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RESEARCH ARTICLE

Interactions of water and nitrogen addition on soil microbial community composition and functional diversity depending on the inter-annual precipitation in a Chinese steppe

SUN Liang-jie[1, 2], QI Yu-chun[1], DONG Yun-she[1], HE Ya-ting[1, 3], PENG Qin[1], LIU Xin-chao[1, 2], JIA Jun-qiang[1, 2], GUO Shu-fang[1, 2], CAO Cong-cong[1, 2]

[1] *Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, P.R.China*
[2] *University of Chinese Academy of Sciences, Beijing 100049, P.R.China*
[3] *Key Laboratory of Crop Nutrition and Fertilization, Ministry of Agriculture/Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R.China*

Abstract

Water and nitrogen are primary limiting factors in semiarid grassland ecosystems. Our knowledge is still poor regarding the interactive effects of water and N addition on soil microbial communities, although this information is crucial to reveal the mechanisms of the terrestrial ecosystem response to global changes. We addressed this problem by conducting a field experiment with a 15% surplus of the average rainfall under three levels of N addition (50, 100, and 200 kg N ha^{-1} yr^{-1}) in two consecutive years in Inner Mongolia, China. Microbial community composition and functional diversity were analyzed based on phospholipid fatty acids (PLFA) and BIOLOG techniques, respectively. The results showed that water addition did not affect the soil microbial community composition, but much more yearly precipitation generally decreased the PLFA concentration, which implied a fast response of soil microbes to changes of water condition. Soil fungi was depressed only by N addition at the high level (200 kg N ha^{-1} yr^{-1}) and without hydrologic leaching, while Gram-negative bacteria was suppressed probably by plant competition at high level N addition but with hydrologic leaching. The study found unilateral positive/negative interactions between water and N addition in affecting soil microbial community, however, climate condition (precipitation) could be a significant factor in disturbing the interactions. This study highlighted that: (1) The sustained effect of pulsed water addition was minimal on the soil microbial community composition but significant on the microbial community functional diversity and (2) the complex interaction between water and N addition on soil microbial community related to the inter-annual variation of the climate and plant response.

Keywords: water addition, nitrogen addition, phospholipid fatty acid (PLFA), BIOLOG-substrate utilization, semiarid steppe

1. Introduction

Precipitation changes and increasing nitrogen (N) deposition are two important components of ongoing global changes (Dore 2005; Gruber and Galloway 2008). It is predicted that precipitation will increase at high latitudes and decrease in most subtropical regions (IPCC 2007). N deposition is

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SUN Liang-jie, E-mail: sunlj-2007@hotmail.com;
Correspondence DONG Yun-she, Tel: +86-10-64856500,
E-mail: dongys@igsnrr.ac.cn

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presently increasing in most terrestrial ecosystems, and the projected rate was estimated to be as double as the current values by 2050 (Galloway *et al.* 2004). It had exceeded the critical loads that have detrimental impacts on the ecosystems (Achermann and Bobbink 2003; Galloway *et al.* 2008). These predicted changes may in turn influence semiarid grasslands because of the limiting role of water and N in these ecosystems. On the other hand, water and N are two coupling factors in grassland ecosystems (Hooper and Johnson 1999; Niu *et al.* 2009). Firstly, the mobility and availability of N depends on water, for example, through wet N deposition and the contents of soil dissolved inorganic N (Fenn *et al.* 2003; Harpole *et al.* 2007). Secondly, soil N addition could enhance the photosynthetic capability of plant through increasing leaf chlorophyll content and the activity of Robisco, which is an important enzyme in photosynthesis (Wang *et al.* 2012; Lin *et al.* 2013), and lead to greater plant transpiration and more rapid loss of soil moisture from the rooting zone. Thirdly, N addition might increase plant productivity as mentioned above, thereby increasing canopy light interception and reducing rates of evaporation at the soil surface (Harpole *et al.* 2007). Therefore, study on the water and N synchronously is important in evaluating the real response of ecosystem to the global change.

Soil microbes are primary mediators of organic matter decomposition and nutrient cycling, and thus play a key role in maintaining function and sustainability of terrestrial ecosystems (Lou *et al.* 2011). Some studies had reported that changes of precipitation and N deposition could affect soil microbial community directly by changing the microbial living environment (Drijber *et al.* 2000; Grayston *et al.* 2004) and indirectly by influencing plants (Lü *et al.* 2011; Gutknecht *et al.* 2012). However, no consistent results had been found in different studies. It was generally believed that the microorganisms themselves had different adaptabilities to changes of environmental water condition. For example, fungi is more tolerant to dry condition than bacteria, while Gram-negative bacteria is more sensitive to soil water change (Nesci *et al.* 2004; Manzoni *et al.* 2012). However, evidences suggested that water addition could increase the relative abundances of soil fungi and Gram-negative bacteria (Bell *et al.* 2014) and resulted in greater fungi/bacteria ratios (Williams and Rice 2007). The unexpected results for fungi had been attributed to the complex relationship between various biotic and abiotic factors in soil under changed water status (Drenovsky *et al.* 2004; Williams and Rice 2007), which still needs more specific studies to figure out the exact responding mechanisms. Similar situation happened in researches about the effects of N addition on soil microbial community. It was generally reported that N addition could shift soil microbial community to a status with relatively lower proportion of fungal groups (Frey *et al.* 2004; Demoling *et al.* 2008; Zhang N *et al.* 2013). However, there also existed studies that reported positive effect (Yevdokimov *et al.* 2012) and non-effect (Rousk *et al.* 2011) of N addition on the fungal proportion in soil microbial community. Moreover, our knowledge about the interaction between water and N addition, which has more realistic significance for our global-change prediction, is still rather poor. Therefore, comprehensive studies with full consideration about effects of water addition, N addition and their interactions on the soil microbial community are in great demand.

Semiarid grassland ecosystems, one of the most extensive ecosystems in China, are notably sensitive to climate variation (Chen *et al.* 2009). Predictions made by model analyses indicate that precipitation would increase by 12–18% in the semiarid steppe in North China (IPCC 2007; Liu *et al.* 2010; Feng *et al.* 2011), and a large amount of N deposition in this region is also observed (Liu *et al.* 2011; Zhang *et al.* 2011). To address how increased precipitation and N deposition, as well as how their interactions, influence microbial communities and soil microbial C utilization profile, we conducted a field experiment in which water and N levels were manipulated to simulate the future changes of precipitation and N deposition. We hypothesized that water addition would increase the richness of bacteria comparing to fungi because of the dry condition in the experimental area, while N addition would suppress the abundance of fungi but different levels of N addition could have different influences, considering there might exist some dose effects for N addition (Sheppard *et al.* 2013; Zhang C *et al.* 2013), and the interactions between water and N will influence soil microbial community composition and functional diversity. Our objectives were to investigate: (1) the impacts of water and N additions at different levels on microbial community composition and function; (2) whether there is any interaction between water and N effects on microbial community characteristics; and (3) how the inter-annual variability of the soil microbial community responds to water and N additions.

2. Results

2.1. Soil physiochemical properties

Significant interactions among water, N addition and sampling year were observed in soil dissolved inorganic N (DIN), moisture and total soil organic carbon (TOC) (Table 1). Soil moisture and TOC were higher in 2012 than in 2011, while DIN was significantly lower in 2012 (Table 1). Water addition decreased soil DIN in 2011, and increased soil TOC in 2012. Soil moisture was improved in both of the years under water addition treatment (Table 1). However, N addition decreased soil moisture in 2012. The soil DIN increased sharply with increasing N addition in both years regardless of the water

addition. No significant treatment or sampling year effect or their interactions was found on the soil bulk density (BD) and dissolved organic carbon (DOC), except for some interactions between water and N on DOC. In 2012, the highest contents of DOC were found in the N200 (200 kg N ha^{-1} yr^{-1}) treatments with no water addition and in N50 (50 kg N ha^{-1} yr^{-1}) treatments with water addition, respectively. In addition, the water and N additions significantly increased the plant aboveground biomass (AGB) in both years, and the plant biomass in 2012 was significantly higher than in 2011 (data not shown).

2.2. Soil microbial community composition

The inter-annual variations of the microbial-community phospholipid fatty acid (PLFA) concentration and ratios were statistically significant. The PLFA concentration of the microbial groups, total PLFA and bacteria/fungi (B/F) ratio were all higher, while Gram-negative/Gram-positive bacteria (G–/G+) ratio was lower in 2011 in comparison with those in 2012. In general, no significant water- and N-addition effect was observed on soil PLFA concentration of microbial groups, except for the ratio of G–/G+ (Table 2). There were significant interactions between N addition and sampling year on soil PLFA concentration of microbial groups, except for fungi and the ratio of B/F, which, however, were affected by the interaction among water, N addition and sampling year. In 2011, the high N addition (N200) lowered the fungal PLFA concentration under the treatments with no water addition, while had no significant effect under water addition. In 2012, N additions also did not significantly impact fungal PLFA concentration no matter whether they were companied with the water addition. The middle- and high-N addition (N100 (100 kg N ha^{-1} yr^{-1}) and N200) significantly decreased the PLFA concentration of G– bacteria but did not change G+ bacteria under no-water-addition treatments. Hence, G–/G+ ratio was decreased. The interactions between water and N addition also lowered the G–/G+ ratio in 2012. In addition,

Table 1 The responses of the soil physicochemical properties to the water and N additions in 2011 and 2012 (mean±SD, *n*=3)

Treatment[1]		DIN (mg kg^{-1})[2]	DOC (mg kg^{-1})[3]	Mois (%)[4]	TOC (%)[5]	BD (g cm^{-3})[6]
2011						
W0	N0	7.94±0.41 e	188.18±17.93 a	3.79±0.40 b	1.58±0.02 b	1.29±0.08 a
	N50	16.80±0.99 d	178.28±21.12 a	3.86±0.59 b	1.30±0.03 e	1.29±0.06 a
	N100	26.10±0.70 c	204.62±12.21 a	3.63±0.42 b	1.45±0.02 cd	1.30±0.03 a
	N200	78.76±1.18 a	209.48±10.99 a	4.43±0.31 b	1.69±0.02 a	1.31±0.02 a
W15	N0	3.50±0.57 f	189.18±23.13 a	6.07±0.75 a	1.49±0.03 c	1.30±0.05 a
	N50	9.01±0.34 e	198.69±8.58 a	6.73±0.59 a	1.35±0.02 f	1.31±0.07 a
	N100	15.20±1.12 d	199.82±12.82 a	6.65±0.24 a	1.44±0.01 d	1.23±0.06 a
	N200	68.06±5.40 b	198.48±22.75 a	6.05±0.38 a	1.36±0.03 f	1.27±0.08 a
2012						
W0	N0	0.49±0.02 f	190.74±15.44 bc	7.89±0.11 b	1.71±0.03 b	1.32±0.05 a
	N50	1.89±0.31 e	165.26±8.40 c	6.94±0.17 d	1.56±0.05 c	1.31±0.12 a
	N100	2.09±0.24 de	180.73±2.10 bc	5.82±0.07 f	1.47±0.02 d	1.21±0.06 a
	N200	9.31±1.02 a	206.44±13.24 ab	6.70±0.07 e	1.82±0.02 a	1.26±0.07 a
W15	N0	2.32±0.12 d	188.29±18.15 bc	8.44±0.11 a	1.66±0.06 b	1.24±0.07 a
	N50	2.42±0.11 d	218.85±24.99 a	7.34±0.11 c	1.70±0.06 b	1.28±0.12 a
	N100	3.50±0.17 c	190.32±10.71 bc	8.06±0.21 b	1.73±0.06 b	1.24±0.03 a
	N200	6.08±0.16 b	184.65±9.66 bc	7.22±0.11 c	1.84±0.05 a	1.18±0.10 a
Significance based on a repeated-measure ANOVA (*P* value)						
Y (year)		**<0.001**	0.245	**<0.001**	**<0.001**	0.073
W (water)		**<0.001**	0.263	**<0.001**	0.873	0.216
N (nitrogen)		**<0.001**	0.420	**0.019**	**<0.001**	0.350
Y×W		**<0.001**	0.346	**<0.001**	**<0.001**	0.582
Y×N		**<0.001**	0.356	**<0.001**	**<0.001**	0.624
W×N		**0.002**	**0.009**	**0.001**	**<0.001**	0.789
Y×W×N		**0.113**	0.305	**0.038**	**<0.001**	0.331

[1] W0, no water addition; W15, 15% more water addition; N0, no N addition; N50, 50 kg N ha^{-1} yr^{-1}; N100, 100 kg N ha^{-1} yr^{-1}; N200, 200 kg N ha^{-1} yr^{-1}.
[2] DIN, soil dissolved inorganic nitrogen.
[3] DOC, soil dissolved organic carbon.
[4] Mois, soil moisture.
[5] TOC, soil total organic carbon.
[6] BD, soil bulk density.
Means with different letters were significantly different (P<0.05) among the different water- and N-addition levels (Duncan's test). The bold *P* values denote statistical significance at P<0.05.
The same as below.

Table 2 The responses of soil microbial phospholipid fatty acids (PLFAs) to the water and N additions in 2011 and 2012 (mean±SD, *n*=3)[1)]

Treatment		Bacteria	Fungi	G–	G+	B/F	G–/G+	ToPLFA
2011								
W0	N0	23.66±0.69 a	0.74±0.04 ab	9.01±0.16 a	10.08±0.39 a	32.14±1.48 bcd	0.89±0.02 a	45.39±0.92 a
	N50	25.18±2.75 a	0.88±0.10 a	9.74±1.11 a	10.49±1.17 a	28.53±1.09 d	0.93±0.01 a	48.09±4.36 a
	N100	26.15±1.77 a	0.89±0.03 a	9.78±0.34 a	11.32±1.18 a	29.30±1.59 cd	0.87±0.06 a	50.13±2.71 a
	N200	21.47±3.43 a	0.52±0.15 c	8.15±1.22 a	8.85±1.50 a	42.42±6.32 a	0.92±0.02 a	39.29±6.16 a
W15	N0	26.20±3.45 a	0.75±0.16 ab	10.20±1.39 a	11.04±1.54 a	35.69±6.14 bc	0.92±0.01 a	49.21±6.16 a
	N50	24.41±2.08 a	0.72±0.11 ab	9.61±0.84 a	10.31±0.89 a	34.12±4.17 bcd	0.93±0.03 a	46.06±3.84 a
	N100	25.89±2.98 a	0.68±0.08 bc	10.36±1.22 a	10.67±1.23 a	38.20±1.29 ab	0.97±0.02 a	50.44±5.54 a
	N200	21.89±2.70 a	0.79±0.08 ab	8.95±0.75 a	8.84±1.50 a	27.84±0.49 d	1.03±0.15 a	40.16±6.24 a
2012								
W0	N0	17.32±1.19 a	0.59±0.02 a	7.49±0.48 a	6.78±0.52 a	29.36±1.85 a	1.11±0.02 b	31.27±2.10 a
	N50	15.91±1.00 ab	0.53±0.01 ab	6.78±0.40 ab	6.31±0.46 ab	29.76±1.07 a	1.07±0.02 bc	28.98±1.54 ab
	N100	13.55±1.09 c	0.58±0.05 a	5.41±0.40 d	5.64±0.48 bc	23.34±0.58 b	0.96±0.01 f	24.96±2.07 c
	N200	15.34±0.94 abc	0.53±0.01 ab	6.13±0.38 bcd	6.32±0.38 ab	29.10±1.64 a	0.97±0.01 f	27.83±1.71 abc
W15	N0	14.29±1.78 bc	0.47±0.03 b	6.36±0.86 bc	5.27±0.61 c	30.27±2.09 a	1.21±0.02 a	25.71±3.10 bc
	N50	15.21±1.53 abc	0.54±0.07 ab	6.45±0.61 bc	6.07±0.64 abc	28.29±0.68 a	1.06±0.02 cd	27.98±2.78 abc
	N100	14.05±0.17 bc	0.46±0.02 b	5.73±0.09 cd	5.74±0.19 bc	30.73±1.38 a	1.00±0.05 ef	25.57±0.40 bc
	N200	15.39±0.95 abc	0.51±0.07 ab	6.39±0.36 bc	6.23±0.42 ab	30.24±2.43 a	1.03±0.01 de	27.86±1.57 abc
Significance based on a repeated-measures ANOVA (*P* value)								
Y		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
W		0.809	0.073	0.414	0.505	0.126	**0.002**	0.755
N		0.201	0.099	0.077	0.233	0.244	**0.009**	0.063
Y×W		0.249	0.409	0.067	0.340	0.437	0.543	0.270
Y×N		**0.011**	0.168	**0.011**	**0.006**	0.190	**<0.001**	**0.002**
W×N		0.948	**0.003**	0.633	0.992	**<0.001**	0.207	0.909
Y×W×N		0.180	**0.012**	0.273	0.132	**<0.001**	0.199	0.240

[1)] Bacteria, fungi, G+ (Gram-positive) bacteria, G- (Gram-negative) bacteria and total PLFA (ToPLFA) shown in the table refer to the PLFA contents in soil (nmol PLFA g^{-1} dry soil). B/F refer to the ratio of bacterial to fungal PLFA content, and G–/G+ refer to the ratio of G+ to G– bacterial PLFA content.

the N addition effects on the bacterial PLFA concentration and G+ bacteria and the ratio of B/F also varied with different sampling years.

A redundancy analysis (RDA) of the PLFA profile showed that the model could explain 74.7 and 51.7% of the total variability in 2011 (Fig. 1-A) and 2012 (Fig. 1-B), respectively. Based on a Monte Carlo permutation test, the variability predictors at the significant level were soil temperature (31% of the variability, *P*=0.002), TOC (21% of the variability, *P*=0.004), DIN (10% of the variability, *P*=0.002), AGB (6% of the variability, *P*=0.03) in 2011, and moisture (23% of the variability, *P*=0.004), TOC (11% of the variability, *P*=0.04), and DIN (15% of the variability, *P*=0.008) in 2012.

2.3. Soil microbial BIOLOG substrate (C) utilization

The RDA of the BIOLOG data indicated that the first two principle components accounted for 12.4 and 8.0% of the total variance, respectively, in 2011 (Fig. 2-A) and 17.4 and 9.0%, respectively, in 2012 (Fig. 2-B). The RDA biplots showed that soil microbial BIOLOG-substrate utilization patterns were separated between water treatments along with the alteration of soil moisture in 2011 (Fig. 2-A). The Monte Carlo permutation test also showed that the soil moisture (10% of the variability, *P*=0.002) was the only environmental factor that significantly distinguished the soil microbial C-utilization profiles among the treatments. However, the samples from different N levels tended to be indistinguishable from each other. In 2012 (Fig. 2-B), the separation of microbial C-utilization patterns by the water addition was remarkable under N additions, and the Euclidean distance in the biplot between W0 (no water addition) and W15 (15% more water addition) in a given N treatment increased with the ascending N-addition level. The samples with no water addition were separated by N additions along with the direction of DIN except for the tangling of N50 and N100, while treatments with water addition were not separated. A Monte Carlo permutation test revealed that soil moisture (*P*=0.014), DIN (*P*=0.036) and AGB (*P*=0.002) could explain the variation of samples at a statistically significant level.

3. Discussion

3.1. Effect of water additions on soil microbial community

Water-addition effects were not obvious on soil PLFA con-

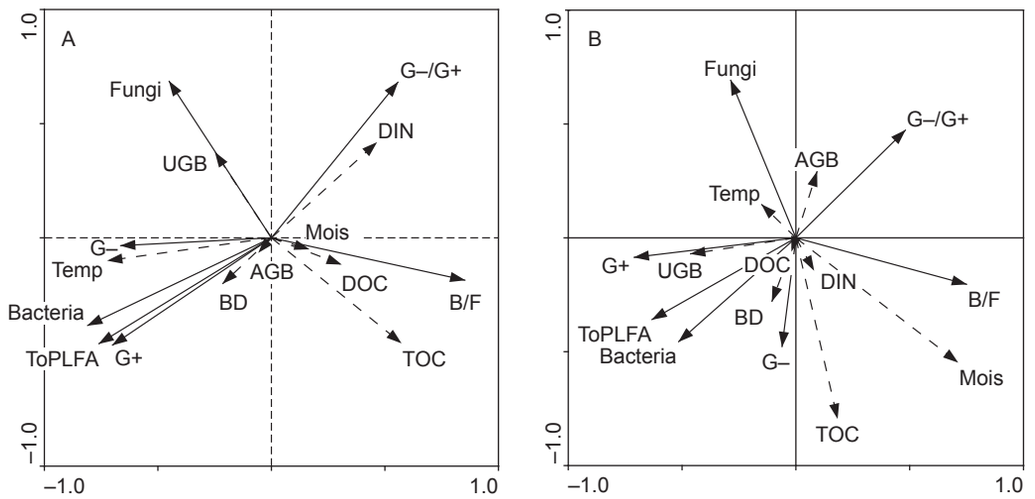

Fig. 1 Redundancy analysis ordination biplots with the phospholopid fatty acids (PLFA) profiles as species variables and the soil and plant characteristics as environmental variables in September 2011 (A) and 2012 (B). The dashed arrows designate environmental variables (Mois, soil moisture; DOC, soil dissolved organic carbon; DIN, soil dissolved inorganic nitrogen; TOC, soil total organic carbon; BD, soil bulk density; UGB, grass underground biomass; AGB, grass aboveground biomass; Temp, soil temperature at 5 cm depth). The solid arrows represent PLFA parameters. The cosine of the angle between two vectors represents their correlations. The same as below.

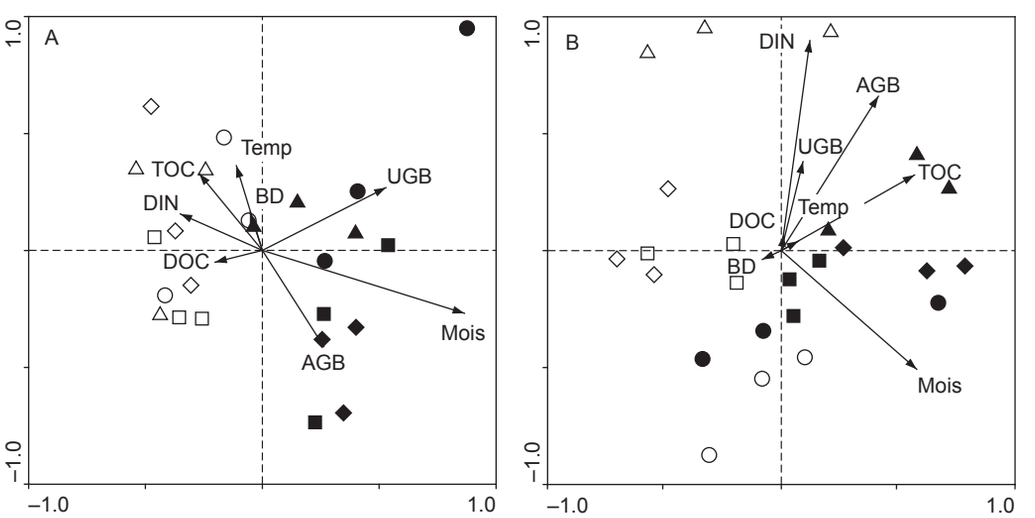

Fig. 2 Redundancy analysis ordination biplots with soil microbial C-utilization profiles as species variables and the soil and plant characteristics as environmental variables in September 2011 (A) and 2012 (B). The symbols indicate treatments (open symbols, zero water added, W0; filled symbols, water added, W15; circle, N0; square, N50; diamond, N100; triangle, N200). The distance of projection of the sample points onto the arrow lines showed the degree of influence of the environmental factors on the microbial C utilization in the different treatments.

centration of microbial groups in our study. The result was not agreed with our hypothesis. Soil microorganisms were thought to be adaptive for local environmental changes by developing their own life history strategies or mechanisms (Schimel *et al.* 2007), such as precipitation (Gutknecht *et al.* 2012), which might be the reason of the nonresponse of soil microbial community to water addition in our study. Our result was consistent with Bi *et al.* (2012), who found little response of microbial community composition to the water manipulation in the typical agro-pastoral ecotone of Inner Mongolia.

However, there may be an alternative explanation that could be easily ignored. On the one side, soil microbes respond quickly to environmental variations. Many studies have concluded that precipitation pulses or drying and rewetting cycles significantly impact the soil microbial community composition (Steenwerth *et al.* 2005; Xiang *et al.* 2008; Landesman and Dighton 2011). After such pluses, the soil

will return to a water-poor condition, which frequently occurs in semiarid steppe ecosystems because of the high evaporation and transpiration. Consequently, the soil microbial community could return to its original adaptation to the environment, which indicated another adaptive life strategy for soil microbes living in the dry environment, i.e., the microorganisms living in the dry environment in our experiment may develop an ability to fast respond to water pulses, like the short-life grasses responding to water deficit. This flexibility of the soil microbial community may be a reason why there was no response of the soil microbial community composition to the water addition, and this hypothesis was supported by the study of Landesman and Dighton (2011), who concluded that microbes were highly resilient and recovered within hours or days of a rain event. On the other side, based on the fast response of soil microbes, sampling time would be an important factor in encountering the changes of soil microbial community. In our experiment, the sampling times were arranged at 5–6 d after the last water addition, which probably missed the fast response of microbes. In addition, there might be a possiblity that the PLFA method was not elaborate enough to reveal the exact changes of soil microbial community, so a genetic-method analysis was demanded to take a further look at the more detailed status of soil microbial communities and find out if there were any changes of microbial community composition or not.

Although there was no response of microbes to the pulsed water addition, we found significant yearly variations in the PLFA concentration of microbial groups and the PLFA ratios, which showed that the biomasses of soil bacteria, fungi, and G– and G+ bacteria, along with the total microbial biomass and B/F ratio, were higher in 2011, while the G–/G+ ratio was higher in 2012. The yearly variation might reflect the sampling time effect. However, the main reason for the yearly difference in microbial PLFA was the large variation in rainfall (65% of the variability, P=0.002, based on the RDA of the two years, data not shown). There was approximately 157% more rainfall in the growing season of 2012 than 2011, which might be an important reason for the dramatic decrease of the mineral N in 2012 (Table 1), because much more rainfall could cause a more serious leaching. Moreover, the greater plant biomass (data not shown) under the relatively abundant precipitation of 2012 also required more mineral N, probably resulting in increased competition between the plants and soil microbes. Hence, the growth of soil microbes was probably suppressed by the deficiency of N in 2012 despite sufficient water. Additionally, N supply could alleviate the negative effects on soil processes caused by water deficits (Wu *et al.* 2012), which could also be a possible reason for the higher PLFA concentration of the microbial groups in 2011. The significant influence of the increasing precipitation suggested that the soil microbial community in semiarid grassland ecosystems might not respond to the increasing precipitation unless the precipitation was enough to sustain the change of microbial communities, such as the dramatic rainfall elevation in 2012 compared to 2011.

In spite of no significant water-addition effect on soil microbial composition, water addition changed the BIOLOG-substrate utilization profile of the microbial community in 2011. Changes in the microbial C-substrate utilization pattern may be caused by variation in the microbial community composition or just a change of phenotypic expression (Williams and Rice 2007). According to our PLFA results, microbial community composition stayed unchanged after water addition, so it was reasonable to infer that the shift of BIOLGO-substrate utilizing profile was likely derived from the change in the physiology of the soil microbe community in our experiment. The RDA results showed that the soil moisture is the most influential environmental factor, which means that the response of the microbial BIOLOG-substrate utilization resulted from the direct influence of water addition in our experiment.

Generally, water addition has both short-term and long-term effects on soil microbial community. The former was mainly referred to the fast response of soil microbes, and it acted quickly and sensitively. Water addition could directly change soil microbial community through altering water and water-soluble nutrient conditions in the short-term effects. However, the long-term effect concerned the indirect influence through the shifts of plant diversity and functional composition (Zak *et al.* 1994; Knapp *et al.* 2002), which would be a slow process for years, decades or even centuries (Bardgett *et al.* 2008). According to these different time-scale effects of water addition on soil microbes, selecting sampling times should be carefully considered if the study aimed to capture microbial changes.

3.2. Effect of N addition on the soil microbial community

Although there were no significant impacts of N addition on soil microbial community composition, our result showed that the high N addition (N200) reduced the fungal PLFA concentration significantly in 2011 and increased the B/F ratio. These results partly affirmed our hypothesis, and indicated that high-level N addition (N200) could inhibit fungal growth and alter the microbial community composition. Our result was similar with those obtained by other studies (Henriksen and Breland 1999; Frey *et al.* 2004; de Vries *et al.* 2006; Docherty *et al.* 2012). The negative response of fungal biomass and the ratio of F/B to the N addition was attributed to many reasons, such as direct suppression on fungi (Donnison *et al.* 2000), shifts in organic-matter quality

and quantity through impacts on plants (Frey *et al.* 2004; Cusack *et al.* 2011), an ecosystem-level limitation of other nutrients (Johnson *et al.* 2003; Menge and Field 2007), and influence on the belowground micro food web (Li *et al.* 2013). In this study, the high N input in 2011 increased the soil TOC (Table 1) and plant biomass (data not shown). Meanwhile, the soil fungal PLFA concentration was highly correlated to TOC and UGB but not to DIN (Fig. 1-A). This result suggested that the influence on fungi might derive from the altered organic matter but not from a direct N effect. However, we cannot be sure if there are other factors that affect soil fungi. Unlike the microbial response in 2011, the medium and high N addition (N100 and N200) reduced the PLFA concentration of G– bacteria in 2012. The plant competition caused by serious N loss and much greater precipitation in 2012 might be responsible for the decline of G– bacteria. The RDA result also supported the viewpoint that the G– bacterial PLFA concentration negatively responded to aboveground plant biomass.

3.3. Interactive effect of water and N addition on soil microbial community

The interaction between water and N addition has been studied widely on aboveground systems (Albrizio *et al.* 2010; Lü *et al.* 2010, 2012), with little information on soil microbial communities. As we supposed in our hypothesis, there was a significant interactive effect of water and N on soil microbial community composition and functional diversity in this study. Under the ambient water condition in 2011, the high N addition decreased fungal biomass, which was consistent with other studies (de Vries *et al.* 2007; Hobbie *et al.* 2012). However, N addition had no significant effect on fungal biomass under water addition in the same year. The results implied an interactive relationship between N and water addition that water addition alleviated the negative effect of high N addition on soil fungi. The alleviating effect might be explained by the reduction of soil DIN under water addition (Table 1), which probably due to the hydrologic leaching and more plant intake. As the results showed, only high N addition suppressed soil fungal biomass, and the other levels of N addition did not significantly influence fungi. Hence, the water addition probably reduced the negative effect of high N addition through washing a part of soil DIN away to a lower level. No significant effect of N addition on fungi found in 2012 under not only water addition but also no water addition further proved our theory, because the much more precipitation in this year could aggravate the N loss in soil and weak the effect of N addition. However, it was likely increased precipitation in 2012 that also simulated a negative effect of N addition on G– bacteria. The increased precipitation improved the growth of vegetation, leading to more serious competition with soil G– bacteria, which was the most nutrient-sensitive microbes comparing to G+ bacteria and fungi (Fierer *et al.* 2003; Kandeler *et al.* 2008). The repeated-measures ANOVA results, which showed significant interactions between sampling year and N addition and between water and N addition on soil microbial PLFA concentrations and the ratios (P<0.01), further proved the interactive relationship between water- and N-addition.

The interaction between water- and N-addition also affected the microbial BIOLOG substrate-utilization profile. As shown in the RDA biplot of 2012, the separation of water treatments was remarkable when they companied with N additions, and their Euclidean distance between W0 and W15 in a given N treatment increased with the ascending N-addition level, which suggested that the difference of the microbial function diversity between W0 and W15 might be simulated by N addition. In contrast, N addition treatments were separated in the biplot when there was no water addition, while they were not separated when water was added, which meant a negative impact of water addition on the microbial function diversity responding to the N addition. In a word, these results indicated that N addition probably induced and amplified the effect of water treatment, while water addition may offset or override the response of microbial BIOLOG-substrate utilization to N application. The exact mechanism about the stimulating effect of N addition on the microbial response to water addition was still unclear. The BIOLOG result reflected the carbon-using strategy of detected soil microbial community, which might depend on the soil microbial substrates that derived from plant litter. As shown in our results, plant aboveground biomass was improved by N addition in 2012, and provided more plant litter and organic substrates for soil microbial community. Accordingly, the effect of water addition could be facilitated based on the more organic substrates under higher N addition. However, it was easy to understand the offsetting or overriding effect of water addition based on the response mechanism of the hydrologic leaching of soil N, which weakened the effects of N addition. Our results suggested a unilateral positive/negative interaction between water and N addition in affecting microbial BIOLOG-substrate utilization in contrast with bilateral positive interactions as Bi *et al.* (2012) had observed. In addition, we did not see any interaction between water and N in 2011, which suggested that the interaction between water and N addition on soil microbial BIOLOG-substrate utilization may only occur in certain climate conditions, such as in a high-rainfall climate.

4. Conclusion

Our results suggested that the effect of pulsed water addition was significant on the functional diversity of microbial

communities but limited over time regarding soil microbial community composition. There could be a microbial fast response to water addition, and sampling time would be an important factor to consider about if the study was aimed to capture microbial changes under water addition. High level N addition could influence soil microbial community composition, but the effect varied with inter-annual precipitation. The interaction between water and N affecting on the soil microbial communities was complex due to plant involvement and the inter-annual variation of climate in the semiarid grassland ecosystem.

5. Materials and methods

5.1. Site description and experimental design

This experiment was established in a typical semiarid steppe (43°26′–44°39′N, 115°32′–117°12′E, 1 265 m a.s.l.) in the Xilin River Basin, Inner Mongolia, China. The mean annual temperature is –0.4°C, ranging from –21.4°C in January to 18.5°C in July, and the mean annual precipitation is approximately 350–450 mm (with 70% falling between July and September). The rainfall in the growing season (from July to September) of 2011 and 2012 varied from 156.0 to 401.5 mm. The soil is classified as chestnut soil in the Chinese classification or calcic-orthic aridisol in U.S. classification, with a texture composed of 60% sand, 21% clay, and 19% silt. The plant community of the experimental site is dominated by *Leymus chinensis*, *Stipa grandis*, *Agropyron michnoi* and *Cleistogenes squarrosa*. The site had been used for fenced grazing for seven years and had never received water treatment or mineral N fertilizer prior to the experiment. The grazing intensity was 2.25 sheep ha^{-1}.

A randomized block design was used in this experiment, with eight treatments replicated five times. A 51 m×78 m fenced steppe ground with flat topography and relatively uniform vegetation distribution was divided into 40 plots (5 rows×8 columns, 8 m×8 m for each plot), which were separated by 1 m buffer zones. The fenced area also included marginal buffers (3.5 m). The water treatments were set as water addition (W15) and a control (W0) with no water addition. Water supplements in each year were carried out during the growing season, from June to September. The total amount of added water in each year was equal to 51.7 mm rainfall, approximately 15% of the mean annual rainfall at the study site, according to the prediction of a future increasing rate of precipitation (IPCC 2007; Liu *et al.* 2010; Feng *et al.* 2011). The water input in each month was determined based on the proportion of rainfall in each month to the total amount over the four months on average. Setting each addition to the medium amount of the daily rainfalls in this area, the amount in each month was split into halves for two applications, except for only one addition in September. The water was evenly irrigated into the blocks with a pump at a flow rate of 10 L min^{-1}. The N treatments included 0 (N0, the control), 50 (N50), 100 (N100), and 200 (N200) kg N ha^{-1} yr^{-1}. The N additions were set based on the current and next 50 years N-deposition amount (Liu *et al.* 2011). According to theory of Dise and Stevens (2005), applying a large dose of N over a short period could effectively mimic a small rate of N deposition over a long period. The total N-input amount under a dose of 200 kg N ha^{-1} yr^{-1} for 5 yr was equal to the amount of N deposition at a rate of 20 kg N ha^{-1} yr^{-1} for 50 yr, closing to the current N deposition rate in China (Qi *et al.* 2014), and setting different doses of N addition was in consideration of observing the effect of the N deposition in period of the next 50 yr, and also there might exist dose effect for N addition (Sheppard *et al.* 2013; Zhang C *et al.* 2013). The N additions were conducted using NH_4NO_3 in late June and early August every year, and the amount of each N addition was half of the N treatment level. The water treatments (W0 and W15) were assigned across the four N treatments for a total of eight treatments.

5.2. Sampling and measurements

Samplings were carried out on September 20th, 2011 and September 23rd, 2012, after the yearly water and N treatments finished. The sampling dates were at the end of growing seasons in the 2nd and 3rd year of the whole experiment, respectively, which focused on the changes of the semiarid steppe under water and N addition. Six soil cores (3.5 cm diameter×10 cm depth) were randomly taken from each plot, mixed thoroughly and immediately transferred to the laboratory using a 4°C portable icebox. The soil samples were sieved through 2-mm mesh to remove roots and small animals. In consideration of the transporting time, subsamples for the analysis of microbial C-utilization potential were analysed within 48 h. Another set of subsamples was stored at –70°C for PLFA determination as soon as possible. The rest of each sample was used to measure soil physicochemical properties. In addition, three 5 cm diameter×5 cm depth cores were taken from each plot to determine the soil bulk density (BD) at the same time. Soil temperature was measured at depths of 5 cm using a SN2202 digital thermo detector (Sinan Instruments Plant of Beijing Normal University, China). The plant AGB was measured using a clipping method by gathering the grass all above the soil surface in an area of 1 m×1 m. Plant UGB was measured by excavating a soil hole with 0.4 m width×0.4 m length×0.4 m depth within the AGB sampling area randomly, and collecting plant roots.

The soil moisture was determined by oven-drying at 105°C for 24 h. 10 g fresh soil with 50 mL distilled water

was shaken, centrifuged, and filtered to extract dissolved organic carbon (DOC), and the extracted solution was analysed using an Elementar C analyzer (Vario TOC cube, Elementar, Hanau, Germany). The total soil organic carbon (TOC) was determined by dry combustion (Vario TOC cube, Elementar, Hanau, Germany). The soil dissolved inorganic N (DIN, including NH_4^+ and NO_3^-) was extracted from 10 g fresh soil with 50 mL 2 mol L^{-1} KCl and determined using a continuous-flow ion auto-analyzer (Bran and Luebbe, Norderstedt, Germany).

The soil microbial C-utilization profile was analyzed using BIOLOG EcoPlates (BIOLOG Inc., Hayward, CA, USA). Fresh soil (10 g) suspended in 100 mL normal saline was vibrated for 20 min at 200 r min^{-1} at 25°C. The suspensions were diluted to 10^{-3} fold through a serial tenfold dilution. Each well of the EcoPlates was inoculated with 150 mL of the 10^{-3} fold dilution. The plates were incubated at 25°C without light for 10 d, and the absorbance at 590 nm was read with a microplate reader (Vmax, Molecular Devices, Oxford, UK) every 24 h during the incubation time. The readings of each well were considered after subtracting the reading of corresponding control, which was the well without any C substrates, to exclude background absorbance. The subtracted readings were set to 0 if they were negative. We used the data from the sample collected at 120 h of incubation in our study.

We evaluated the microbial community composition using PLFA (Budge *et al.* 2011). The extraction steps for PLFA were based on the procedure of Frostegård *et al.* (1991). In brief, phospholipids in the soil samples were extracted using a one-phase chloroform-methanol-phosphate buffer solution (Macnaughton *et al.* 1997), then separated from neutral lipids and glycolipids using a solid-phase extraction system (Supelco Inc., Bellefonte, PA). After methylation of the polar lipids, fatty acid methyl esters were analyzed using an Agilent 6850 Network Gas Chromatography System (GC, Agilent Technologies Co., USA). A standard EUKARY chromatographic program (MIDI, Microbial ID, Inc., Newark, DE, USA) was used to auto-identify our result, and the result was also determined quantitatively by comparing with a standard mixture ranging from C9 to C30. The nomenclature of PLFAs is based on the description of Frostegård *et al.* (1993). We used the sum of all of the PLFAs to represent the total microbial lipid biomass (ToPLFA) (Liang *et al.* 2012). The fatty acid signatures i14:0, 15:0, i15:0, a15:0, 16:0, i16:0, 16:1 2OH, 16:1ω5, 16:1ω7, 16:1ω9, i17:0, a17:0, cy17:0, 17:1ω8, 18:1ω5, and cy19:0 were used to indicate bacteria (Frostegård and Bååth 1996; Zak *et al.* 1996; Hill *et al.* 2000). Terminal branched lipid acids i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0 were used to indicate G+ bacteria, and 16:1 2OH, 16:1ω5, 16:1ω7, 16:1ω9, cy17:0, 17:1ω8, and 18:1ω5, and cy19:0 indicated G– bacteria (Frostegård and Bååth 1996; Zelles 1999). Finally, 18:2ω6 represented fungi (Vestal and White 1989; Frostegård and Bååth 1996).

5.3. Statistical analysis

The soil physicochemical properties and microbial PLFA biomass across the two years were analyzed to determine the effects of water, N and time, using a repeated-measures analysis of variance (ANOVA) and Duncan's multiple-range test in the software package SPSS 20.0 for Windows. A redundancy analysis (RDA) was performed to analyze the relationship between the edaphic physicochemical properties, plant biomass and soil microbial community using CANOCO software (ver. 4.5). A Monte Carlo permutation test (499 permutations with automatic forward selection) was used to test the statistical significance of the effects of the studied environment variables on the microbial community. The significance level was set at $P<0.05$ throughout unless otherwise stated.

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