand under
Ca(H$_2$PO$_4$)$_2$ addition (Figure S2c) that are usually associated with low levels of Olsen
P and high levels of PME activities (Antibus et al., 1992). Soil P parameters have been
reported to positively (Colvan et al., 2001; Tian et al., 2016) and negatively (Olander
& Vitousek, 2000; Phoenix et al., 2004) affect PME activity, where responses may
depend on effects of initial levels of soil P, plant productivity, intensity of P uptake by
plants, and soil properties on abiotic P fixation (Tian et al., 2016; Margalef et al., 2017).
In our study, the increase in alkaline PME activity, even with exogenous P inputs,
indicated that microbial P demand was stimulated with nutrient addition. We found a
lack of response in aboveground biomass to addition of P (Figure S1b). Nevertheless,
the increase in microbial P demand and uptake, as supported by the observed rise in
MBP under fertilization with the two forms of P, could have diminished the ability of
root biomass to successfully outcompete microbes for P (Marschner et al., 2011).
Inconsistent changes in MBC concentrations and enzymatic activities indicate a
decoupling of the size and activity of the microbial community under elevated nutrient
inputs (Lori et al., 2017), and asymmetric changes in MBC with MBP concentrations
indicate that soil microorganisms may preferentially immobilize P (Bünemann et al., 2012) and are stoichiometrically plastic (Xu et al., 2013) in response to nutrient inputs. Chemical form of P only affected alkaline PME activity, partially supporting our second hypothesis, where we found greater levels of alkaline PME activity in the Ca(H$_2$PO$_4$)$_2$ treatment that were associated with lower levels of Olsen P. The RDA indicated that drivers of enzymatic activity differed between the two forms of P (Figure 5) and the overall contrasting effects of P form were likely caused by differences in soil environments and soil-plant interactions, such as the rate and intensity of P uptake.

**Effects of N on enzymatic activities**

We found that BG activity increased with elevated N, indicating that greater availability of N alleviated microbial N limitation and stimulated microbial BG activity, thus supporting our first hypothesis. Although evidence that availability of N increases BG activity has been reported from other grassland and forest ecosystems (Henry et al., 2005; Keeler et al., 2009), our results contrast with those from semi-arid steppe grasslands in Inner Mongolia, where BG activity decreased with N addition (Wei et al., 2013; Yang et al., 2017). This discrepancy may be due to differences in effects of temperature, precipitation and soil fertility on the decomposition of plant residues and supply of C to microorganisms. For example, the meadow steppe is less water-limited than the semi-arid steppe, and an increase in soil moisture in Inner Mongolian grasslands has been reported to alleviate soil acidification, due to a reduction in leaching of basic cations (Cai et al., 2017), and physiological stress in soil microorganisms.
caused by atmospheric N deposition (Zhang et al., 2015; Yang et al., 2017). Thus, improved water conditions in wetter meadow steppes may interact with higher N availability to promote microbial growth and BG activity. Our finding that acid PME activity increased with elevated N indicated associated increases in P limitation and microbial and plant demand for P. Given that mineralization of C is the first step in P mineralization, where the hydrolysis of large C polymers facilitates the enzymatic catalysis of P-C and N-C hydrolysis, it is likely that increased BG activity may lead to subsequent P mineralization.

Although the increase in NAG activity with N addition was unexpected, positive effects of N on NAG activity have been detected in bulk soil (Yang et al., 2017) and soil fractions (Wang et al., 2015) in a semi-arid steppe ecosystem. The addition of N may have enhanced plant N uptake that increased plant productivity (Hodge et al., 2000) and microbial N demand. According with this, increases in the rates of litter decomposition associated to soil enzyme activities (including N-cycle enzymes) has been observed in response to N-addition (Wang et al., 2011). A recent meta-data analysis indicated how N fertilization increases the activities of hydrolase and oxidase enzymes, related to an increase in litter production due to higher plant production under higher levels of N-availability (Jian et al., 2016). Increased NAG activity may derive from increases in mycorrhizal biomass for higher P transportation, possibly in response to higher plant P demand under elevated N (Miller et al., 1998; Henry et al., 2005). However, the increase in aboveground plant biomass (Figure S1b) coupled with a decrease in AMF biomass (Figure S2a) under the addition of N indicated more effective...
competition by plants for N, resulting in N-limitation among the soil microorganisms, especially AMF, that then led to increased NAG production with greater plant density and productivity.

The divergent responses of acid and alkaline PME activities to N addition in this study may be due to greater levels of plant productivity (Figure S2b) and plant P uptake (Figure S2c) and indicate that PME production may have derived from different sources; for example, acid PME is produced by plant roots and soil microbes, whereas alkaline PME is principally produced by soil microbes (Tabatabai, 1994). Therefore, stable acid PME activity may be the consequence of a tradeoff between plant and soil microbial demand for P due to N enrichment. The greater levels of alkaline PME activity under N addition infer greater microbial P demand as a result of superior competition by plants for P (Marschner et al., 2011), as supported by the greater levels of plant biomass and plant P uptake (Figures S2b and c) and unaffected MBC (Table S2) and MBP concentrations (Figure 1) in response to elevated N.

Stoichiometric traits of soil enzymes

The extracellular enzyme model (Moorhead et al., 2012) and data collected from globally distributed soils and freshwater sediments (Sinsabaugh et al., 2009) have demonstrated that the ratios of the activities of C-, N- and P-acquiring enzymes approximately converge to 1. Usually, soil microbial growth is more limited by C than N or P (Allison et al., 2010); however, enzymatic activity is not always correlated with nutrient requirements for microbial growth, as indicated by our data. The SMA analysis
indicated that microbial activity in the grassland was more co-limited by N and P than the global average (Figure 3; Table S4). Indeed, it has been shown that N limitation constrains grassland productivity (Ren et al., 2017) and microbial activity (Henry et al., 2005), whereas P limitation of productivity may be gradual, as indicated by the globally decreasing soil P pool across grassland soils, due to intensified forage production and food supply (Sattari et al., 2012, 2016). Thus, N and P fertilization may be necessary to maintain fertility in grassland soils (Sattari et al., 2012), because increases in atmospheric N and P deposition may not be sufficient. Under this scenario, it is likely that greater levels of large-scale ecosystem nutrient inputs would facilitate microbial activity that may then affect plant nutrition and soil C sequestration. The optimal amounts of P addition in this grassland ecosystem is suggested to be 6 g P m\(^{-2}\) yr\(^{-1}\) as shown by the relatively higher extracellular enzyme activities and potentially enhanced nutrient cycling rates at this P input level. Enzymatic stoichiometry may be a more reliable indicator of microbial nutrient limitation than microbial biomass C:N:P ratios (Xu et al., 2013), due to the functional role of enzymes in the uptake and cycling of nutrients that sustain ecosystem productivity (Sinsabaugh & Shah, 2011).

Conclusions

Availability of N and P elicited positive effects on the activities of BG, NAG and alkaline PME; alkaline PME activity was lower under the more soluble KH\(_2\)PO\(_4\). Elevated N input stimulated plant productivity and P uptake and led to soil microbial P limitation that was greater in the Ca(H\(_2\)PO\(_4\))\(_2\), as indicated by higher levels of alkaline
PME activity. Addition of N increased activities of BG, NAG and alkaline PME by increasing substrate availability, potentially increasing plant-microbe competition for C and N and intensity of plant P uptake. Our data indicated that KH$_2$PO$_4$ mediated changes in enzymatic activities tended to be highly and positively associated with soil P availability and intensity of plant P uptake, while Ca(H$_2$PO$_4$)$_2$ mediated changes in soil pH played a more essential role in enzymatic activities than plant P uptake. The activities of soil enzymes in the study grassland were principally determined by P availability and plant P content, indicating anthropogenic changes in ecosystem N and P levels may elicit similar effects on soil enzymes, but that will likely depend on the chemical form of P fertilizer.

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Data accessibility

Data sets may be obtained from the corresponding author.

References

Allison, S.D., Weintraub, M.N., Gartner, T.B., Waldrop, M.P., 2010. Evolutionary-
economic principles as regulators of soil enzyme production and ecosystem function. In: Shukla, G., Varma, A. (Eds). Soil enzymology. Springer-Verlag Berlin Heidelberg, pp. 229-243.

Allison, S.D., Lu, Y., Weihe, C., Goulden, M.L., Martiny, A.C., Treseder, K.K., Martiny, J.B., 2013. Microbial abundance and composition influence litter decomposition response to environmental change. Ecology 94, 714-725.

Alvarez-Clare, S., Mack, M.C., Brooks, M., 2013. A direct test of nitrogen and phosphorus limitation to net primary productivity in a lowland tropical wet forest. Ecology 94, 1540–1551.

Bai, Y., Wu, J., Clark, C. M., Naeem, S., Pan, Q., Huang, J., Zhang, L., Han, X., 2010. Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from inner Mongolia Grasslands. Global Change Biology 16, 358-372.

Bilyera, N., Blagodatskaya, E., Yevdokimov, I., Kuzyakov, Y., 2018. Towards a conversion factor for soil microbial phosphorus. European Journal of Soil Biology 87, 1-8.

Binkley, D., Högberg, P., 2016. Tamm review: revisiting the influence of nitrogen deposition on Swedish forests. Forest Ecology and Management 368, 222-239.

Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1982. Measurement of microbial biomass phosphorus in soil. Soil Biology & Biochemistry 14, 319-329.

Bünemann, E.K., Oberson, A., Liebisch, F., Keller, F., Annaheim, K.E., Huguenin-Elie, O., Frossard, E., 2012. Rapid microbial phosphorus immobilization dominates
gross phosphorus fluxes in a grassland soil with low inorganic phosphorus availability. Soil Biology & Biochemistry 51, 84-95.

Cai, J., Luo, W., Liu, H., Feng, X., Zhang, Y., Wang, R., Xu, Z., Zhang, Y., Jiang, Y., 2017. Precipitation-mediated responses of soil acid buffering capacity to long-term nitrogen addition in a semi-arid grassland. Atmospheric Environment 170, 312-318.

Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A., Parkhurst, D.F., 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81, 2359-2365.

Cenini, V.L., Fornara, D.A., McMullan, G., Ternan, N., Lajtha, K., Crawley, M.J., 2015. Chronic nitrogen fertilization and carbon sequestration in grassland soils: evidence of a microbial enzyme link. Biogeochemistry 126, 301-313.

Chen, J., Luo, Y., Li, J., Zhou, X., Cao, J., Wang, R. W., Wang, Y., Shelton, S., Jin, Z., Walker L. M., Feng, Z., Niu, S., Feng, W., Jian, S., Zhou, L., 2017. Costimulation of soil glycosidase activity and soil respiration by nitrogen addition. Global Change Biology 23, 1328-133.

Colvan, S., Syers, J., O'Donnell, A., 2001. Effect of long-term fertiliser use on acid and alkaline phosphomonoesterase and phosphodiesterase activities in managed grassland. Biology and Fertility of Soils 34, 258-263.

Craine, J.M., Morrow, C., Stock, W.D., 2008. Nutrient concentration ratios and co-limitation in South African grasslands. New Phytologist 179, 829-836.

Craine, J.M., Jackson, R.D., 2010. Plant nitrogen and phosphorus limitation in 98 North
American grassland soils. Plant and Soil 334, 73-84.

Fernández-Martínez, M., Vicca, S., Janssens, I.A., Sardans, J., Luyssaert, S., Campioli, M., Chapin III, F.S., Ciais, P., Malhi, Y., Obersteiner, M., Papale, D., Piao, S., Reichstein, M., Peñuelas, J., 2014. Nutrient availability as the key regulator of global forest carbon balance. Nature Climate Change 4, 471-476.

Henry, H.A., Juarez, J.D., Field, C.B., Vitousek, P.M., 2005. Interactive effects of elevated CO\textsubscript{2}, N deposition and climate change on extracellular enzyme activity and soil density fractionation in a California annual grassland. Global Change Biology 11, 1808-1815.

Hobbie, S.E., 2005. Contrasting effects of substrate and fertilizer nitrogen on the early stages of litter decomposition. Ecosystems 8, 644-656.

Hodge, A., Robinson, D., Fitter, A., 2000. Are microorganisms more effective than plants at competing for nitrogen? Trends in Plant Science 5, 304-308.

IUSS Working Group WRB: World Reference Base for Soil Resources 2014. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106, FAO, Rome, 2014.

Jian, S., Li, J., Chen, J., Wang, G., Mayes, M. A., Dzantor, K.E., Hui, D., Luo, Y., 2016. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. Soil Biology & Biochemistry 101, 32-43.

Jing, X., Yang, X., Ren, F., Zhou, H., Zhu, B., He, J.S., 2016. Neutral effect of nitrogen addition and negative effect of phosphorus addition on topsoil extracellular enzymatic activities in an alpine grassland ecosystem. Applied Soil Ecology 107,
Keeler, B.L., Hobbie, S.E., Kellogg, L.E., 2009. Effects of long-term nitrogen addition on microbial enzyme activity in eight forested and grassland sites: implications for litter and soil organic matter decomposition. Ecosystems 12, 1-15.

Liebisch, F., Keller, F., Huguenin-Elie, O., Frossard, E., Oberson, A., Bünemann, E. K., 2014. Seasonal dynamics and turnover of microbial phosphorus in a permanent grassland. Biology and Fertility of Soils 50, 465-475.

Li, H., Yang, S., Xu, Z., Yan, Q., Li, X., van Nostrand, J.D., He, Z., Yao, F., Han, X., Zhou, J., Deng, Y., Jiang, Y., 2017. Responses of soil microbial functional genes to global changes are indirectly influenced by aboveground plant biomass variation. Soil Biology & Biochemistry 104, 18-29.

Lori, M., Symnaczik, S., Mäder, P., De Deyn, G., Gattinger, A., 2017. Organic farming enhances soil microbial abundance and activity-A meta-analysis and meta-regression. PLoS ONE 12, e0180442.

Margalef, O., Sardans, J., Fernández-Martínez, M., Molowny-Horas, R., Janssens, I.A., Ciais, P., Goll, D., Richter, A., Obersteiner, M., Peñuelas, J., 2017. Global patterns of phosphatase activity in natural soils. Scientific Reports 7, 1337.

Marschner, P., Crowley, D., Rengel, Z., 2011. Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis–model and research methods. Soil Biology & Biochemistry 43, 883-894.

Mganga, K. Z., Razavi, B. S., Kuzyakov, Y., 2015. Microbial and enzymes response to nutrient additions in soils of Mt. Kilimanjaro region depending on land
Marschner, P., Crowley, D., Rengel, Z., 2011. Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis–model and research methods. Soil Biology & Biochemistry 43, 883-894.

Miller, M., Palojärvi, A., Rangger, A., Reeslev, M., Kjøller, A., 1998. The use of fluorogenic substrates to measure fungal presence and activity in soil. Applied and Environmental Microbiology 64, 613-617.

Moorhead, D.L., Lashermes, G., Sinsabaugh, R.L., 2012. A theoretical model of C-and N-acquiring exoenzyme activities, which balances microbial demands during decomposition. Soil Biology & Biochemistry 53, 133-141.

Mori, T., Lu, X., Aoyagi, R., Mo, J., 2018. Reconsidering the phosphorus limitation of soil microbial activity in tropical forests. Functional Ecology 32:1145-1154.

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta 27, 31-36.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2018. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biology and Fertility of Soils 54, 11-19.

Niklas, K.J., 2006. A phyletic perspective on the allometry of plant biomass-partitioning patterns and functionally equivalent organ-categories. New Phytologist 171, 27-40.

Niu, S., Classen, A. T., Dukes, J.S., Kardol, P., Liu, L., Luo, Y., Rustad, L., Sun, J., Tang, J., Templer, P.H., Thomas, R.Q., Tian, D., Vicca, S., Wang, Y., Xia, J., Zaehle, S., 2016. Global patterns and substrate-based mechanisms of the terrestrial nitrogen
cycle. Ecology Letters 19, 697-709.

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. Biogeochemistry 49, 175-190.

Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183-1190.

Peñuelas, J., Sardans, J., Rivas-ubach, A., Janssens, I.A., 2012. The human-induced imbalance between C, N and P in Earth's life system. Global Change Biology 18, 3-6.

Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., Boucher, O., Godderis, Y., Hinsinger, P., Llusia, J., Nardin, E., Vicca, S., Obersteiner, M., Janssens, I.A., 2013. Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. Nature Communications 4, 2934.

Phillips, L.A., Ward, V., Jones, M.D., 2014. Ectomycorrhizal fungi contribute to soil organic matter cycling in sub-boreal forests. The ISME Journal 8, 699.

Phoenix, G.K., Booth, R.E., Leake, J.R., Read, D.J., Grime, J.P., Lee, J.A., 2003. Effects of enhanced nitrogen deposition and phosphorus limitation on nitrogen budgets of semi-natural grasslands. Global Change Biology 9, 1309-1321.

Poeplau, C., Bolinder, M.A., Kirchmann, H., Kätterer, T., 2016a. Phosphorus fertilisation under nitrogen limitation can deplete soil carbon stocks: evidence from Swedish meta-replicated long-term field experiments. Biogeosciences 13, 1119-1127.

Poeplau, C., Herrmann, A.M., Kätterer, T., 2016b. Opposing effects of nitrogen and
phosphorus on soil microbial metabolism and the implications for soil carbon storage. Soil Biology & Biochemistry 100, 83-91.

Ren, H., Xu, Z., Isbell, F., Huang, J., Han, X., Wan, S., Chen, S., Wang, R., Zeng, D., Jiang, Y., Fang, Y., 2017. Exacerbated nitrogen limitation ends transient stimulation of grassland productivity by increased precipitation. Ecological Monographs 87, 457-469.

Sardans, J., Peñuelas, J., Estiarte, M., 2007. Seasonal patterns of root-surface phosphatase activities in a Mediterranean shrubland. Responses to experimental warming and drought. Biology and Fertility of Soils 43, 779-786.

Sardans, J., Alonso, R., Janssens, I. A., Carnicer, J., Vereseglou, S., Rillig, M. C., Fernández-Martínez, M., Sanders, T.G.M., Peñuelas, J., 2016. Foliar and soil concentrations and stoichiometry of nitrogen and phosphorous across European Pinus sylvestris forests: relationships with climate, N deposition and tree growth. Functional Ecology 30, 676-689.

Schimel, J.P., Weintraub, M.N., 2003. The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. Soil Biology & Biochemistry 35, 549-563.

Shukla, G., Varma, A., 2011. Soil enzymology. Springer-Verlag, Berlin, Heidelberg.

Sinsabaugh, R. L., Lauber, C. L., Weintraub, M. N., Ahmed, B., Allison, S. D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008.
Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252-1264.

Sinsabaugh, R.L., Hill, B.H., Shah, J. J. F., 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462, 795-798.

Sinsabaugh, R.L., Shah, J.J.F., 2011. Ecoenzymatic stoichiometry of recalcitrant organic matter decomposition: the growth rate hypothesis in reverse. Biogeochemistry 102, 31-43.

Sinsabaugh, R.L., Belnap, J., Findlay, S.G., Shah, J.J.F., Hill, B.H., Kuehn, K.A., Kuske, C.R., Litvak, M.E., Martinez, N.G., Moorhead, D.L., Warnock, D.D., 2014. Extracellular enzyme kinetics scale with resource availability. Biogeochemistry 121, 287-304.

Sattari, S.Z., Bouwman, A.F., Giller, K.E., van Ittersum, M.K., 2012. Residual soil phosphorus as the missing piece in the global phosphorus crisis puzzle. Proceedings of the National Academy of Sciences 109, 6348-6353.

Sattari, S.Z., Bouwman, A.F., Rodríguez, R.M., Beusen, A.H.W., Van Ittersum, M.K., 2016. Negative global phosphorus budgets challenge sustainable intensification of grasslands. Nature Communications 7, 10696.

Sterkenburg, E., Clemmensen, K.E., Ekblad, A., Finlay, R.D., Lindahl, B.D., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. The ISME Journal 12, 2187-2197.

Tabatabai, M., 1994. Soil enzymes. In: Bottomley, P.S., Angle, J.S., Weaver, R.W. (Eds.), Methods of soil analysis: Part 2-microbiological and biochemical properties,
4th edn. Soil Science Society of America, Madison, pp 775-833.

Tian, L., Dell, E., Shi, W., 2010. Chemical composition of dissolved organic matter in agroecosystems: correlations with soil enzyme activity and carbon and nitrogen mineralization. Applied Soil Ecology 46, 426-435.

Tian, J., Wei, K., Condron, L. M., Chen, Z., Xu, Z., Chen, L., 2016. Impact of land use and nutrient addition on phosphatase activities and their relationships with organic phosphorus turnover in semi-arid grassland soils. Biology and Fertility of Soils 52, 675-683.

Trivedi, P., Delgado-Baquerizo, M., Trivedi, C., Hu, H., Anderson, I.C., Jeffries, T. C., Zhou, J., Singh, B.K., 2016. Microbial regulation of the soil carbon cycle: evidence from gene–enzyme relationships. The ISME Journal 10, 2593-2604.

Turner, B.L., Wright, S.J., 2014. The response of microbial biomass and hydrolytic enzymes to a decade of nitrogen, phosphorus, and potassium addition in a lowland tropical rain forest. Biogeochemistry 117, 115-130.

Vance, E.D., Brooks, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass-C. Soil Biology & Biochemistry 19, 703-707.

Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Global Change Biology 15, 1631-1639.

Wallenstein, M.D., Weintraub, M.N., 2008. Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. Soil Biology & Biochemistry 40, 2098-2106.
Wang, C., Han, G., Jia, Y., Feng, X., Guo, P., Tian, X., 2011. Response of litter decomposition and related soil enzyme activities to different forms of nitrogen fertilization in a subtropical forest. Ecological Research 26, 505-513.

Wang, D., Ba, L., 2008. Ecology of meadow steppe in northeast China. The Rangeland Journal 30, 247-254.

Wang, R., Filley, T. R., Xu, Z., Wang, X., Li, M. H., Zhang, Y., Luo, W., Jiang, Y., 2014. Coupled response of soil carbon and nitrogen pools and enzyme activities to nitrogen and water addition in a semi-arid grassland of Inner Mongolia. Plant and Soil 381, 323-336.

Wang, R., Dorodnikov, M., Yang, S., Zhang, Y., Filley, T. R., Turco, R. F., Zhang, Y., Xu, Z., Li, H., Jiang, Y., 2015. Responses of enzymatic activities within soil aggregates to 9-year nitrogen and water addition in a semi-arid grassland. Soil Biology & Biochemistry 81, 159-167.

Wang, Y., Ciais, P., Goll, D., Huang, Y., Luo, Y., Wang, Y.P., Bloom, A.A., Broquet, G., Hartmann, J., Peng, S., Peñuelas, J., Piao, S., Sardans, J., Stocker, B.D., Wang, R., Zechle, S., Zechmeister-Boltenstern, S., 2018. GOLUM-CNP v1.0: a data-driven modeling of carbon, nitrogen and phosphorus cycles in major terrestrial biomes. Geoscientific Model Development 11, 3903-3928.

Waring, B.G., Weintraub, S.R., Sinsabaugh, R.L., 2014. Ecoenzymatic stoichiometry of microbial nutrient acquisition in tropical soils. Biogeochemistry 117, 101-113.

Wei, C., Yu, Q., Bai, E., Lü, X., Li, Q., Xia, J., Kardol, P., Liang, W., Wang, Z., Han, X., 2013. Nitrogen deposition weakens plant–microbe interactions in grassland
ecosystems. Global Change Biology 19, 3688-3697.

Wright, A.L., Reddy, K.R., 2001. Phosphorus loading effects on extracellular enzyme activity in Everglades wetland soils. Soil Science Society of America Journal 65, 588–595.

Xu, X., Thornton, P.E., Post, W.M., 2013. A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. Global Ecology and Biogeography 22, 737-749.

Xu, W., Luo, X.S., Pan, Y., Zhang, L., Tang, A. H., Shen, J.L., Zhang, Y., Li, K., Wu, Q., Yang, D.W., Zhang, Y.Y., Xue, J., Li, W.Q., Li, Q.Q., Tang, L., Lu, S.H., Liang, T., Tong, Y.A., Liu, P., Zhang, Q., Xiong, Z.Q., Shi, X.J., Wu, L.H., Shi, W.Q., Tian, K., Zhong, X.H., Shi, K., Tang, Q.Y., Zhang, L.J., Huang, J.L., He, C.E., Kuang, F.H., Zhu, B., Liu, H., Jin, X., Xin, Y.J., Shi, X.K., Du, E.Z., Dore, A.J., Tang, S., Collett Jr., J.L., Goulding, K., Sun, Y.X., Ren, J., Zhang, F.S., Liu, X. (2015). Quantifying atmospheric nitrogen deposition through a nationwide monitoring network across China. Atmospheric Chemistry and Physics 15, 12345-12360.

Yang, S., Xu, Z., Wang, R., Zhang, Y., Yao, F., Zhang, Y., Turco, R.F., Jiang, Y., Zou, H., Li, H., 2017. Variations in soil microbial community composition and enzymatic activities in response to increased N deposition and precipitation in Inner Mongolian grassland. Applied Soil Ecology 119, 275-285.

Zhang, N., Wan, S., Guo, J., Han, G., Gutknecht, J., Schmid, B., Yu, L., Liu, W., Bi, Y., Wang, W., Ma, K., 2015. Precipitation modifies the effects of warming and nitrogen addition on soil microbial communities in northern Chinese grasslands. Soil
Biology & Biochemistry 89, 12-23.
Table 1: Literature review of effects of phosphorus (P) addition on soil β-glucosidase (BG), N-acetyl-glucosaminidase (NAG) and acid and alkaline phosphomonoesterases (PMEs) activity in grassland ecosystems.

| Grassland type and location                  | P form            | BG   | NAG  | Acid PME | Alkaline PME | Reference          |
|----------------------------------------------|-------------------|------|------|----------|-------------|--------------------|
| Meadow grassland, UK                         | Triple superphosphate | -    | -    | 0        | ↑           | Colvan et al. 2001 |
| Mesic grassland, Switzerland                 | Superphosphate    | -    | -    | ↓        | -           | Bünemann et al. 2012 |
| Mesic grassland, Switzerland                 | Superphosphate    | -    | -    | ↓        | -           | Liebsch et al. 2014 |
| Savannah, Tanzania                           | KH₂PO₄            | ↑    | ↑    | ↑        | -           | Mganga et al. 2015  |
| Semi-natural grassland, Tanzania             | KH₂PO₄            | ↑    | ↑    | ↑        | -           |                    |
| Alpine grassland, China                      | Triple superphosphate | 0    | 0    | 0        | -           | Jing et al. 2016   |
| Semi-arid steppe, China                      | Superphosphate    | -    | -    | ↑        | ↑           | Tian et al. 2016   |
| Old field, China                             | Superphosphate    | -    | -    | ↓        | ↓           |                    |

Effects annotated as †, ↓, 0 or - indicate positive, negative, no significant change or lack of data, respectively.
Figure captions

Figure 1 Effect of addition of KH$_2$PO$_4$ (a) or Ca(H$_2$PO$_4$)$_2$ (b) with 0 and 10 g N m$^{-2}$ y$^{-1}$ nitrogen (N) on concentration of microbial biomass phosphorus (MBP) (mean ±SE, n = 5). Upper- and lowercase letters indicate differences among KH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$ treatments with and without added N, respectively.

Figure 2 Boxplots of activity of BG (a, b), NAG (c, d), acid PME (e, f) and alkaline PME (g, h) with addition of KH$_2$PO$_4$ (a, c, e, g) and Ca(H$_2$PO$_4$)$_2$ (b, d, f, h) with 0 (blue) and 10 g N m$^{-2}$ y$^{-1}$ (red). Different letters indicate differences among KH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$ treatments with or without added N, and asterisks indicate differences between N treatments for the rates of KH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$. Error bars indicate the 10th and 90th percentiles; black lines within the boxes represent median activity and the box limits indicate activity within the 25-75th percentile range.

Figure 3 Regression analyses of activities of NAG and BG, PME and BG and PME and NAG. All data are Ln-transformed. Dashed line: line of unity.

Figure 4 Mean litter biomass (±SE, n = 5) with addition of KH$_2$PO$_4$ (a) or Ca(H$_2$PO$_4$)$_2$ (b) under ambient and added nitrogen (N). Relationship between BG activity and litter biomass with addition of KH$_2$PO$_4$ (c) or Ca(H$_2$PO$_4$)$_2$ across N treatments (d). Upper- and lowercase letters indicate differences among KH$_2$PO$_4$ and Ca(H$_2$PO$_4$)$_2$ treatments with and without N addition, respectively. Asterisk indicates within P rate and type.
differences between N treatments.

Figure 5 Redundancy analysis of the relationship between soil enzyme activity (BG, NAG, acid PME and alkaline PME) and explanatory parameters (plant P uptake, pH, plant biomass and TP, TIN, SOC and Olsen-P concentrations) (left) and their contributions to the variation in overall activity (right) under addition of KH$_2$PO$_4$ (a) or Ca(H$_2$PO$_4$)$_2$ (b) addition.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5