Responses of enzymatic activities within soil aggregates to 9-year nitrogen and water addition in a semi-arid grassland

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\textbf{A B S T R A C T}

Soil microorganisms secrete enzymes used to metabolize carbon (C), nitrogen (N), and phosphorus (P) from the organic materials typically found in soil. Because of the connection with the active microbial biomass, soil enzyme activities can be used to investigate microbial nutrient cycling including the microbial response to environmental changes, transformation rates and to address the location of the most active biomass. In a 9-year field study on global change scenarios related to increasing N inputs (ambient to 15 g N m\textsuperscript{-2} yr\textsuperscript{-1}) and precipitation (ambient to 180 mm yr\textsuperscript{-1}), we tested the activities of soil β-glucosidase (BG), N-acetyl-glucosaminidase (NAG) and acid phosphomonoesterase (PME) for three soil aggregate classes: large macroaggregates (>2000 μm), small macroaggregates (250–2000 μm) and microaggregates (<250 μm). Results showed higher BG and PME activities in micro-vs. small macroaggregates whereas the highest NAG activity was found in the large macroaggregates. This distribution of enzyme activity suggests a higher contribution of fast-growing microorganisms in the micro-compared with the macroaggregates size fractions. The responses of BG and PME were different from NAG activity under N addition, as BG and PME decreased as much as 47.1% and 36.3%, respectively, while the NAG increased by as much as 80.8%, which could imply better adaption of fungi than bacteria to lower soil pH conditions developed under increased N. Significant increases in BG and PME activities by as much as 103.4 and 75.4%, respectively, were found under water addition. Lower ratio of BC:NAG and higher NAG:PME underlined enhanced microbial N limitation relative to both C and P, suggesting the repression of microbial activity and the accompanied decline in their ability to compete for N with plants and/or the accelerated proliferation of soil fungi under elevated N inputs. We conclude that changes in microbial activities under increased N input and greater water availability in arid- and semi-arid grassland ecosystems where NPP is co-limited by N and water may result in substantial redistribution of microbial activity in different-sized soil particles. This shift will influence the stability of SOM in the soil aggregates and the nutrient limitation of soil biota.

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\textbf{1. Introduction}

Extracellular enzymes are secreted by soil microorganisms to mineralize organic carbon (C), nitrogen (N), and phosphorus (P) from soil organic matter (Waring et al., 2014). Extracellular activities are distinct from intracellular ones as they can be stabilized by abiotic soil components (Dilly and Nannipieri, 1998). Measured enzyme activities represent the apparent catalytic history of a soil as continuously modified by soil microorganism in response to environmental changes (Dilly and Nannipieri, 2001). As a result, enzyme activities can be used to assess microbial nutrient demands (Schimel and Weintraub, 2003; Moorhead and Sinsabaugh, 2006) and used to formulate an ecosystem response index that reflect environmental changes (Ajwa et al., 1999; Sinsabaugh et al., 2008). For example, β-glucosidase (BG) has been used to assess the microbial response to long-term N amendments in a tall-grass prairie
soil (Aijwa et al., 1999) and N-acetyl-glucosaminidase (NAG) has been utilized to quantify N-limitation affecting woody plant encroachment into grasslands (Creamer et al., 2013). Enzymatic process stoichiometry is suggested as a means to understand the C and N limitations in soil processes as it is demonstrated that soil enzymatic activities and stoichiometry are related to substrate availability, soil pH, and climatic factors (e.g., precipitation and temperature) (Sinsabaugh et al., 2008, 2009; Waring et al., 2013). However, the effects of environmental factors, such as elevated N inputs and precipitation on enzymatic stoichiometry are unclear. Various global change scenarios have suggested that increased inputs of reactive N from fertilization and fossil fuel combustion and altered precipitation regimes will become common (Knapp et al., 2002; Liu et al., 2009, 2013). As soil enzyme activities are sensitive to ecosystem fluctuations, they can serve as indicators of various responses of the plant-soil system to changes in N deposition (Sinsabaugh et al., 2005), elevated atmospheric CO₂ (Dorodnikov et al., 2009a,b), and precipitation intensity (Henry et al., 2005; Bardgett et al., 2008; Fiddall et al., 2008).

Clearly, nitrogen and water availability are two driving factors affecting grassland net primary productivity (NPP) (Xu et al., 2012), especially in semi-arid grasslands where evaporation greatly exceeds annual precipitation inputs (Heisler-White et al., 2008). Recent grassland-related field studies in Inner Mongolia suggested a plant NPP of about 1.5 tons ha⁻¹ is both N- and water-limited, because N addition above 5.25–17.5 g N m⁻² yr⁻¹ of background increased NPP by 13%–62% (Bai et al., 2010), while water addition increased above- and belowground NPP by 32.9% and 38.3%, respectively (Xu et al., 2010). However, soil microorganisms are not limited by the same factors that limit plant systems (Hobbie et al., 2005; Wei et al., 2013). For example, Wei et al. (2013) reported different N saturation levels (threshold levels for N demand) for plants and soil microorganisms highlighting that microbes can be limited by C or P while plants are N limited (Treseder, 2008). Additionally, reduction in both the size and activity of soil microbial biomass were shown under higher N availability in temperate grasslands (Gutknecht et al., 2012; Wei et al., 2013), which indicates microorganisms are not always limited by N. On the other hand, positive effects of added N were also observed (Zeglin et al., 2007; Keeler et al., 2009). Keeler et al. (2009) found N addition to increase the activity of phosphatase and cellulohydrolase by 13% and 17%, respectively. Similar findings were also reported by Zeglin et al. (2007) where N increased both cellulolytic activities (BG and cellobiohydrolase) and phosphatase activity. Other studies showed that added water in grassland ecosystems stimulated (Zhou et al., 2013) or suppressed microbial activity (Hendy et al., 2005) depending on the study site. For instance, increased water availability resulted in increases of NAG, leucine aminopeptidase, and alkaline phosphomonoesterase (PME) in an Inner Mongolia grassland (Zhou et al., 2013), while water addition resulted in decreases of BG, NAG, and PME in a California grassland soil (Henry et al., 2005).

Aggregate structure can affect microbial activities as fluxes of water and oxygen (Six et al., 2004) and accessibility of SOM will differ between aggregate-size classes (Jastrow et al., 2007). Jastrow et al. (2007) report most labile SOM is concentrated in macroaggregates and more recalcitrant, or less accessible SOM is resident in microaggregates resulting in overall higher enzyme activities in macro- vs. microaggregates (Dorodnikov et al., 2009b). The study of soil microbial enzyme activities on an aggregate level could provide insight into soil C and N cycling in response to increased N input and precipitation.

The objectives of this study were to examine the effects of elevated N inputs and precipitation intensity on the distribution and activity of C-, N-, and P-acquiring enzymes by evaluating aggregate size fractions for soils collected from the semi-arid grasslands of Inner Mongolia, China. A prior field manipulation experiments, had demonstrated significant increase of NPP in response to four 4-year N and water addition (Xu et al., 2010) and SOC in response to 7-year water addition (Wang et al., 2014). We hypothesized that (i) microbial biomass and enzyme activities would increase in macroaggregates because of presumably higher amount of labile SOM; (ii) N-acquiring enzymes would respond to N additions in a way that is different from C- and P-acquiring enzymes because N addition would decrease the substrate C:N ratio and increase the N:P ratio; and (iii) increasing moisture inputs for ecosystems under water limitation should stimulate microbial activity resulting in higher overall enzyme production for C-, N-, and P-acquisition. As N amendment may potentially cause C and P limitation, we predict that the ratio of β-glucosidase to N-acetyl-glucosaminidase will increase while N-acetyl-glucosaminidase to phosphomonoesterase ratio will decrease under higher N availability.

2. Materials and methods

2.1. Field site and experimental design

The study site is located in Duolun County, Inner Mongolia in northern China (116° 17'E and 42°02'N, elevation 1324 m a.s.l.). The mean annual temperature is 2.1 °C with mean monthly temperature ranging from −17.5 °C in January to 18.9 °C in July, and the mean annual precipitation is 379.4 mm with approximately 86% occurring from May to September. The plant community at the site is a typical temperate grassland dominated by prairie sagewort (Artemisia frigida Willd.), wheatgrass (Agropyron cristatum Gaertn.), and needlegrass (Stipa krylovii Roshev.). The soil type is classified as Haplic Calcisols according to the FAO classification with 63% sand, 20% silt, and 17% clay, respectively (Li et al., 2009).

In April 2005, a split-plot experiment design was applied to the site. Twelve 8 m × 8 m plots were established in each treatment block (107 m × 8 m) with a 1 m buffer zone between any two adjacent plots; each block was replicated seven times. The blocks were divided into two main plots based on water treatment (ambient precipitation and 180 mm of water addition) and then each main plot was divided into six subplots. The 180 mm of water addition is an approximately 50% increase above mean annual precipitation based on meteorological record for the site (Xu et al., 2012). This experiment is part of an on-going project designed to investigate the effects of increased N and water on ecosystem responses in the Inner Mongolia grassland (Xu et al., 2010, 2012). The N addition plots were randomly assigned to subplots within each main plot. The water addition plots received 15 mm of precipitation weekly by sprinkling irrigation during the growing season (12 consecutive weeks from June to August). Nitrogen (in the form of urea) was applied at four 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) in four of six subplots, half of which was applied in early May and the other half in late June from 2005 to 2013. Background N inputs (atmospheric deposition plus fertilizer application) in this area are about 5 g m⁻² yr⁻¹ so this manipulation represents a 5–10 g m⁻² yr⁻¹ increase in N addition.

2.2. Soil aggregate-size fractionation and other soil physicochemical properties

In September 2013, a composite soil sample from the top 0–10 cm soil layer was taken from five randomly selected locations at each plot from four out of seven blocks in both the N and water treatment main plots using a 5-cm diameter corer. Fresh soil
samples were placed in hard plastic containers to maintain their primary structures during transportation to the laboratory. To minimize the disruption in microbial communities and activities, soil aggregates were isolated by modified dry-sieving method according to Dorodnikov et al. (2009a,b). The field moisture of these soil samples was optimal (10–15%) for dry-sieving which allowed limited mechanical stress to induce maximum brittle failure along the natural planes of weakness. The recovered soil was gently sieved through a 5 mm screen and visible plant residues and stones were removed. The recovered soil samples (500 g) were transferred to a nest of sieves (2000 and 250 μm) on a Retsch AS200 Control (Retsch Technology, Düsseldorf, Germany). The sieves were mechanically shaken (amplitude 1.5 mm) for 2 min to separate the aggregates >2000 μm (large macroaggregates class), 250–2000 μm (small macroaggregates), and <250 μm (microaggregates class). Samples were used immediately for inorganic nitrogen and dissolved organic carbon (DOC) analyses and frozen at −20 °C for use on enzyme assays.

Soil moisture of the separated aggregates was determined as mass loss after drying the isolates at 105 °C for 24 h. Nitrate and ammonium were determined colorimetrically from 1 M KCl soil extracts from the fresh soil aggregate samples using an Auto-analyser III continuous Flow Analyzer (Bran & Luebbe, Norderstedt, Germany). Additionally, soil pH was determined in a 1:5 (w/v) soil-to-water extract of soil aggregate samples from treatments and controls with a PHS-3G digital pH meter (Precision and Scientific Corp., Shanghai, China). Soil DOC was extracted by adding 50 ml of 0.5 M potassium sulfate (K2SO4) to subsamples of 25 g homogenized soil fractions, and agitated on an orbital shaker at 120 rpm for 1 h (Wei et al., 2013). After filtering through 0.45 μm cellulose acetate filter paper, the filtrate was analyzed by a TOC analyzer (High TOC, Elementar).

2.3. Microbial biomass C analysis and enzyme assays

Microbial biomass C (MBC) was determined using the fumigation-extraction method (Vance et al., 1987). Briefly, 10 g (dry weight equivalent) of each soil aggregate fraction was fumigated with ethanol-free chloroform (CHCl3) for 24 h at 25 °C. Simultaneously, another subsample was kept at the same conditions without fumigation. After complete removal of CHCl3, organic C from fumigated and unfumigated soil samples were extracted with 0.5 M K2SO4 with a soil:extractant ratio of 1:4 (w/v) and shaken at 150 rpm for 1 h. After filtration with Whatman no. 2 filter paper, the extractable organic C in soil extracts was analyzed by a TOC analyzer (High TOC, Elementar). Microbial biomass C was calculated as the difference between fumigated and non-fumigated samples and normalized to the weight of the soil fraction. To correct for incomplete extraction, we used efficiency factor of 0.38 (Vance et al., 1987) to calculate the actual MBC concentration as described by Zhang et al. (2013a) for the soils in this area.

Frozen (at −20 °C) field moist soil aggregate samples were thawed to 4 °C one week prior to the start of any enzyme assay. The measurement of activities of β-glucosidase (BG), N-acetyl-β-D-glucosaminidase (NAG) and acid phosphomonoesterase (PME) was performed on the basis of p-nitrophenol (PNP) released after cleavage of enzyme-specific synthetic substrates according to method of Tabatabai (1994). The specific substrates (Sigma, St. Louis, USA) were p-nitrophenyl-β-D-glucopyranoside for BG, p-nitrophenyl-N-acetyl-β-D-glucosaminide for NAG and p-nitrophenyl-phosphate for acid phosphomonoesterase (PME). For BG activity, 10 g of soil aggregates was weighed into Erlenmeyer flask to mix with a pH 6.0 modified universal buffer consisting of 0.1 M trihydroxymethyl aminomethane, 0.067 M citric acid monohydrate compound, and 0.1 M boric acid. The indicator substrate, 0.05 M p-nitrophenyl-β-D-glucopyranoside was added to the reaction system followed by 1 h incubation. Reactions were stopped by adding 0.5 M CaCl2 and 0.1 M trihydroxymethyl aminomethane which was buffered to pH 12. Controls were performed with the substrate being added after the reactions were stopped. The products were filtered through Whatman no. 2 filter paper and measured colorimetrically at 410 nm (Tabatabai, 1994) with a UV-VIS spectrophotometer (UV-1700, Shimadzu). The procedures for the assays of NAG and PME activities were the same as for BG except using p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-phosphate as the substrate and buffering pH of reaction systems to 5.5 (Parham and Deng, 2000) and 6.5 (Tabatabai, 1994), respectively. The activities of BG, NAG and PME were expressed in mg PNP released per kg dry soil fraction per hour.

2.4. Statistical analysis

ANOVA was used to determine the effects of factor on the (between-subjects) and their interactions on the pH values, concentrations of MBC and DOC, the activities of BG, NAG and PME and the ratios of BG:NAG, BG:PME and NAG:PME. The effects of N addition rates on the activities of BG, NAG and PME and the ratio of BG:NAG, BG:PME and NAG:PME were determined by one-way ANOVA and run separately for ambient precipitation and water addition. Pearson correlation analysis was used to examine the relationship among soil parameters. All statistical analyses were performed in SPSS 16.0 (SPSS, Inc., Chicago, IL, U.S.A) and statistical significance was accepted at P < 0.05.

3. Results

3.1. Soil pH, DOC and MBC values among soil aggregates

Soil pH decreased significantly (P < 0.05) with increasing N input under either normal or elevated water (Fig. 1a). Water additions (W) significantly increased soil pH in N5, N10 and N15 treatments of large-macroaggregates and microaggregates, and in N5 treatment of small macroaggregates (Fig. 1a). Neither aggregate size nor interactive N, W and aggregate size (A) effects was detected in changes of soil pH (Table S1). The DOC concentration was not affected by water addition or soil aggregate sizes but significantly increased by the highest N (N15) for both large- and small-macroaggregates and by N additions at N5 and N15 for microaggregates under high level of water conditions (Table S1, Fig. 1b).

Under normal level of precipitation, N addition significantly decreased MBC in small macroaggregates for the N5 and N15 treatments and in the microaggregates for N15 treatments. In contrast to normal level of precipitation, under high levels of precipitation, MBC was reduced in the N15 treatment for large macroaggregates (Fig. 1c). Water addition significantly increased MBC in N5 of small macroaggregates (Fig. 1c). The concentration of MBC in aggregates increased as follows: small macroaggregates < large macroaggregates < microaggregates.

3.2. Response of enzyme activities in different soil aggregate classes to N and water addition

In general, soil β-glucosidase activity was affected by water and N treatments, and was different across the aggregate fractions; there was a significant W × N × A interaction (Table S1). Across the three aggregate factions, the activity of BG decreased significantly (P < 0.05) at the highest N addition rate (N15) by 31.5–471 % as compared to respective control plots under normal-level water conditions (Fig. 2a). Under elevated water inputs, the BG activity was significantly increased at the highest N addition rate (N15) by 10–34 % compared with control treatments; however, there was no significant differences in the activity of BG under normal-level water conditions.
activity was significantly higher within each N treatment and soil fraction as compared to normal level of water conditions by 45.5–103.4%, however the decrease of BG activity with increasing N supply was much more pronounced (Fig. 2a). The BG activity was distributed differently through aggregate-size classes: under normal level of water regime the lowest activity was measured in small macroaggregates followed by comparable activities in large macro- and microaggregates; under higher level of water treatment, however, BG activity increased in the order large macro- > micro- > small macroaggregates with the highest being CK (without N additions) (Fig. 2a).

Our results showed that there were significant N, aggregate size, W × N, and N × A effects on N-acetyl-β-α-glucosaminidase activity, whereas no significant water, W × A, and W × N × A effects were found (Table S1). N addition significantly increased the NAG activity in the three soil aggregates by as much as 80.8%, except for small macroaggregates and microaggregates under normal-level water conditions (Fig. 2b). The NAG activity was significantly higher in water addition plots of N5 in large macroaggregates and of N15 in microaggregates while it was lower for N15 in large macroaggregates as compared to normal-level water conditions (Fig. 2b).

Across three soil fractions, the NAG activity in large macroaggregates was the highest under N treatments of N10 and N15 for both water treatments.

While water treatment substantially stimulated phosphomoesterase activity, N addition significantly decreased PME activity under both water regimes throughout three soil aggregate classes by 0.8–36.3% (Fig. 2c). Among three aggregate-size classes, the PME activity was the highest in microaggregates followed by large macro- and small macroaggregates. This trend was especially pronounced under high-level water treatment (Fig. 2c).

3.3. Response of enzymatic ratios to N and water addition among soil aggregate classes

The ratio of β-glucosidase: N-acetyl-β-α-glucosaminidase was significantly decreased by N addition under both normal and high level of water treatments (Table S1 vs. Fig. 3a). Water addition increased (P < 0.05) the ratio of BG:NAG across the three soil aggregate fractions (Fig. 3a). Aggregate sizes significantly influenced the distribution of BG:NAG ratio (Table S1). This ratio was the highest in microaggregates under normal-level water treatment
and with increased N additions. However, under N control treatment and high level of precipitation, the highest BG:NAG ratio was detected in large macroaggregates (Fig. 3a). Significant W × N, and N × A effects were detected on the ratio of BG:NAG (Table S1). The ratio of BG:NAG ranged from 4.6 to 14.2 under normal level of precipitation conditions and from 8.7 to 26.9 under water addition treatments throughout three aggregate classes (Fig. 3a).

The highest value of \( \beta \)-glucosidase: phosphomonoesterase ratio was observed under high level of water supply of CK treatment in large macroaggregates (Fig. 3b). Addition of water significantly increased BG:PME ratio in CK of small macroaggregates, and N0 and N15 of microaggregates, whereas other differences were not significant (Table S1, Fig. 3b). Elevated N significantly increased the NAG:PME ratio in three soil aggregate-size classes under both ambient precipitation and water addition treatments (Fig. 3c).

Water addition significantly decreased the ratio of NAG:PME in all treatments of large macroaggregates, in CK and N10 of small macroaggregates, and in CK, N5, and N10 of microaggregates (Fig. 3c). Significant interaction of N with water or aggregate size was detected on NAG:PME ratio (Table S1).

3.4. Correlations between soil chemical and biological characteristics

Soil pH had significant positive correlations with MBC, BG activity, and PME activity (Table S2) while it had significant negative correlations with NAG activity (Table S2). MBC positively correlated with BG and PME activities but negatively correlated with NAG activity (Table S2). No significant correlation between DOC and MBC as well as enzyme activities was found (Table S2).

4. Discussion

4.1. Distribution of microbial biomass and enzyme activities among soil aggregates

Given that fresher plant material, presumably enriched in labile OM should accumulate in large-size fractions (Jastrow et al., 2007), we expected to observe more accumulation of MBC in the larger fraction. However, we found that MBC was higher in the microaggregates than in the macroaggregates (Fig. 1c), which is similar to
the finding reported by Dorodnikov et al. (2009a) and Zhang et al. (2013b). This could be due to the fact that smaller aggregate sizes have higher specific surface because of a larger portion of clay and silt to which microbial cells are attached (Amato and Ladd, 1992; Van Gestel et al., 1996). Additionally, smaller pore sizes in microaggregates (Jastrow et al., 2007) protect microorganisms from predation by protozoa or from desiccation (Zhang et al., 2013b) and allow for accumulation in particles with longer mean residence time.

The distribution of MBC did not coincide with activities of BG, NAG, and PME among the aggregate fractions (Fig. 2). Thus, the relatively higher BG and PME activities in microaggregates versus small macroaggregates could result from a higher contribution of fast-growing microorganisms in microaggregates (Dorodnikov et al., 2009a). Indeed, previous investigations found that BG activity correlated well with fast-growing Gram-negative bacteria and PME with the whole community (Waldrop et al., 2000). In contrast, Dilly et al. (2001) found microbial mass and BG were representing better for integral microbiological characteristics which may be due to the discrepancies between media cultivation and field studies. However, in contrast to BG and PME, and in line with our initial hypothesis, NAG activity was the highest in large macroaggregates which is abundant in fresher particulate organic carbon (Fig. 3b). As NAG activity is thought to be mainly driven by the activity of the fungal community (Miller et al., 1998; Chung et al., 2007), we interpret the increasing trend of NAG with aggregate size as a preference of habitat by fungi in this system rather than a response to N availability (Dorodnikov et al., 2009a,b).

In this system, we surmised that a high microbial demand for P relative to both C and N, and high demand for C relative to N would persist in microaggregates, with higher mineral content, regardless of N or water treatment. We found this to be the case, evidenced by...
both BG:PME and NAG:PME ratios in microaggregates being lower than in macroaggregates, and the BG:NAG ratio showing an opposite response (Fig. 3). Phosphorus-deficiency of microorganisms might derive from P sequestration through mineral interactions of clays (Waring et al., 2014), and iron- and aluminum-oxide coatings (Khare et al., 2005). Lower C availability relative to N, as evidenced by lower proportions of extractable DOC to dissolved inorganic nitrogen (DIN) in microaggregates (Fig. S1), could explain the elevated BG to NAG ratio in our study.

4.2. N and water addition induces divergent responses of activities of C-, N- and P-acquisition

Consistent with our hypothesis, N addition altered the soil microbial community such that the soil recorded a divergent response of N- from C- and P- acquisition enzymes (Fig. 2). The activities of BG and PME, as well as MBC content, were generally repressed by increased N supply (Figs. 1 and 2). A reduction in overall soil microbial activity under N addition has been documented in both field and lab-based studies (Treseder, 2008) although variable responses have been documented including no net impact (Thomas et al., 2012). When respiration exhibits a suppressed response to chronic N addition, microbial biomass was found to be strongly related to the duration and amount of N added (Treseder, 2008), but not necessarily dependent on the form of N added (Ramirez et al., 2010). An overall decrease in microbial activity with N was suggested to be due to the increase of copiotrophic microorganisms that rely on more labile C sources, thus relying less on the need for extracellular enzyme secretion (Ramirez et al., 2012). Indeed, in the present study we detected positive N effects on dissolved organic C pool (Fig. 1b), which might lead to an increase in copiotrophic groups (Fierer et al., 2007). The lack of correlation between DOC and MBC as well as enzyme activities (Table S2) indicated that, overall, the repression of microbial activities was not caused by DOC limitation. Consistent with this conclusion is the increase of the NPP/SOC ratio (Fig. S2) under N addition.

Significant positive correlations between soil pH and MBC, BG and PME were observed in our study (Table S2). Soil pH is suggested to be a primary control on microbial activity, enzyme kinetics (Rousk et al., 2009; Wang et al., 2014), and microbial diversity (Fierer and Jackson, 2006). Because soil pH strongly influences the denaturation of enzyme active center as well as enzyme folding (Frankenberger and Johnson, 1982; Wang et al., 2006), C and nutrient availabilities (Andersson et al., 2000; Kemmitt et al., 2006; Aciego Pietri and Brookes, 2008), and the concentration of DOC (Wei et al., 2013). The negative correlation between NAG and soil pH (Table S2) was consistent with the study of Sinsabaugh et al. (2008) who analyzed global enzymatic database from 40 ecosystems. Additionally, the opposite responses of BG and NAG to N addition, as well as the associated lowering of soil pH, might suggest that soil acidification favors fungi over bacteria consistent with pH-fungi relationships (e.g., Miller et al., 1998; Rousk et al., 2009).

The NAG activity may also be induced under a variety of scenarios that result in soil microbial N limitation, including chemical sequestration of N through humification in SOM (Creamer et al., 2013) and increases in NPP which transfer N from the soil to plant biomass. This latter case could explain NPP of plants is frequently limited by N (LeBauer and Treseder, 2008) and as N addition increased NPP (Xu et al., 2012), and N concentration of plant biomass at our field site (unpublished data). The activity of NAG may be induced under a NPP driven microbial N limitation.

In line with our initial hypothesis, water addition significantly increased (P < 0.05) MBC and the activities of BG and PME in all three soil aggregate fractions (Table S1, Fig. 2a and c). Our results are consistent with previous surveys in this semi-arid area that showed positive water effects on MBC and enzyme activities in bulk soils (Zhou et al., 2013; Zhang et al., 2013a). Other studies have demonstrated that under improved water conditions, soil nutritional compounds were brought into soil solution activating microorganisms that up-regulated enzymatic production (Edwards et al., 2007; Geisseler et al., 2011) which is a likely mechanism to explain our findings. With the documented increase in plant density and NPP with water addition at this study site (Xu et al., 2010, 2012), changes in plant quantity and quality could be indirect drivers of microbial activity enhancement (Zak et al., 2003; Milcu et al., 2010; Zhang et al., 2013a).

4.3. Responses of enzymatic ratios to elevated N and water availability

In contrast to our hypothesis, the ratio of BG:NAG decreased under elevated N availability across aggregate classes while NAG:PME increased (Fig. 3a and c) suggesting N addition elevated microbial demand for N relative to C and P. Higher microbial N demand relative to C could result from plants outcompeting microbes for N while allocating N-poor carbon compounds back to the rhizosphere (especially mycorrhizal fungi) (Allen, 1991; Treseder and Allen, 2002). Microbial N limitation could also be induced by deficiency of accessible organic N as heterotrophic microorganisms prefer simple organic N monomers to inorganic N sources (Nasholm et al., 1998; Schimmel and Bennett, 2004; Dunn et al., 2006; Creamer et al., 2013). Previous studies suggested that N addition could induce microbial C limitation through a lower allocation by plants to fine root production resulting in less C to the soil (Treseder, 2008). Our conclusion of higher microbial N demand relative to C, however, was in contrast to the proposed microbial C-limitation cases under N amendment.

Nitrogen addition increased microbial P limitation relative to C (suggested by BG:PME, Fig. 3b). Since new P is derived primarily from rock weathering (Walker and Syers, 1976), it may not keep pace with the supply of C under higher N and water availability (Vitousek et al., 2010). Similar to N limitation, microbial P limitation may result from increased plant P uptake as revealed in the present site by a higher leaf P concentration (unpublished data). In fact, we observed a decrease in soil aggregate P under N addition (unpublished data). Our results suggest that enhanced N inputs have accelerated microbial P limitation and could ultimately place a potential constraint on ecosystem productivity at this site.

Significantly higher BG:NAG and BG:PME ratios under higher water availability suggest a relatively higher microbial C limitation relative to N and P as compared to normal level of water plots (Waring et al., 2014). With unchanged DOC concentration, however, the increase in microbial growth, discerned from overall positive water effects on MBC (Table S1), would cause C limitation under increase water availability. Also, enhanced microbial respiration under water addition (Niu et al., 2009) might be responsible for microbial C limitation.

5. Conclusions

After 9-year of N and water field amendment in a semi-arid grassland, we observed significant changes in soil enzyme activities, physicochemical properties like pH, and MBC among specific soil size fractions. Overall, macroaggregates retained higher BG and PME activities than small macroaggregates suggesting greater levels of fast-growing microbes therein regardless of treatment. With N addition, however, BG activity decreased and NAG activity increased, which was concomitant with a decrease in soil pH; a response consistent with a selective proliferation of soil fungi over bacteria. As expected, water addition increased activities of BG and
PME in all soil fraction sizes, consistent with an up-regulating effect of activated microorganisms under improved water and nutrient conditions that also sparked an increase in NPP. Enzymatic ratios indicated, however, higher microbial P limitation in micro-aggregates, while higher N limitation under N addition and higher C limitation under water addition over all soil fractions. Under projected global change scenarios for this region of China, we can expect changes of microbial activity and chemistry at the multiple spatial scales of the soil continuum that is linked to concomitant changes in ecosystem NPP. These changes could have important impacts related to the localization of microbial community function in aggregate-size fractions and a change in the nutrient cycling capacity of these soils. Overall, identifying how microbes respond (i.e. biomass and functional activity) among soil particles that are responsible for stabilization of different pools of soil C in systems under coupled water-N changes will enhance our capability to predict ecosystem resilience to future global change.

Acknowledgments

We would like to thank Professor Lijun Chen for providing protocols of enzyme assays and laboratory facilities. We also acknowledge support provided by the China-U.S. EcoPartnership for Environmental Sustainability. This work was supported by the National Natural Science Foundation of China (41371251), the National Key Basic Research Program of China (2011CB403204), and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15010100).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.11.015.

References


For the full list of references, please see the original publication.


