**The effects of a 9-year nitrogen and water addition on soil aggregate
phosphorus and sulfur availability in a semi-arid grassland**

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Abstract

Previous studies have demonstrated that higher nitrogen (N) and water availability affect both above- and below-ground communities, soil carbon and N pools, and microbial activity in semi-arid grasslands of Inner Mongolia. However, how soil phosphorus (P) and sulfur (S) pools, and related soil enzyme activities (as indicators of P and S cycles) respond to long-term N and water addition has still remained unclear. Since 2005, a field experiment with urea and water amendments has been conducted to examine their effects on total and available P and S concentrations and alkaline phosphomonoesterase (PME) and aryl-sulfatase (ArS) activities in three soil aggregate fractions: large macroaggregates (>2 mm), small macroaggregates (0.25-2 mm), and microaggregates (<0.25 mm) in an Inner Mongolia semi-arid grassland. Normalized to aggregate mass, microaggregates retained the highest total P and S concentrations. Both N and water additions increased the available P (by up to 84.5%) and the available S (by up to 150%) in the soil aggregate fractions. Soil acidification, as a result of the N addition, decreased both alkaline PME and ArS activities by up to 62.9% and 39.6%, respectively, while the water addition increased their activities. Our observations revealed that soil acidification (under the N addition) and elevated enzyme activity (under the water addition) played important roles in the levels of soil available P and S. The depression of P- and S-acquiring enzymes with soil acidification may decrease P and S availability, potentially impacting ecosystem processes and limiting the restoration of these grassland systems. The water addition was shown to be a more effective practice than the urea amendment for improving

36 soil structure, supplying available P and S, and maintaining the sustainability of this
37 semi-arid grassland.

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39 *Key words:* nitrogen deposition, precipitation increment, soil extracellular enzymes,
40 soil phosphorus and sulfur, global change

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Introduction

Increasing nitrogen (N) inputs and changing precipitation regimes are two important and interacting factors influencing terrestrial ecosystems under global change scenarios (Bai et al., 2010; Zhang et al., 2013), especially for semi-arid grasslands which are co-limited by N and water. In China, over 78% of grasslands are distributed in the northern temperate and semi-arid zones, like Inner Mongolia (Kang et al., 2007). Previous studies in these semi-arid grasslands have demonstrated that N and water addition can affect net primary production (NPP) (Bai et al., 2010), species turnover, plant community composition (Xu et al., 2012), and even soil organic carbon (C) content and microbial community composition (Zhang et al., 2013; Zhou et al., 2013a,b; Wang et al., 2014). Nitrogen fertilization can result in grassland acidification (Yang et al., 2012; Wang et al., 2014), changes in the microbial community composition (Wei et al., 2013), and reductions in microbial activity (Wang et al., 2014). In contrast, water addition can increase microbial activity or mediate suppressive effects of N addition, and can influence the abundance, richness, and composition of bacterial communities (Zhou et al., 2013a; Wang et al., 2014; Zhang et al., 2014). Though numerous studies have concentrated on responses of above- and below-ground communities to N and water addition in these areas, little is known about the effects of N and water amendment on soil phosphorus (P) and sulfur (S) dynamics.

The turnover of soil carbon and nutrients is influenced by P and S availability (Mooshammer et al., 2012; Manzoni et al., 2010; Parton et al., 1988). Nitrogen addition can reduce the sorption capacity of soil P ions (Stroia et al., 2011) which can potentially increase P availability, particularly in calcareous soils (Tunesi et al., 1999). Observations of the long-term effects of N fertilization on P availability have produced contrasting results, mainly because high N inputs may enhance P uptake (in the form of H_2PO_4^- and HPO_4^{2-}) by plants (Olander and Vitousek, 2000), but also reduce phosphatase activity due to soil acidification (Wang et al., 2014). In this case, the amount of phosphate ions in soil solution depends upon the balance between sorption capacity of PO_4^{3-} to soil minerals and phosphate ions absorbed by plants (Tunesi et al., 1999). Under N addition, mineralization of soil organic P might decrease due to phosphatase suppression (Olander and Vitousek, 2000). Under elevated water regimes, an increase in soil moisture enhances P availability for uptake by plants and microbes as the diffusivity of P increases (Lambers et al., 2006). Similarly, N fertilization can also intensify the mineralization of C-bonded S resulting in higher S availability (in the form of $\text{SO}_4\text{-S}$) (De Bona and Monteiro, 2010). How soil available S responds to water addition is still unclear and related studies are rarely seen.

As soil P and S cycling is largely associated with soil organic matter (SOM) dynamics, factors controlling SOM stabilization will impact soil P and S storage and influence biologically-associated P and S pools in ecosystems (Fonte et al., 2014; McGill and Cole, 1981). Soil aggregation, driven by binding agents such as SOM,

arbuscular mycorrhizal fungi, and roots, has been long considered as a key factor in providing physical protection for SOM (Jastrow et al., 1998). In particular, microaggregates (< 0.25 mm) are thought to play a dominant role in SOM storage (O'Brien and Jastrow, 2013). Macroaggregates (> 0.25 mm) are responsible for the early stages of SOM protection, which provide a preferential site for the formation and stabilization of C within microaggregates (Six et al., 2004). Though many studies have concentrated on soil C and N dynamics (Gunina and Kuzyakov, 2014; O'Brien and Jastrow, 2013; Wilson et al., 2009) or microbial community composition and activity (Dorodnikov et al., 2009) within soil aggregates, studies concerning aggregate-scale P and S storage and dynamics are still rarely seen, especially in these nutrient-poor grasslands under global change scenarios of enhanced N input and changing precipitation regimes.

Microbial extracellular enzymes play a controlling role over SOM decomposition (Henry et al., 2005) and are widely used to indicate responses of belowground processes to exogenous disturbances (Sinsabaugh et al., 2002). The activities of phosphatases are responsible for organic P hydrolysis and available P supply to plants (Wei et al., 2014). Aryl-sulfatase is secreted to release soil available S from organic S (mainly ester sulfates) for plant growth (Tabatabai, 1994). Previous studies have shown that N fertilization increases acid and alkaline phosphomonoesterases (PME) activities while soil P availability is negatively correlated with acid PME (Colvan et al., 2001). Also, aryl-sulfatase activity can decrease under conditions of soil

acidification (Brockett et al., 2012). However, studies of how these enzyme activities respond to N fertilization in presence of elevated water inputs are still rarely seen.

In previous works we showed that a prolonged N fertilization with urea and water amendment enhanced soil aggregate stability (Wang et al., 2015a,b). In this study we hypothesized that microaggregates would have higher total P and S contents when normalized to aggregate mass due to better SOM protection, and that both N and water additions would increase the availability of P and S in the soil. We also expected that soil aggregate P- and S-cycling enzyme activities would decrease under elevated N inputs due to a suppressive effect of lower soil pH, while enzyme activities would increase under higher water availability. We tested these hypotheses by analyzing soils from a nine-year long field trial which has important implications for the sustainability of these vulnerable and essential ecosystems.

Materials and methods

Study sites and experimental design

In April 2005, a field trial was established at the south of Duolun County of Inner Mongolia, China (42°02'27"N, 116°17'59"E, elevation 1,324 m a.s.l.) on a sandy loam soil (Calcisorthic Aridisol, in the U.S. Soil Taxonomy classification). The trial area has undergone severe degradation due to overgrazing during the last 50 years, but the experimental plots of the present study were fenced in 2000 to investigate the impacts of grassland management practices (N fertilization and irrigation) on plant diversity

and soil properties (Wang et al., 2014). The site has a semi-arid climate with mean annual precipitation of 379 mm and mean annual temperature of 2.1 °C. The steppe is dominated by *Stipa krylovii* (C₃ grass), *Agropyron cristatum* (C₃ grass), and *Artemisia frigida* (C₃ forb).

Treatments were established on 8 × 8 m plots arranged in a split-plot design (water and N addition) with seven replicates per treatment and 1 m buffer zone between any two adjacent plots. Each treatment block was divided into two main plots where water treatments (ambient precipitation and ambient plus water addition) were assigned. As a weekly 15-mm event, a total of 180 mm of additional water (+ 50 % of mean annual precipitation) was sprinkled during growing season from June to August. Each main plot was divided into six subplots where four were randomly treated with one of four N addition levels: 0 (CK), 5 g N m⁻² yr⁻¹ (N₅), 10 g N m⁻² yr⁻¹ (N₁₀), and 15 g N m⁻² yr⁻¹ (N₁₅). Nitrogen was added as urea pellets with half applied in early May and the other half in late June. This study is part of an on-going project designed to investigate the effect of increased N and water on ecosystem responses in the Inner Mongolia grassland (Xu et al., 2012).

Soil sampling and aggregate isolation

In September 2013 (i.e. after 9 years of treatments), five randomly placed soil cores (0-10 cm) were taken from four replicate plots of the N and water treatments, using a 10-cm-deep corer (5 cm in diameter). Fresh soil samples were stored at 4 °C in hard

plastic containers (to maintain their primary structures) during transportation to the laboratory. To minimize the impact on microbial activity and nutrient availability, aggregates were isolated by a dry-sieving method as described by Wang et al. (2015a). Soil samples were isolated into three aggregate size classes: >2 mm (large macroaggregates class), 0.25-2 mm (small macroaggregates class), and <0.25 mm (microaggregates class). No correction was made for sand (e.g. Elliott et al., 1991) as sand grains can be embedded in soil aggregates. Although this prevents direct comparisons of mineral-associated OM between aggregates, it allows for the potential that OM coatings on sand grains may be important for SOM turnover, and that sand may be an important part of the aggregate structure. This was preferred to examine whole aggregate (including sand) P and S dynamics. However, as a result differences in aggregate sizes cannot be attributed solely to mineral associations. The chemical characteristics of the soil aggregates (0-10 cm) are given in Table 1 (Wang et al., 2015a,b).

Analyses of soil chemical and biological parameters

Soil pH was determined in a 1:5 (w/v) soil-water extract of soil aggregate samples by a PHS-3G digital pH meter (Precision and Scientific Crop., Shanghai, China).

Total P in the aggregate fractions was determined by perchloric acid (HClO₄) digestion (Sommers and Nelson, 1972) and analyzed by the molybdenum blue colorimetric method (Murphy and Riley, 1962). Briefly, 0.5 g of the air-dried soil (<

100-mesh) was weighed into separate digestion tubes and mixed with 7.5 ml of 70% HClO₄. The tubes containing reaction solutions were placed in digestion blocks at 203 °C for 75 min with pyrex funnels on the top. After digestion, the digests were diluted with distilled water to 50 ml into volumetric flasks and then filtered. The amount of orthophosphate was determined by the molybdenum blue colorimetric method at a wavelength of 700 nm with an UV-VIS spectrophotometer (UV-1700, Shimadzu) (Murphy and Riley, 1962). The Olsen P of soil aggregates was quantified by colorimetric analysis following extraction of soil with 0.5 M NaHCO₃ (pH 8.5) (Olsen et al., 1954). Briefly, 2.5 g of the air-dried soil were mixed with 50 ml 0.5 M NaHCO₃ into a 150 ml Erlenmeyer flask. Approximately 5 g of phosphorus-free charcoal was added into the mixture and the flasks were shaken at 150 rpm for 30 min. After filtration, the extracts were analyzed by the molybdenum blue colorimetric method (Murphy and Riley, 1962). Total S concentrations in soil fractions were measured using the acid digestion method developed by Tabatabai (1982). Briefly, 2.0-g of finely ground soil were placed in a digestion tube and digested with 3 ml of 69% HNO₃ for 1h. Then 3 ml of 60% HClO₄ and 7 ml of H₃PO₄ were added to the mixture and digested around 200 °C until white fumes of HClO₄ were visible. After the mixture cooled, 2 ml of 37% HCl were added to the mixture and heated again until fumes of HClO₄ visible again. The digests were transferred to volumetric flasks and the volume was adjusted with 1 M HCl. The concentration of total S in the digest was determined by turbidimetry with 0.5 g BaCl₂ crystals. Available S within soil aggregates was determined by turbidimetry after extraction with ammonium acetate

(Bardsley and Lancaster, 1960). Briefly, 10.0 g of the air-dried soil was extracted with 0.25 M ammonium acetate and available S concentration was measured by a turbidimetry method using 0.5 g of BaCl₂ crystals.

Enzyme assays for alkaline phosphomonoesterase (PME) and aryl-sulfatase (ArS) were performed on frozen, fresh sieved soil aggregate samples. Enzyme substrates were p-nitrophenyl phosphate for alkaline PME and p-nitrophenyl sulfate for ArS. For alkaline PME activity, 1.0 g of soil aggregates were weighted into Erlenmeyer flask and mixed with a modified universal buffer (0.1 M trihydroxymethyl aminomethane + 0.067 M citric acid monohydrate compound + 0.1 M boric acid at pH 11.0). The substrate, 0.05 M p-nitrophenyl phosphate, was added to the reaction system, gently shaken, and incubated at 37 °C for 1h. Reactions were terminated by adding 0.5 M CaCl₂ and 0.5 M NaOH. The procedure for the assay of ArS activity was the same as for alkaline PME activity, except p-nitrophenyl sulfate was used as the substrate and the pH of the reaction system was adjusted to 5.8 by using an acetate buffer (Tabatabai, 1994). The activities of alkaline PME and ArS were expressed as mg p-nitrophenyl released per kg of soil fraction per hour after colorimetry at 410 nm with a UV-VIS spectrophotometer (UV-1700, Shimazu) (Tabatabai, 1994). As the activity of acid PME was reported in the study of Wang et al. (2015a), we compared these two enzyme activities by presenting the ratio of alkaline to acid PME.

Statistical analyses

The normality of data was tested using the Kolmogorov-Smirnov test, and homogeneity of variances using Levene's test. ANOVAs with a split-plot design were executed to determine the effect of water (between-subject), N addition and soil aggregate sizes (within-subject), and their interactions on the soil total P and S, available P and S, and the activities of alkaline PME and ArS. The effects of N addition rates on concentrations of total P and S, available P (Olsen-P) and S, and the activities of PME and ArS were determined by one-way ANOVA run separately for the ambient precipitation and the water addition treatments. Within each N addition rate, student's t-tests were conducted to determine differences in the concentrations of total P and S, available P and S, and the activities of alkaline PME and ArS between N amendments alone and N in combination with water addition. Pearson correlation analysis was used to determine the relationship among soil parameters. All statistical analyses were performed in SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and statistical significance was accepted at $P < 0.05$.

Results

Effect of N and water addition on soil aggregate P and S concentrations

Normalized to aggregate mass, total P concentrations differed among soil aggregate fractions, with the highest concentration in microaggregates for the CK treatment (378.5 mg kg aggregate⁻¹) and the lowest in small macroaggregates for the N₁₅

treatment ($189.2 \text{ mg kg aggregate}^{-1}$) (Fig. 1a). Nitrogen addition significantly decreased total soil P by 25.5% in the N_{15} treatment of small macroaggregates under ambient precipitation, by 22.9% in N_{10} of small macroaggregates under water addition, and by 21.8% in N_5 of microaggregates under water addition (Fig. 1a). No water effect on soil total P was detected (Table 2). Soil Olsen-P concentrations ranged from 3.7 to $10.0 \text{ mg kg aggregate}^{-1}$, and increased by up to 84.5% with N addition in large macro- and microaggregates under both ambient and elevated water levels (Fig. 1b). However, soil Olsen-P decreased with N addition in small macroaggregates under ambient precipitation (Fig. 1b).

When normalizing the data to whole soil mass, the lowest soil total P concentration was found in the N_5 treatment of microaggregates ($41.0 \text{ mg kg soil}^{-1}$) (Fig. S1a). N addition decreased soil total P in the N_{15} treatment of small macroaggregates under ambient precipitation, in N_{10} of small macroaggregates under water addition, and in N_5 of microaggregates under water addition (Fig. S1a). Water addition showed no impact on soil total P concentration when normalizing the data to whole soil basis (Fig. S1a). Ranging from 1.1 to $5.4 \text{ mg kg soil}^{-1}$, soil Olsen-P significantly increased with N addition in large macroaggregates under water addition, and in microaggregates under ambient precipitation (Fig. S1b). Nitrogen addition significantly decreased soil Olsen-P concentration (from 2.5 to $1.5 \text{ mg kg soil}^{-1}$) in small macroaggregates under ambient precipitation (Fig. S1b).

The soil aggregate total S was the highest in microaggregates (varying from 696.4 to $1336.9 \text{ mg kg aggregate}^{-1}$), while no difference was detected between large and

small macroaggregates when averaging all the N and water treatments (Fig. 2a). Nitrogen addition alone or in combination with water, had no effect on the soil total S concentration (Fig. 2a, Table 2). On average, available S was significantly lower in small macroaggregates relative to large macroaggregates and microaggregates (Fig. 2b). The soil available S, ranging from 7.8 to 28.5 mg kg aggregate⁻¹ significantly increased with both N and water amendments within all three aggregates by up to 150% (Fig. 2b).

When the data was normalized to whole soil basis, there was no difference in soil aggregate total S among three soil fractions (Fig. S2a). Both N and water had no effect on soil total S concentration (Fig. S2a). However, soil aggregate available S was the lowest in microaggregates (varying from 3.0 to 6.1 mg kg soil⁻¹) when normalized the data to whole soil basis (Fig. S2b). Both N and water addition significantly increased soil available S (on a whole soil basis) within all soil aggregates (Fig. S2b).

Response of enzyme activities to N and water addition within soil aggregates

The activity of alkaline PME varied from 259.6 to 736.7 mg PNP kg aggregate⁻¹ h⁻¹ across three soil aggregate fractions (Fig. 3a) and the activity was the highest in microaggregates of the CK treatment (Fig. 3a). The alkaline PME activity decreased with N by up to 62.9%, while it increased with water addition by up to 30.9%, except for small macroaggregates under N₁₀ treatment (Fig. 3a, Table 2). The effects of N and

water addition on acid PME activity were described previously in Wang et al. (2015a). Under ambient precipitation, alkaline PME activity was higher than acid PME (the ratio higher than 1) in CK, N₅, and N₁₀ treatments across three soil fractions, except N₁₀ of large macroaggregates (Fig. S3). Under water addition treatments, acid PME was significantly higher than alkaline PME in N₁₀ of large macroaggregates, while lower in N₅ of microaggregates (Fig. S3). Averaging all the N and water treatments together, the highest aryl-sulfatase activity was observed in microaggregates (6.8-28.4 mg PNP kg aggregate⁻¹ h⁻¹), followed by large macroaggregates (6.3-17.4 mg PNP kg aggregate⁻¹ h⁻¹) (Fig. 3b). The N addition significantly decreased the ArS activity under ambient precipitation by up to 39.6% across three soil aggregates (Fig. 3b). Under elevated water conditions, lower N addition rates (5 g N m⁻² yr⁻¹) significantly increased ArS activity within all three aggregates (Fig. 3b). A significant positive effect of water was seen on ArS activity (Fig. 3b, Table 2).

On a whole soil basis, alkaline PME activity was the lowest in microaggregates (ranging from 64.4 to 198.7 mg PNP kg soil⁻¹ h⁻¹) (Fig. S4a). Nitrogen addition significantly decreased while water addition increased alkaline PME activity within all soil fractions (Fig. S4a). Ranging from 1.3 to 7.8 mg PNP kg soil⁻¹ h⁻¹, soil ArS activity was the highest in large macroaggregates, while no difference was detected in ArS activity between small macroaggregates and microaggregates when averaging all N and water treatments (Fig. S4b). Soil ArS activity was significantly decreased by N addition under ambient precipitation across three soil aggregates (Fig. S4b). Under water addition, N₅ treatment significantly increased soil ArS activity within three soil

fractions (Fig. S4b). Significant and positive water effect was found on soil ArS activity (Fig. S4b).

Correlation between soil properties

Soil pH was negatively correlated with available P under both ambient precipitation and water addition treatments in large macroaggregates, while in microaggregates a negative correlation was only seen under ambient precipitation (Table 3). Soil pH was positively correlated with available P in small microaggregates (Table 3). Soil pH and available S were negatively correlated across all aggregate classes and water treatments (Table 3).

Soil Olsen-P was negatively correlated with alkaline PME activity under ambient precipitation in both large macroaggregates and microaggregates (Table 3). Within all soil aggregates, the soil total P concentration was significantly and positively correlated with both SOC and soil total N (TN) ($P < 0.01$), while the soil total S was not (Table 4).

Discussion

Nutrient distribution differs among soil aggregate fractions

Compared to mineral surfaces, the sand matrix plays a minimal role in soil C stabilization and nutrient turnover within aggregates, as sand does not adsorb organic

C like mineral surfaces (Elliott et al., 1991). In this manuscript, we compared nutrient concentrations among soil aggregates (including the sand) instead of making comparisons of mineral-associated OM. Recent work has emphasized the importance of microbial accessibility to OM turnover (Dungait et al., 2012), and removing sand from aggregate nutrient dynamics ignores its potential role in microbial spatial isolation from mineral surfaces, as well as the contributions of organic coatings to OM dynamics. As a result, the data were normalized to aggregate mass, and not corrected for sand. Therefore, results between aggregate size classes should be attributed to the influences of both the sand and mineral matrix. As reported by our previous study, the proportions of large macro-, small macro-, and microaggregates were 30.3%-44.8, 38.3%-42.8%, and 15.3%-31.1%, respectively, across all N and water treatments (Wang et al., 2015b). When normalizing the data to a whole soil basis, soil macroaggregates tended to have the highest nutrient concentrations and enzyme activities, which was mainly due to the higher proportions of both large and small macroaggregates relative to microaggregates (Wang et al. 2015b). As a result, normalization to the whole soil would primarily reflect changes in aggregate proportional distributions and examine whole soil P and S storage, instead of reflecting stabilization across soil aggregates, which was the focus of this manuscript.

In line with our initial hypothesis, we observed the highest total P and S in the smallest aggregate-size fraction on the basis of soil aggregate mass (Fig. 1a, Fig. 2a). The mechanism of retaining higher total P and S levels in microaggregates was likely through reactions with minerals (Wright, 2009), better protection of SOM due to

limited water and oxygen diffusion (Fonte et al., 2014), or higher SOM stability (Angers et al., 1997). There is also the potential that if the soils had greater coarse sand (> 0.25 mm) relative to fine sand (< 0.25 mm) external to but isolated with the aggregate fractions, that the higher P and S levels in microaggregates could be due to differences in sand. However, the higher P and S levels in microaggregates were confirmed by a similar distribution pattern of total P, SOC, and TN among soil aggregates (see Wang et al., 2015b), as well as significant correlations between soil total P and SOC and TN (Table 4). In line with our findings, previous studies have reported the coupling effects of oxygen and soil water dynamics on soil biochemical reactions and substrate utilization (Kremen et al., 2005; Rubol et al., 2013; Freixa et al., 2015). For example, Freixa et al. (2015) showed that anoxic condition inhibited microbial utilization of SOM which might be the reason of higher total P and S concentrations in soil microaggregates. If water was supplied by water-table or deep irrigation systems, soil organic P and S mineralization might be slowed down resulting in lower soil P and S availabilities and higher soil total P and S accumulation, as SOM destruction would be slower than its accumulation in submerged soil (Freixa et al., 2015). However, our findings contradicted Fonte et al. (2014) who suggested that macroaggregates (> 0.25 mm) were the dominant site for both soil C and P accumulation in pasture areas of Colombia. The discrepancy could mainly result from the different distribution of SOM among soil aggregates in different ecosystems. Higher soil total S concentration within microaggregates has been attributed to the stabilization of S by clay (Eriksen et al., 1995). We also detected higher total P

concentrations in large macroaggregates than in small macroaggregates (Fig. 1a) which could result from higher organic residue incorporation (Tisdall and Oades, 1982). The above observations suggest that microaggregates play an important role in long-term retention of P and S.

Enzymes involved in soil P and S cycling were still higher in microaggregates than in macroaggregates (Fig. 3), although water and oxygen diffusion rates were shown to be limited within microaggregates (Six et al., 2004). Our results contradicted Freixa et al. (2015) who found negative effect of anoxic conditions on phosphatase activity in a sediment study. The discrepancy might be explained by higher mineral content of soil microaggregates which could stabilize soil extracellular enzymes (Wang et al., 2015a). The ratio of alkaline to acid PME suggested alkaline PME activity played a more dominant role than acid PME in soil P cycling under low N additions (Fig. S1). Thus, microbial decomposition was one of the main reasons for higher P and S availability in microaggregates as well as in large macroaggregates (as indicated by the activities of alkaline PME and ArS). Additionally, higher P availability in microaggregates could result from soil acidification promoting the dissolution of mineral-bound phosphates (Stroia et al., 2011). Similarly, this could also be the reason for higher available S levels in microaggregates (Eriksen et al., 1995). Our results contradicted the findings of Zhang et al. (2013) who found a reduction in soil total and available P under N addition in meadow steppe in northern China. This could be due to higher plant P demand in meadow steppe where productivity was less restrained by water as compared to the semi-arid grassland.

Higher soil P and S availabilities resulted from abiotic dissolution under the N addition and decomposition under the water addition

According to Fonte et al. (2014), aggregate-associated SOM is important for maintaining organic P in degraded grasslands. Thus, we expected to detect unchanged soil total P concentrations in response to N and water treatments as our previous research indicated no significant N or water effects on aggregate SOC concentrations (Wang et al., 2015b). However, we found that soil total P decreased under the N addition (Fig. 1a), which might result from an enhanced plant P uptake as indicated by increased foliar P (unpublished data) and the slower decomposition of the plant tissue P, where P existed as phospholipid and phytates (Zhang et al., 2013). Indeed, decreased P-cycling enzyme activity under the N amendment (Fig. 3a) might reduce the release of P from the decomposition of plant residues. Under soil acidification, a continuous dissolution of phosphate from inorganic P pools (such as mineral-bound P) as well as an enhanced P uptake and retention by plants could decrease total P pools. Unfortunately, our current study cannot distinguish the relative contributions of organic vs. inorganic P pools to the observed decrease in the total P concentration.

Higher availability of soil S under N addition might be a consequence of SO_4^{2-} exchanged by excessive PO_4^{3-} , as mono-calcium phosphate solution could be used as a reagent to extract soil inorganic sulfate (Li et al., 2001).

The suppression of both acid PME (Wang et al., 2015a) and alkaline PME (Fig. 3a) should decrease soil organic P decomposition and consequentially decrease soil P availability. However, we observed an increase in available P under the N amendment, indicating the abiotic release of PO_4^{3-} ions played a more essential role in the available P supply than biotic mineralization. Abiotic release processes could result from the dissolution of mineral-bound (for instance, crystalline Ca-bound) phosphates under soil acidification (Faurie and Fardeau, 1990; Wright, 2009) or a reduction in soil P sorption capacity resulting in a higher concentration of diffusive PO_4^{3-} ions (Stroia et al., 2011).

An increase in soil aggregate stability will help to retain soil nutrients and sustain primary production (Six et al., 2004). The higher availability of the soil P (Fig. 1b) and S (Fig. 2b), as well as improved aggregate stability or higher macroaggregate proportions (Wang et al., 2015b) indicates that the water amendment is an efficient way to improve soil structure and increase the available P and S supply. Water addition increased both available P and S concentrations (Fig. 1b and 2b) as well as soil P- and S-acquiring enzyme activities (Fig. 3) suggesting that increased microbial decomposition is the major factor for higher available nutrients. Increased water levels can enhance substrate diffusion (Allison, 2005; Sinsabaugh et al., 2008) and the motility of microorganisms or extracellular enzymes (Kemmitt et al. 2008; Dungait et al., 2012). In terms of SOM turnover, accessibility of the SOM to decomposer organisms or catalytic enzymes is even more important than the chemical composition of SOM pools, especially in this semi-arid ecosystem (Dungait et al., 2012). As a

result, water addition can alleviate P- and S-limitations in this nutrient-poor and water-limited grassland.

The combination of N and water addition accelerated available P release in the soil, revealing that the interactive effects on available P release were stronger than each separately (see also Fig. 1b). A higher moisture input might promote the transformation of H_2PO_4^- and HPO_4^{2-} to PO_4^{3-} (Stroia et al., 2011). This process together with the decrease of soil aggregate total P pool (Fig. 1a) would have far-reaching consequences on long-term P supply in this nutrient-poor ecosystem.

The biological release of available P and S as affected by enzymatic suppression

The decrease of alkaline PME across three soil aggregates might be due to a decreased soil pH as suggested by positive correlations between these two parameters within all soil fractions (Table 3). Actually, the optimal soil pH for alkaline PME is around 11 (Tabatabai, 1994), while the soil pH of our study site is 6.1-7.2 (Wang et al., 2014). Under ambient precipitation, the soil pH dropped from 7.10 to 5.58 for large macroaggregates, from 7.08 to 5.84 for small macroaggregates, and from 7.06 to 5.90 for microaggregates as N addition increased (see Wang et al., 2015a). Under the water addition, soil pH values ranged from 7.27 in CK to 6.11 in N_{15} plot for large macroaggregates, from 7.47 (CK) to 6.20 (N_{15}) for small macroaggregates, and from 7.40 (CK) to 6.23 (N_{15}) for microaggregates (Wang et al., 2015a). Additionally, microbial activity could be suppressed owing to increases in the osmotic potential as a

result of exogenous ions from the fertilizer application (Treseder, 2008) and /or the leaching of nutrients of magnesium and calcium under a lower soil pH (Vitousek et al., 1997). Indeed, we found decreases of both DTPA- extractable magnesium and calcium within soil aggregates under the N addition and significant positive correlations of enzymes with DTPA-extractable magnesium as well as with extractable calcium (Table S1). Additionally, the increase in available nutrients also caused a reduction in alkaline PME production, as interpreted from the negative relationship between the available P and PME activity, although this relationship depended on the soil aggregate size and water treatments (Table 3).

The significant correlation between ArS activity and soil pH was only seen under elevated water levels in large macroaggregates and under ambient precipitation in microaggregates (Table 3). This suggests that the ArS activity was less affected by soil acidification than the alkaline PME activity, despite the significant correlation between ArS and alkaline PME activity (Table 3). Although it is difficult to interpret the reason for the decrease in ArS activity under ambient precipitation across three soil aggregates and the increase in ArS activity under low N-addition rate ($5 \text{ g m}^{-2} \text{ yr}^{-1}$) in small macro- and microaggregates under elevated water inputs (Fig. 3b), it has a potential impact on the S availability in these systems and therefore should be investigated further.

This study highlights the weakening role of the biological release of available P and S under soil acidification. Even with increased NPP under both N and water additions in the study site, our findings suggested that the water addition was a better

restoration procedure for this semi-arid grassland, particularly when considering belowground processes (microbial activities and nutrient availability), while N addition would cause soil acidification and the suppression of microbial activities. Our findings suggested that the alkaline PME could serve as a sensitive indicator of the soil P cycle responding to environmental changes which decreased with N additions but increased with precipitation in this temperate steppe.

Conclusion

Our results showed that water addition is an effective practice in sustaining grassland productivity because of a higher available P and S supply. Relative to aggregate mass, microaggregates had higher P- and S-acquisition enzyme activities, resulting in relatively higher available P and S levels, and were essential for long-term P and S supply. Increased P and S availability was mainly due to the greater dissolution of phosphate from mineral surfaces under the N addition, and a combination of greater dissolution and enzyme activity under the combined N and water addition. Even in soils with a pH value lower than 7, the alkaline PME was important for organic P mineralization, and could serve as an indicator of the response of soil P cycle to external disturbances. The repressive effect of soil acidification on enzyme activities could cause a decrease in the biological release of the available P and S supply. These findings indicated increased soil moisture, but not increased N, will potentially ameliorate both soil structure and soil fertility issues in this region.

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Tables

Table 1 The chemical characteristics of soil aggregates without field-manipulated treatments. SOC, TN, and C/N represent soil organic C, total N, and the ratio of SOC to TN, respectively (data from Wang et al., 2015a,b)

	Soil aggregates (mm)		
	> 2	0.25-2	< 0.25
SOC (g kg soil ⁻¹)	18.2 ± 1.0	15.1 ± 2.0	21.6 ± 1.4
TN (g kg soil ⁻¹)	1.9 ± 0.1	1.5 ± 0.2	2.2 ± 0.1
C/N	9.5 ± 0.5	10.4 ± 0.4	10.0 ± 0.2
pH	7.1 ± 0.2	7.1 ± 0.2	7.1 ± 0.3
NO ₃ ⁻ -N (mg kg soil ⁻¹)	4.2 ± 0.1	31.6 ± 2.2	34.4 ± 3.8
NH ₄ ⁺ -N (g kg soil ⁻¹)	17.0 ± 1.3	17.9 ± 2.5	22.9 ± 1.0

Table 2 Results (*F* values) of three-way ANOVAs with a split-plot design on the effect of water addition (W), N addition (N), aggregate size (S) and their interactions on soil total phosphorus (P), Olsen-P, available sulfur (AS), alkaline phosphomonoesterase (PME) and aryl-sulfatase (ArS)

	Total P	Total S	Olsen-P	AS	Alkaline PME	ArS
W	1.36	0.11	75.03**	195.70**	16.18**	166.00**
N	7.79**	0.32	11.01**	47.52**	103.56**	24.97**
S	36.77**	20.51**	8.69**	5.09**	10.60**	43.37**
W×N	2.7	1.02	5.70**	2.71	5.44**	26.96**
W×S	3.468	0.05	6.71**	1.89	0.21	6.50**
N×S	0.36	1.06	3.57**	1.88	0.80	2.86*
W×N×S	0.37	1.21	0.96	1.35	0.27	1.49

* and ** indicate the significance level at $P < 0.05$ and 0.01 , respectively

Table 3 Correlation analyses (*R* value) between soil physicochemical and biological characteristics within three soil aggregates under ambient and elevated water levels. P, S, AS, PME, and ArS represent phosphorus, sulfur, available sulfur, phosphomonoesterase, and aryl-sulfatase, respectively

			Total P	Olsen P	Total S	AS	Alkaline PME	ArS
>2 mm	Ambient precipitation	pH	0.42	-0.62*	-0.31	-0.84**	0.93**	-0.47
		Total P		-0.69**	-0.17	-0.24	0.43	0.08
		Olsen-P			0.16	0.56*	-0.60*	-0.35
		Total S				0.39	-0.54*	-0.29
		AS					-0.83**	-0.15
	Water addition	pH	0.44	-0.63**	0.19	-0.66**	0.94**	0.53*
		Total P		-0.34	0.15	-0.29	0.40	0.29
		Olsen P			-0.53*	0.72**	-0.48	-0.36
		Total S				-0.21	0.04	0.08
		AS					-0.60*	-0.31
0.25-2 mm	Ambient precipitation	pH	0.47	0.53*	-0.42	-0.80**	0.87**	0.49
		Total P		0.21	-0.20	-0.58*	0.56*	0.39
		Olsen-P			-0.05	-0.20	0.40	0.36
		Total S				0.53*	-0.48	0.12
		AS					-0.82**	-0.36
	Water addition	pH	0.20	-0.14	-0.29	-0.59*	0.68**	0.34
		Total P		0.27	-0.16	-0.04	0.47	0.18
		Olsen P			0.15	0.19	0.09	0.21
		Total S				0.02	-0.08	-0.17
		AS					-0.47	0.09
<0.25 mm	Ambient precipitation	pH	0.48	-0.60*	0.001	-0.67**	0.87**	0.63**
		Total P		-0.16	0.01	-0.20	0.41	0.36
		Olsen P			0.006	0.66**	-0.72**	-0.42
		Total S				-0.039	0.08	0.40
		AS					-0.77**	-0.32
	Water addition	pH	0.21	-0.42	0.20	-0.76**	0.73**	0.12
		Total P		-0.42	0.14	-0.16	0.04	-0.28
		Olsen P			-0.39	0.41	-0.18	0.18
		Total S				-0.21	0.21	-0.02
		AS					-0.70*	-0.37

* and ** indicate the significance level at $P < 0.05$ and 0.01 , respectively

Table 4 Correlation analyses between SOC and TN and total P and total S within

three soil aggregates

		Total P	Total S
>2 mm	SOC	0.61**	0.08
	TN	0.80**	-0.04
0.25-2 mm	SOC	0.78**	0.05
	TN	0.82**	0.01
<0.25 mm	SOC	0.66**	0.05
	TN	0.72**	0.03

* and ** indicate significant level at $P < 0.05$ and 0.01 , respectively

Figure Legends

Fig. 1 Concentrations of soil total phosphorus (P) (a) and available P (b) in three soil aggregate-size classes of the control (CK) and three N addition treatments ($\text{g m}^{-2} \text{yr}^{-1}$) under ambient (unshaded bars) and elevated (+50%, shaded bars) water levels. Values are averages of four replicates ($\pm \text{SE}$). Letters indicate a significant difference among means for the N addition under ambient precipitation or the water addition treatments separately (lowercase letters) and differences among soil aggregate-size classes (capital letters). Asterisks indicate a significant difference between water treatments for each N and CK treatment ($P < 0.05$)

Fig. 2 Concentrations of soil total sulfur (S) (a) and available S (b) in three soil aggregate-size classes of the control (CK) and three N addition treatments ($\text{g m}^{-2} \text{yr}^{-1}$) under ambient (unshaded bars) and elevated (+50%, shaded bars) water levels. Values are averages of four replicates ($\pm \text{SE}$). Letters indicate a significant difference among means for the N addition under ambient precipitation or the water addition treatments separately (lowercase letters) and differences among soil aggregate-size classes (capital letters). Asterisks indicate a significant difference between water treatments for each N and CK treatment ($P < 0.05$)

Fig. 3 Activities of soil alkaline phosphomonoesterase (alkaline PME, mg PNP kg aggregate⁻¹ h⁻¹) (a) and aryl-sulfatase (ArS, mg PNP kg aggregate⁻¹ h⁻¹) (b) in three soil aggregate-size classes of the control (CK) and three N addition treatments (g m⁻² yr⁻¹) under ambient (unshaded bars) and elevated (+50%, shaded bars) water levels. Values are averages of four replicates ($\pm$ SE). Letters indicate a significant difference among means for the N addition under ambient precipitation or the water addition treatments separately (lowercase letters) and differences among soil aggregate-size classes (capital letters). Asterisks indicate a significant difference between water treatments for each N and CK treatment ($P < 0.05$).

Fig. 1

Fig. 2

Fig. 3