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

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Summary

Increased inputs of ecosystem nitrogen (N) and phosphorus (P) may affect the activity of soil enzymes that play essential roles in the metabolization 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 (NH_4NO_3), and two forms of P with contrasting solubility, comprising monopotassium phosphate (KH_2PO_4) that is more soluble than triple superphosphate ($Ca(H_2PO_4)_2$, 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 KH_2PO_4 increased activity of BG, NAG and alkaline PME, but had no impact on acid PME activity. Addition of $Ca(H_2PO_4)_2$ 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 KH_2PO_4. 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~~BG~~, 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~~) 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;

105 Waring *et al.*, 2014; Mori *et al.*, 2018). Indeed, N-induced soil acidification has been
106 found to decrease BG and PME activities by inhibiting microbial growth, but higher
107 levels of N availability led to reduced limitation of microbial N (Yang et al., 2017).
108 Effects of P addition, particularly those related to chemical form of P, on enzymatic
109 activities (Table 1) and potential ecosystem responses in grasslands are less clear than
110 those of N. For example, the addition of the relatively soluble monosodium phosphate
111 (NaH_2PO_4) increased plant productivity on average by 22% in 98 grassland soils across
112 North America (Craine & Jackson, 2010), while the less soluble triple superphosphate
113 ($Ca(H_2PO_4)_2$) elicited positive (+59%) and neutral effects in South African grasslands
114 (Craine *et al.*, 2008), and. And additions of NaH_2PO_4 and $Ca(H_2PO_4)_2$ decreased
115 (Phoenix *et al.*, 2003) and increased (Tian *et al.*, 2016) PME activity in natural
116 calcareous grasslands, respectively.

117 These contrasting effects of different chemical forms of P on enzyme activity may
118 be due to differences in soil pH, P-absorption capacity, time since application and their
119 varying use efficiencies by plants and soil microorganisms related to P compound
120 chemistry (Mori *et al.*, 2018). For example, KH_2PO_4 addition was found to increase BG,
121 NAG and acid PME activities in both a savannah and a semi-natural grassland (Mganga
122 *et al.*, 2015), while superphosphate application had no impact on the activity of the three
123 enzymes in an alpine grassland (Jing *et al.*, 2016). However, no comparison has been
124 made for the differential effects of soluble and less soluble P forms on enzyme activities
125 in the same grassland ecosystem with the same environmental conditions and
126 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<u>s</u> 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) $Ca(H_2PO_4)_2$ ~~would~~ ha<u>s</u>~~ve~~ less impact on enzyme activities than KH_2PO_4 where increases in BG and NAG activities and

149 decreases in PME activities are more pronounced under the more soluble form of P.

150

151 **Materials and methods**

152 ***Study site and experimental design***

153 The field experiment was conducted at the Erguna Forest-Steppe Ecotone Research
154 Station, Chinese Academy of Sciences, located at the southern boundary of the Eurasian
155 permafrost region in Inner Mongolia. The climate of this area belongs to the transition
156 zone between cold- and mid-temperate climates, with mean annual temperature and
157 precipitation of -3 °C and 375 mm, respectively. The meadow steppe ecosystem is
158 dominated by *Leymus chinensis* (Trin.) Tzvel, *Stipa baicalensis* Roshev and *Carex*
159 *duriuscula* C.A.Mey, and soils are classified as Chernozem (IUSS Working Group
160 WRB 2014). The relatively low background inputs of N and P render this an ideal
161 location for the study of ecosystem responses to global environmental change and
162 nutrient management. The bulk soil pH was 6.8 ± 0.07. Elemental analysis showed the
163 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.

164 The experiment started in May 2014, and annual applications of fertilizers in May
165 comprised KH_2PO_4 or $Ca(H_2PO_4)_2$ applied at 0, 2, 4, 6, 8 and 10 g P m^{-2} y^{-1}, with
166 (elevated) and without (ambient) N, applied as NH_4NO_3 at 0 and 10 g N m^{-2} y^{-1},
167 arranged as three factors in a randomized block design, with five replicates; blocks were
168 separated by 2-m buffer strips, and the 24 plots (8 × 8 m) within a block were separated
169 by 1-m buffer strips (Figure S1). Fertilizers were applied to the soil surface as pellets.
170 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~~commonly used P fertilizers of KH_2PO_4 and $Ca(H_2PO_4)_2$ to compare their effects on soil enzyme activities. We used KCl in the KH_2PO_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 ºC 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 $K_2Cr_2O_7$ oxidation (Nelson & Sommers, 1982) and the Kjeldahl method (Bremner, 1996), respectively, and soil TP concentration was determined using molybdenum-blue colourimetry 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_2SO_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 colourimetry 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 colourimetry. 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 16:1ω5 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

237 temperature was not adjusted to the mean annual temperature (-3 °C) that may have
238 been a rate-limiting factor and obscured any treatment effects (Nannipieri *et al.*, 2018).
239 For BG activity, 1 g of soil was mixed with 0.25 ml of toluene, 4 ml of modified
240 universal buffer (comprising 0.1 M tris(hydroxymethyl)aminomethane, 0.067 M citric
241 acid monohydrate and 0.1 M boric acid; pH 6.0) and 1 ml of 0.5 M *p*-nitrophenyl-β-D-
242 glucopyranoside (CAS:2492-87-7) substrate; the mixture was incubated at 37 °C for 1
243 h, and the reaction was stopped by adding 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M
244 tris(hydroxymethyl)aminomethane (pH 12.0). Then, the mixture was filtered and
245 production of *p*-nitrophenol (PNP) was measured colourimetrically using a
246 spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 410 nm. Activities of NAG
247 and acid/alkaline PME were measured as for BG activity, except we used a different
248 substrate and pH for the reaction system: we used *p*-nitrophenyl-*N*-acetyl-β-D-
249 glucosaminidine, *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphate as substrates
250 buffered at pHs of 5.5 (Parham & Deng, 2000), 6.5 and 11.0 (Tabatabai *et al.*, 1994),
251 respectively. Activities were expressed as the rate of PNP production (mg PNP kg $soil^{-1}$
252 ^{1} h^{-1}). We acknowledge that our enzyme "activity" measurements only provide an
253 indication of enzyme concentrations and do not represent actual soil enzyme activities
254 (Wallenstein and Weintraub 2008).
255
256 ***Statistical analyses***
257 Data were tested for normality using the Kolmogorov-Smirnov test, and homogeneity
258 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 $Ca(H_2PO_4)_2$ under ambient and elevated levels of N

input ($P < 0.01$; Tables S1 and S2) due to release of H^+ during hydrolysis of $Ca(H_2PO_4)_2$. There was a tendency towards a decrease in SOC concentration with increasing rate of KH_2PO_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_2PO_4, irrespective of addition of N, and with addition of $Ca(H_2PO_4)_2$, 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_2PO_4 than $Ca(H_2PO_4)_2$ ($P < 0.01$; 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~~BG~~ activity increased with higher rates of KH_2PO_4 under ambient N ($P < 0.01$; Figure 2a) and at 4, 8 and 10 g m^{-2} y^{-1} KH_2PO_4 and 6 g m^{-2} y^{-1} $Ca(H_2PO_4)_2$ 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_2PO_4, irrespective of N (Fig. 2e), and increased with addition of $Ca(H_2PO_4)_2$ with elevated N (Figure 2f). Alkaline PME activity increased with increasing rate of KH_2PO_4, irrespective of N

(Figure 2g) and with rate of $Ca(H_2PO_4)_2$ under elevated N ($P < 0.01$; Figure 2h). Chemical form of P affected alkaline PME activity ($P < 0.05$; Table S3), where average activity across rates of P and N was greater with added $Ca(H_2PO_4)_2$ than KH_2PO_4. We found positive associations between BG and NAG ($P < 0.01$; Figure 3a, Table S4) and between NAG and acid PME ($P = 0.02$; Figure 3f, Table S4), and all slopes in the SMA regression differed from 1.0, indicating allometric relationships between enzymatic activities (Table S4).

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_2PO_4 than $Ca(H_2PO_4)_2$ ($P = 0.036$; Figure S2b). Phosphorus uptake in the three plant species was greater with addition of KH_2PO_4 than $Ca(H_2PO_4)_2$ ($P = 0.02$; 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).

β-glucosidase~~BG~~ 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_2PO_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 ~~NAG~~ 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_2PO_4 and positively correlated in the $Ca(H_2PO_4)_2$ treatment ($P < 0.05$; Figure S3i). Alkaline PME activity was positively correlated with Olsen P concentration with addition of KH_2PO_4 ($P = 0.001$; Figure S3j).

Under addition of KH_2PO_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 $Ca(H_2PO_4)_2$ 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_2PO_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 $Ca(H_2PO_4)_2$ (Figure 5b).

Discussion

Microbial biomass P~~MBP~~ 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_2PO_4, but unaffected by $Ca(H_2PO_4)_2$. This finding was supported by the positive correlations of BG activity with soil TP and with Olsen P concentrations when KH_2PO_4 was added, and the lack of such associations under addition of $Ca(H_2PO_4)_2$ (Figure S3a and b). It is also possible that optimal BG activity decreased with the lower pH levels recorded under the addition of $Ca(H_2PO_4)_2$. The addition of the less soluble $Ca(H_2PO_4)_2$ may have reduced decomposition rates, because microbial BG activity was lower than in soils treated with the more soluble KH_2PO_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 ~~increase~~ improve substrate quality, such as ~~r~~lower educed 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_2PO_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

391 ecosystems (Waring *et al.*, 2014). Paradoxically, microbial enzyme activities may be
392 constrained by P in relatively fertile chernozems, but not in highly weathered and P-
393 limited tropical soils, and it is possible these contrasting results may be due to
394 differences in data synthesis from large-scale ecosystems and small-scale field-
395 manipulative experiments (Niu *et al.*, 2016). Contrasting correlations between Olsen P
396 concentration and acid PME activity under addition of KH_2PO_4 (negative) and
397 $Ca(H_2PO_4)_2$ (positive) may have been due to the greater levels of plant P demand under
398 $Ca(H_2PO_4)_2$ addition (Figure S2c) that are usually associated with low levels of Olsen
399 P and high levels of PME activities (Antibus *et al.*, 1992). Soil P parameters have been
400 reported to positively (Colvan *et al.*, 2001; Tian *et al.*, 2016) and negatively (Olander
401 & Vitousek, 2000; Phoenix *et al.*, 2004) affect PME activity, where responses may
402 depend on effects of initial levels of soil P, plant productivity, intensity of P uptake by
403 plants, and soil properties on abiotic P fixation (Tian *et al.*, 2016; Margalef *et al.*, 2017).
404 In our study, the increase in alkaline PME activity, even with exogenous P inputs,
405 indicated that microbial P demand was stimulated with nutrient addition. We found a
406 lack of response in aboveground biomass to addition of P (Figure S1b). Nevertheless,
407 the increase in microbial P demand and uptake, as supported by the observed rise in
408 MBP under fertilization with the two forms of P, could have diminished the ability of
409 root biomass to successfully outcompete microbes for P (Marschner *et al.*, 2011).
410 Inconsistent changes in MBC concentrations and enzymatic activities indicate a
411 decoupling of the size and activity of the microbial community under elevated nutrient
412 inputs (Lori *et al.*, 2017), and asymmetric changes in MBC with MBP concentrations

413 indicate that soil microorganisms may preferentially immobilize P (Bünemann *et al.*,
414 2012) and are stoichiometrically plastic (Xu *et al.*, 2013) in response to nutrient inputs.
415 Chemical form of P only affected alkaline PME activity, partially supporting our
416 second hypothesis, where we found greater levels of alkaline PME activity in the
417 $Ca(H_2PO_4)_2$ treatment that were associated with lower levels of Olsen P. The RDA
418 indicated that drivers of enzymatic activity differed between the two forms of P (Figure
419 5) and the overall contrasting effects of P form were likely caused by differences in soil
420 environments and soil-plant interactions, such as the rate and intensity of P uptake.
421
422 ***Effects of N on enzymatic activities***
423 We found that BG activity increased with elevated N, indicating that greater availability
424 of N alleviated microbial N limitation and stimulated microbial BG activity, thus
425 supporting our first hypothesis. Although evidence that availability of N increases BG
426 activity has been reported from other grassland and forest ecosystems (Henry *et al.*,
427 2005; Keeler *et al.*, 2009), our results contrast with those from semi-arid steppe
428 grasslands in Inner Mongolia, where BG activity decreased with N addition (Wei *et al.*,
429 2013; Yang *et al.*, 2017). This discrepancy may be due to differences in effects of
430 temperature, precipitation and soil fertility on the decomposition of plant residues and
431 supply of C to microorganisms. For example, the meadow steppe is less water-limited
432 than the semi-arid steppe, and an increase in soil moisture in Inner Mongolian
433 grasslands has been reported to alleviate soil acidification, due to a reduction in leaching
434 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

457 competition by plants for N, resulting in N-limitation among the soil microorganisms,
458 especially AMF, that then led to increased NAG production with greater plant density
459 and productivity.

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

471

472 ***Stoichiometric traits of soil enzymes***

473 The extracellular enzyme model (Moorhead *et al.*, 2012) and data collected from
474 globally distributed soils and freshwater sediments (Sinsabaugh *et al.*, 2009) have
475 demonstrated that the ratios of the activities of C-, N- and P-acquiring enzymes
476 approximately converge to 1. Usually, soil microbial growth is more limited by C than
477 N or P (Allison *et al.*, 2010); however, enzymatic activity is not always correlated with
478 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~~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_2PO_4. Elevated N input stimulated plant productivity and P uptake and led to soil microbial P limitation that was greater in the $Ca(H_2PO_4)_2$, 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_2PO_4 mediated changes in enzymatic activities tended to be highly and positively associated with soil P availability and intensity of plant P uptake, while $Ca(H_2PO_4)_2$ 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.

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766

767 **Table 1** Literature review of effects of phosphorus (P) addition on soil β-glucosidase
768 (BG), *N*-acetyl-glucosaminidase (NAG) and acid and alkaline phosphomonoesterases
769 (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	–	–	↓	–	Liebisch *et al*. 2014
Savannah, Tanzania	KH_2PO_4	↑	↑	↑	–	Mganga *et al*. 2015
Semi-natural grassland, Tanzania	KH_2PO_4	↑	↑	↑	–	
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	–	–	↓	↓	

770 Effects annotated as ↑, ↓, 0 or - indicate positive, negative, no significant change or lack of data,
771 respectively.

772 **Figure captions**

773 **Figure 1** Effect of addition of KH_2PO_4 (a) or $Ca(H_2PO_4)_2$ (b) with 0 and 10 g N m^{-2} y^{-1}
774 nitrogen (N) on concentration of microbial biomass phosphorus (MBP) (mean ±SE, *n*
775 = 5). Upper- and lowercase letters indicate differences among KH_2PO_4 and $Ca(H_2PO_4)_2$
776 treatments with and without added N, respectively.

777

778 **Figure 2** Boxplots of activity of BG (a, b), NAG (c, d), acid PME (e, f) and alkaline
779 PME (g, h) with addition of KH_2PO_4 (a, c, e, g) and $Ca(H_2PO_4)_2$ (b, d, f, h) with 0 (blue)
780 and 10 g N m^{-2} y^{-1} (red). Different letters indicate differences among KH_2PO_4 and
781 $Ca(H_2PO_4)_2$ treatments with or without added N, and asterisks indicate differences
782 between N treatments for the rates of KH_2PO_4 and $Ca(H_2PO_4)_2$. Error bars indicate the
783 10th and 90th percentiles; black lines within the boxes represent median activity and
784 the box limits indicate activity within the 25-75th percentile range.

785

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

788

789 **Figure 4** Mean litter biomass (±SE, *n* = 5) with addition of KH_2PO_4 (a) or $Ca(H_2PO_4)_2$
790 (b) under ambient and added nitrogen (N). Relationship between BG activity and litter
791 biomass with addition of KH_2PO_4 (c) or $Ca(H_2PO_4)_2$ across N treatments (d). Upper-
792 and lowercase letters indicate differences among KH_2PO_4 and $Ca(H_2PO_4)_2$ treatments
793 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_2PO_4 (a) or $Ca(H_2PO_4)_2$ (b) addition.

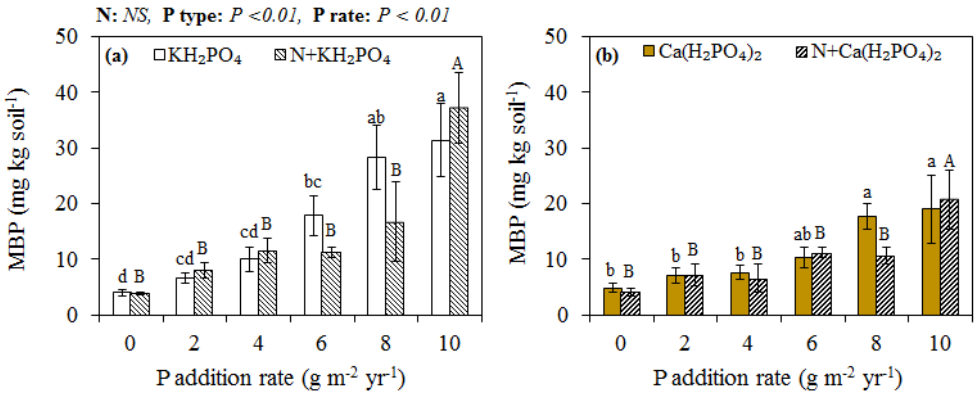

N: NS, P type: P <0.01, P rate: P < 0.01
(a) KH2PO4 N+KH2PO4
(b) Ca(H2PO4)2 N+Ca(H2PO4)2
MBP (mg kg soil-1)
P addition rate (g m-2 yr-1)
0
10
20
30
40
50
0
2
4
6
8
10
d B
cd B
cd B
bc B
ab B
a A
b B
b B
b B
ab B
a B
a A

Figure 1

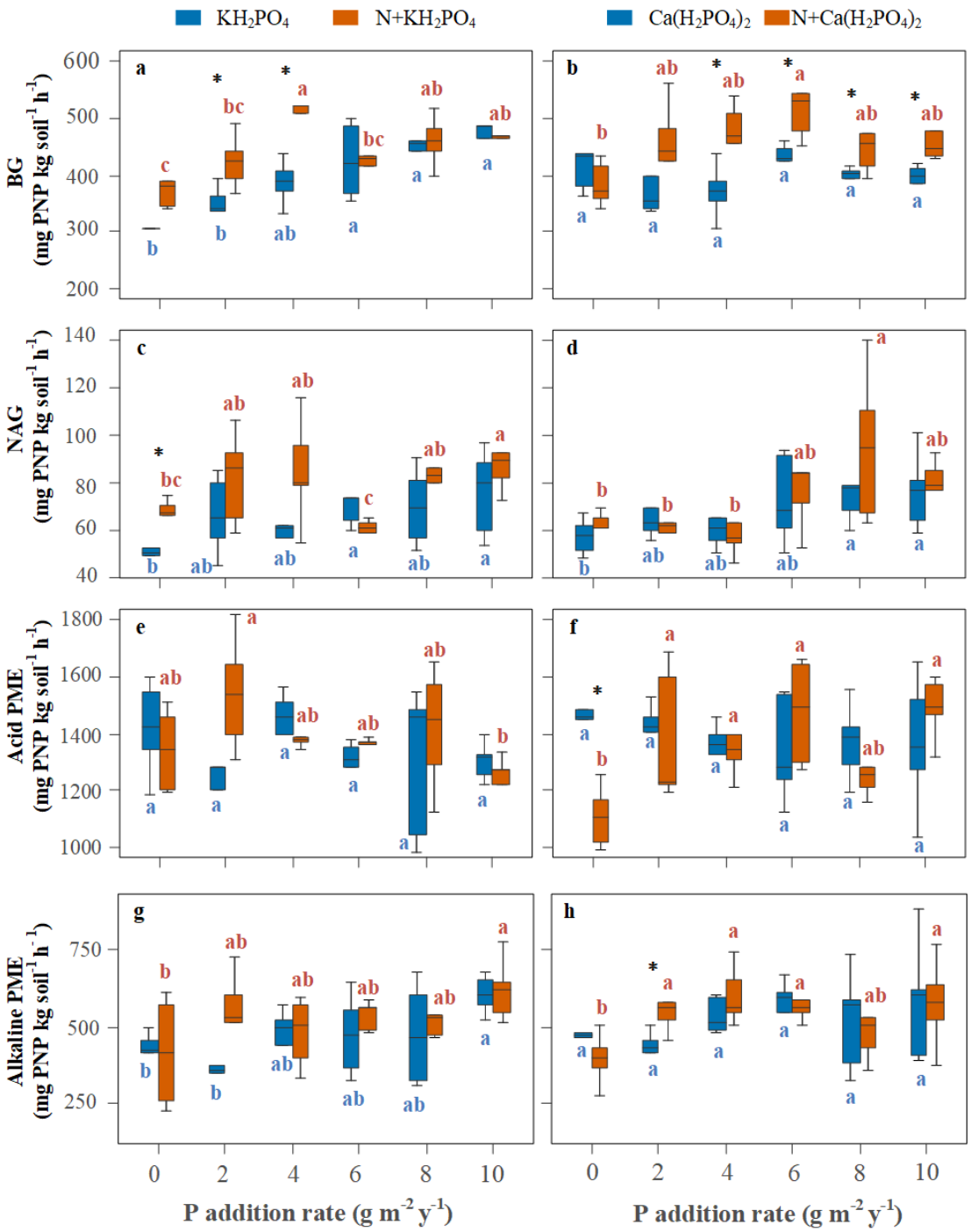

KH2PO4
N+KH2PO4
Ca(H2PO4)2
N+Ca(H2PO4)2
BG (mg PNP kg soil-1 h-1)
NAG (mg PNP kg soil-1 h-1)
Acid PME (mg PNP kg soil-1 h-1)
Alkaline PME (mg PNP kg soil-1 h-1)
P addition rate (g m-2 y-1)
P addition rate (g m-2 y-1)

Figure 2

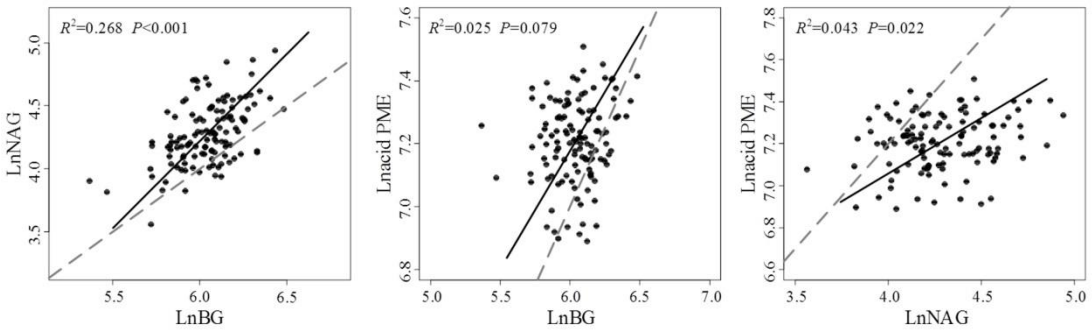
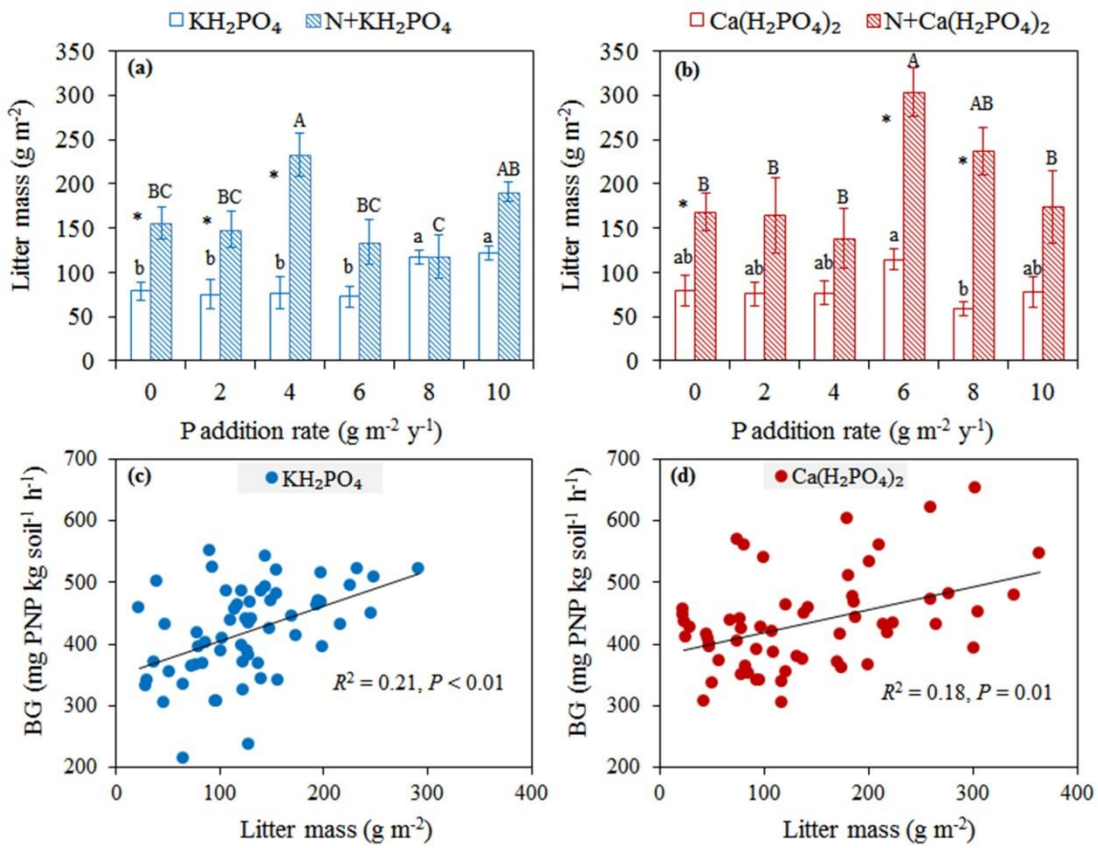

R^2=0.268 P<0.001
LnNAG
LnBG
R^2=0.025 P=0.079
Lnacid PME
LnBG
R^2=0.043 P=0.022
Lnacid PME
LnNAG

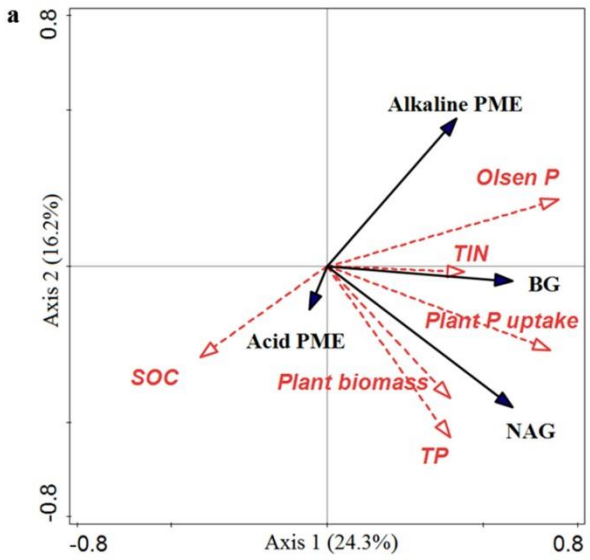
Figure 3



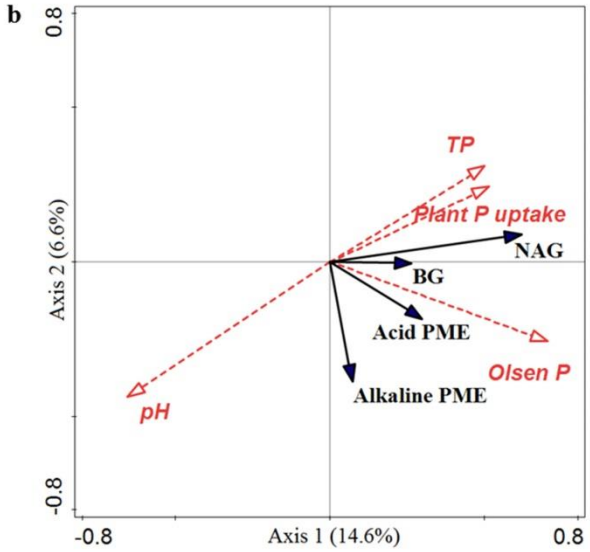
811

812 **Figure 4**

813



	Explanatory parameters		
	%	*F*	*P*
Olsen P	14.4	9.7	0.001
Plant P uptake	13.7	9.2	0.001
TP	8.9	5.7	0.002
Plant biomass	6.8	4.3	0.011
SOC	5.5	3.4	0.031
TIN	5.0	3.0	0.037



	Explanatory parameters		
	%	*F*	*P*
Olsen P	7.9	5.0	0.002
pH	7.6	4.8	0.002
Plant P uptake	5.4	3.3	0.02
TP	4.6	2.8	0.033

Figure 5