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Interactive effects of water and nitrogen addition on soil microbial communities in a semiarid steppe

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Abstract

Aims

Better understanding of microbial compositional and physiological acclimation mechanisms is critical for predicting terrestrial ecosystem responses to global change. The aim is to assess variations in soil microbial communities under future scenarios of changing precipitation and N deposition in a semiarid grassland of northern China.

Methods

In order to explicitly estimate microbial responses, a field experiment with water and N addition was established in April 2005 and continuously conducted for 4 years. Specifically, soil microbial community composition and microbial C utilization potential were determined by phospholipid fatty acid (PLFA) and community-level physiological profiles, respectively.

Important Findings

Water addition had no effects on the PLFA concentrations of gram-positive (GP) and negative bacteria (GN), total bacteria and fungi. However, N addition caused significant reductions in the PLFA concentrations of GP, GN, total bacteria and fungi and thus decreased total PLFA of microbial communities. Moreover, there were interactive effects of water and N addition on GN/GP and the ratio of fungal to bacterial PLFA (F/B). In addition, synergistic effects were found between water and nitrogen in affecting microbial C utilization potentials, which implies that microbial C utilization potentials tend to be enhanced when both N and water availability are sufficient. Overall, the microbial responses to water and N addition support our hypothesis that water and N addition may be combined together to affect microbial communities in the semiarid grassland.

Keywords: microbial PLFA composition • microbial C utilization potentials • N addition • water addition • semiarid grassland

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INTRODUCTION

Changes in global- and regional-scale precipitation patterns along with altered run-off and water availability have been observed in terrestrial ecosystems (IPCC 2007). Precipitation has been predicted to increase in the populous regions in East and South-East Asia (IPCC 2007). As another widespread global change, nitrogen deposition and consequential N enrichment in soils become an expanding problem. In the past few years, considerable efforts have been devoted to investigate aboveground vegetation in response to precipitation change (Adler and Levine 2007; Sternberg *et al*. 1999; Yang *et al*. 2011a, 2011b) and N deposition (Hutchison and Henry 2010; Treseder 2008; van den Berg *et al*. 2011; Zhang *et al*. 2008a). However, lack of the knowledge on soil microbial communities hampers our ability to reveal the underlying mechanisms of terrestrial ecosystems in response to the future global change.

Changes in precipitation can influence soil microbial community composition (Clark *et al*. 2009; Hawkes *et al*. 2011; Steenwerth *et al*. 2005; Williams and Rice 2007) and trigger changes in the responses of microbial C utilization potentials (Bell *et al*. 2008; McCulley *et al*. 2008). Microbial community composition may be reshaped through different responses of microbial groups with specific characteristics responding to altered precipitation (Hawkes *et al*. 2011; Williams and Rice 2007). Drenovsky *et al*. (2004) found increased precipitation can stimulate the relative proportion of fungi compared to bacteria. Additionally, it has been shown that increased precipitation can stimulate microbial C utilization potentials through improving enzyme activities in dryland ecosystems (Williams

and Rice 2007). However, naturally increased or added precipitation has no positive impacts on microbial C utilization in some arid ecosystems (Bell *et al*. 2008; Sherman and Steinberger 2009). While altered soil parameters and plant compositions under manipulative or natural precipitation change can partially explain the responses of microbial community composition (Drenovsky *et al*. 2004; Williams and Rice 2007; Zhang *et al*. 2011), mechanisms underlying microbial responses to changes in precipitation are still poorly known and need further investigation.

A growing body of evidence suggests that N deposition can stimulate primary productivity (Bassin *et al*. 2007; Clark *et al*. 2007) and cause plant species loss in terrestrial ecosystems with widespread N limitation (Clark and Tilman 2008; Lebauer and Treseder 2008). However, we know little about how N deposition influence soil microbial communities. Although some study provides convincing evidence that microbial biomass can be reduced by N deposition (Treseder 2008), there are inconsistent results in different studies, indicating N deposition may have different impacts on soil microbial community composition and microbial C utilization potentials in different ecosystems (Bradley *et al*. 2006; Mamilov and Dilly 2002; Nemergut *et al*. 2008; Rousk *et al*. 2011; Zhang *et al*. 2008a). Nemergut *et al*. (2008) found N application did not alter the ratio of fungi to bacteria in a field experiment of an alpine tundra dry meadow. Bradley *et al*. (2006), however, found that 18 years N application decreased the relative proportion of fungi and increased the proportion of bacteria. In a pasture of The Netherlands, N addition also caused reductions in fungi but had no effects on bacteria (de Vries *et al*. 2006). Synthetic sheep urine did not caused fungal responses but induced changes in bacteria in a field study of grassland (Singh *et al*. 2009). As to microbial C utilization potential, N may have different effects in different ecosystems. Non-linear relationship between N addition and microbial C utilization potential was found and the optimum N addition amount was between 16 and 32 g N m^{-2} $year^{-1}$ in a semiarid grassland ecosystem (Zhang *et al*. 2008a). However, microbial C utilization potential was not affected by N addition in a desert ecosystem (Zhou *et al*. 2011).

Arid and semiarid ecosystems, with the low plant productivity, may be particularly sensitive to altered precipitation and N deposition (Austin *et al*. 2004; Christensen *et al*. 2004). Water and N are generally considered as two limiting factors in arid and semiarid ecosystems (Liu *et al*. 2009; Zhang *et al*. 2008a). Modeling studies have suggested that increasing precipitation would occur in semiarid grasslands of north China (Gao *et al*. 2003), and N deposition was also observed in these grassland ecosystems (Zhang *et al*. 2008b). The variability in water and N may induce changes in soil characteristics (Bååth and Anderson 2003; Bell *et al*. 2008; Clark *et al*. 2009; McCulley *et al*. 2008; Zhang *et al*. 2008a) and plant communities (Chung *et al*. 2007; Jaatinen *et al*. 2007; Rinnan *et al*. 2007), which would undoubtedly modify microbial composition and C utilization potentials. It has been well documented that plant community structure and composition were sensitive to climate change and N deposition in these grasslands of north China (Bai *et al*. 2010; Christensen *et al*. 2004; Yang *et al*. 2011a). Lack of underground data of soil microbial communities to altered water and N make it impossible to predict ecosystem responses to global change.

A field experiment manipulating water and N levels was conducted from 2005 to 2008 in a semiarid grassland of Duolun County of Inner Mongolia. Soil microbial community composition and microbial C utilization potential were determined. The specific object of this study is to elucidate whether water addition, N addition and their interactions affect the composition and physiology of soil microbial community in the semiarid grassland ecosystem.

MATERIALS AND METHODS

Site description and experimental design

The study was conducted in Duolun County of Inner Mongolia, China (42°02′N, 116°17′E, 1324 m a.s.l.), which belongs to the semiarid monsoon climate of moderate temperate zones. Mean annual temperature was 2.1°C and mean annual precipitation was 385.5 mm in the study site. Soil is classified as Haplic Calcisols according to the Food and Agriculture Organization (FAO) classification. Sand, silt and clay in soils were 62.8 ± 0.04, 20.3 ± 0.01 and 16.9 ± 0.01%, respectively. Mean bulk density and pH were 1.3 ± 0.02 g cm^{-3} and 7.1 ± 0.07, respectively. N deposition in this area was, on average, 20 kg ha^{-1} $year^{-1}$ from 2005 to 2006 (Zhang *et al*. 2008b). The dominant plant species of the temperate steppe include *Stipa krylovii*, *Artemisia frigida*, *Potentilla acaulis*, *Cleistogenes squarrosa*, *Allium bidentatum* and *Agropyron cristattum*.

This study was a part of the Duolun Global Change Multifactor Experiment and conducted from April 2005 to 2008. The experimental design has been described by Niu *et al*. (2009). Four pairs of 44 × 28 m plots of control and N supplement treatments were randomly assigned with four replicates per treatment. We established pairs of 15 × 10 m subplots within each plot, where the control or water supplement treatments were applied. Nitrogen supplementation (10 g N m^{-2} $year^{-1}$) was applied with urea in 2005 and NH_4NO_3 from 2006 to 2008. The water-supplemented subplots each had six sprinklers arranged in two rows in order to cover the 15 × 10 m area. For water addition treatments, 15 mm of water was added to each subplot each week from July to August, and totally, 120 mm precipitation was added (about 30% of mean annual precipitation in the study site). We have four treatments in our experiment: control (CK), N addition (N), water addition (W) and water plus N addition (WN).

Soil sampling and measurements

Soil sampling was conducted in early August from 2006 to 2008. Precipitation during the week before sampling were 0, 57.14 and 13.46 mm in 2006, 2007 and 2008, respectively. A composite sample of three soil cores (4.5 cm in diameter and 15 cm in depth) was collected from each subplot. Soil samples were sieved through 2-mm mesh to remove roots and stones

and stored at 4°C in an icebox and immediately transferred to the lab for further analysis. Subsamples from each raw soil sample were used to determine soil microbial C utilization potentials within 24 h after soil sampling. The rest of each sample was used to measure phospholipid fatty acid (PLFA) profiles and soil physicochemical properties.

The hydrometer method was used to measure sand, silt and clay contents. Fresh soil (10 g) was dried for 24 h at 105°C for determining soil moisture (SM). Soil organic C (SOC) and total N (TN) were measured using the dichromate oxidation and titration method (Kalembasa and Jenkinson 1973) and Kjeldahl digestion method with an Alpkem auto-analyzer (Kjektec System 1026 Distilling Unit, Sweden). To estimate soil dissolved inorganic N (DIN, including NH_4^+ and NO_3^-), N was extracted from 10 g of fresh soil with 50 ml 2 M KCl on a reciprocating shaker. After shaking for 1 h, the soil solution was filtered and DIN content of the filtrate was analyzed on a continuous-flow ion auto-analyzer (Scalar SAN^{plus} segmented flow analyzer, The Netherlands). Soil pH was measured using glass pH electrodes (1:2.5 soil to water ratio (w/v), Thermo Orion T20, USA).

The Biolog redox technology, a rapid, community-level measure of microbial metabolic potential, was used to characterize physiological profiles of microbial communities (Garland and Mills 1991). This method is useful for characterizing fast-growing and culturable microorganisms. In this study, Biolog EcoPlates™ with the ecologically relevant C sources were used. Carbon substrates in the EcoPlates™ can be assigned to six types, including amines, amino acids, carbohydrates, carboxylic acids, polymers and phenolic compounds (Insam 1997). Detailed procedures were described by Classen *et al*. (2003). Microbes were extracted from 4 g of fresh soil into 36 ml of 50 mM K_2HPO_4 buffer (pH = 6) by shaking for 30 min on a reciprocal shaker and settling for 30 min. The soil suspension was further diluted 10^{-3} fold into an inoculation solution (containing 0.40% NaCl and 0.03% Pluronic F-68). A volume of 150 µl diluted suspension was pipetted into each EcoPlate™ well. In order to avoid desiccation, the plates were then covered by polyethylene bags after the inoculation. All plates were incubated in the dark at 25°C. All solutions, glassware and equipment were sterilized in advance and all operations were conducted on a clean bench. Optical density (OD) was measured at 595 nm every 24 h using an enzyme-linked immunosorbent assay plate reader. We chose OD values at 96 h for further statistical analysis because the greatest response of microbial metabolic potential was predicted to occur at 96 h of incubation (Choi and Dobbs 1999; Salomo *et al*. 2009).

PLFA profiles were used to assess soil microbial community composition. PLFAs were extracted, fractionated, quantified and analyzed in the amount of fresh soil equivalent to 8 g of dry soil as described by Bossio and Scow (1998). From these samples, fatty acid methyl esters (FAMEs) were identified by chromatographic retention time using the standard EUKARY chromatographic program (MIDI, Microbial ID, Inc., Newark, DE, USA). The FAME profiles were compared with analytical standard mixtures ranging from C9 to C30 FAME (Microbial ID, Inc.) in order to determine the mole percentage of PLFAs. The nomenclature used for PLFAs is as described by Frostegård *et al*. (1993). We chose i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, 16:1ω7c, 16:1ω9c and cy19:0 as the bacterial markers (Frostegård and Bååth 1996; Ringelberg *et al*. 1997; Zak *et al*. 1996; Zelles 1997; Zogg *et al*. 1997). Terminally branched PLFAs, i.e. i15:0, a15:0, i16:0, i17:0 and a17:0, were used as indicators of gram-positive (GP) bacteria and cy17:0, cy19:0, 16:1ω7c and 16:1ω9c were used as indicators of gram-negative (GN) bacteria (Frostegård and Bååth 1996; Ringelberg *et al*. 1997; Zak *et al*. 1996; Zelles 1997; Zogg *et al*. 1997). Polyunsaturated PLFAs, i.e. 18:2ω6c and 18:3ω6c, represented fungi (Frostegård *et al*. 1993; Madan *et al*. 2002; Pinkart *et al*. 2002; Ringelberg *et al*. 1997; Vestal and White 1989; Zak *et al*. 1996; Zelles 1997; Zogg *et al*. 1997). Normal saturated PLFAs, including 15:0, 16:0 and 17:0, and monounsaturated PLFAs, including 16:1ω5c, 17:1ω8c, 18:1ω5c, 18:1ω9c and 20:1ω9c, were also taken into consideration for assessing the variability of soil microbial community composition.

Plant measurements

A permanent quadrat (1 × 1 m) was set up in each subplot in June 2005 for the measure of plant coverage and frequency. Three plant functional groups, i.e. grass, non-gramineous forb and legume, were estimated. Before measurements were conducted, the 1 × 1 m frame with 100 grids (10 × 10 cm) was placed above the canopy of each subplot. Plant coverage [i.e. grass coverage (CG), non-gramineous forb coverage (CF), and legume coverage (CL)] was estimated based on the percentage of all the grids occupied by plants. Plant group frequency (i.e. grass frequency (FG), non-gramineous forb frequency (FF), and legume frequency (FL)) was calculated according to the occurrence of plant species in the quadrats.

Statistical analyses

The net OD of each Biolog EcoPlate™ substrate well was obtained by subtracting the control well OD from the raw substrate well OD (Garland and Mills 1991). Any yielded value that was negative or <0.06 was considered to be 0 or below the system's detection limit (Miguel *et al*. 2007). The net OD values were used to analyze changes in microbial C utilization potentials. Average well color development (AWCD), which can reflect soil microbial metabolic potential, was calculated as follows (Garland and Mills 1991):

$$\mathrm{AWCD} = \sum_{i=1}^{n} (x_i - c)/31,$$

where x_i is the OD value measured at 595 nm in substrate i in each Biolog Ecoplate™, respectively, and c is the OD value of the control well.

To evaluate the responses of C source utilization to water and N addition, two indices: water response t index (WRTI) and nitrogen response t index (NRTI) were used. The WRTI is defined as

$$\mathrm{WRTI} = (\bar{X}_{W+} - \bar{X}_{W-})/S_{TOT},$$

where $\bar{X}_{W-}$ and $\bar{X}_{W+}$ are the means of C source utilization parameters in the ambient and water addition plots, respectively, and S_{TOT} is the total standard error.

The NRTI is defined as

$$\mathrm{NRTI} = (\bar{X}_{N+} - \bar{X}_{N-})/S_{TOT},$$

where $\bar{X}_{N-}$ and $\bar{X}_{N+}$ are the means of C source utilization parameters without and with N addition treatments and S_{TOT} is the total standard error. In order to show the impact of N addition on WRTI and water addition on NRTI of C source utilization parameters of microbial communities, WRTI of control (N−) and N added (N+) treatments as well as NRTI of ambient (W−) and water addition (W+) treatments were calculated, respectively.

Soil physicochemical properties and microbial parameters were analyzed using a three-way analysis of variance (ANOVA) using software R 2.9.2 and Duncan's multiple-range test using software SPSS 13.0. Redundancy analysis (RDA) was conducted using the vegan package of R to assess the variability in microbial community composition under water and N addition. The function 'envfit' in the vegan package of R was used to determine correlations of microbial community composition with soil and plant parameters across 2006–2007 and estimate association of microbial C utilization potentials with soil and plant parameters across 2006–2008.

RESULTS

Soil parameters

Water addition significantly enhanced SOC (Table 1). Neither water nor N addition had effect on TN across the experimental years (Table 1). Water addition did not affect DIN, but N addition increased DIN significantly in plots with N addition (N) and water plus N addition (WN, Table 1). In contrast to DIN, soil pH decreased under N addition and water plus N addition (Table 1). Water addition and water plus N addition both elevated SM (Table 1). N addition did not alter SM across the 3 years (Table 1).

Soil microbial community compositions

The PLFA concentration of GN bacteria declined with year ($P < 0.05$) and thus led to the lower GN/GP ratio ($P < 0.001$) in 2007 than in 2006 (Table 2). The PLFA concentrations of other microbial groups were, however, independent of year (all $P > 0.05$, Table 2). Water addition had no effects on the PLFA concentrations of GP bacteria, GN, total bacteria and fungi (all $P > 0.05$, Table 2). However, N addition significantly reduced the PLFA concentrations of GP ($P < 0.01$), GN ($P < 0.05$), total bacteria ($P < 0.01$) and fungi ($P < 0.05$) and consequentially caused decrease in total PLFA ($P < 0.01$) across the 2 years (Table 2). Furthermore, marginal interactions between water and N addition affecting the PLFA of GN and fungi were observed (both $P < 0.10$), which coupled with significant interaction effects of water and N addition on GN/GP and the ratio of fungal to bacterial PLFA (F/B) (both $P < 0.05$, Table 2). Water addition counteracted the reduced trends of GN/GP and F/B caused by N addition (Table 2).

Soil microbial C utilization potentials

The main effects of water and N addition and their interactions were observed on the AWCD ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively, Table 3). The utilization of amino acids, carboxylic acids and phenolic compounds contribute to the variability in AWCD under combined water and N addition in 2006 (Table 3). The utilization of carbohydrates and carboxylic acids in 2007 and all of the six C source types in 2008 contribute to changes in AWCD (Table 3).

Table 1: effects of water and N addition on soil physicochemical parameters (mean ± SE, $n = 4$)

	pH	SM (%)	DIN (mg kg^{-1})	SOC (g kg^{-1})	TN (g kg^{-1})
CK	7.19 ± 0.02^{a}	0.13 ± 0.01^{b}	12.90 ± 0.54^{b}	15.52 ± 0.43^{a}	1.53 ± 0.06^{a}
W	7.23 ± 0.03^{a}	0.17 ± 0.01^{a}	15.09 ± 0.74^{b}	17.09 ± 0.83^{a}	1.68 ± 0.14^{a}
N	6.61 ± 0.06^{b}	0.13 ± 0.01^{b}	36.58 ± 3.54^{a}	15.06 ± 0.55^{a}	1.49 ± 0.07^{a}
WN	6.73 ± 0.06^{b}	0.15 ± 0.01^{ab}	37.45 ± 3.69^{a}	15.71 ± 1.05^{a}	1.59 ± 0.10^{a}
Significance of three-way ANOVA (P value)					
N	**<0.001**	0.494	**0.002**	0.395	0.593
W	0.064	**<0.001**	0.549	**0.022**	0.068
Y	0.336	**<0.001**	0.074	0.662	**<0.001**
$N \times W$	0.315	0.382	0.795	0.320	0.722
$N \times Y$	**<0.001**	0.578	0.113	0.237	0.445
$W \times Y$	0.956	**<0.001**	0.565	0.803	0.612
$N \times W \times Y$	0.914	0.527	0.809	0.542	0.839

Results from three-way ANOVA were shown in the lower panels of the table, and significant P values were shown in bold. CK, control; N, N addition; W, increased precipitation; WN, N addition plus increased precipitation; pH, soil pH. Different letters denote significant differences at $P < 0.05$ (Duncan's multiple-range test).

Table 2: effects of water and N addition on the PLFAs of microbial communities across 2006 and 2007 (mean ± SE, $n = 4$)

	GP	GN	Bacteria	Fungi	GN/GP	F/B	Total PLFA
2006							
CK	403 ± 12^{a}	279 ± 23^{a}	682 ± 35^{a}	83 ± 10^{a}	0.69 ± 0.04^{ab}	0.12 ± 0.01^{a}	3340 ± 398^{a}
W	394 ± 30^{a}	273 ± 19^{a}	667 ± 46^{a}	79 ± 9^{a}	0.70 ± 0.04^{ab}	0.12 ± 0.01^{a}	2733 ± 225^{a}
N	167 ± 15^{b}	98 ± 11^{b}	265 ± 18^{b}	29 ± 4^{b}	0.60 ± 0.08^{b}	0.11 ± 0.01^{a}	1189 ± 67^{b}
WN	159 ± 29^{b}	130 ± 15^{b}	290 ± 43^{b}	41 ± 7^{b}	0.85 ± 0.06^{a}	0.14 ± 0.01^{a}	1237 ± 163^{b}
2007							
CK	427 ± 18^{a}	248 ± 25^{a}	675 ± 37^{a}	81 ± 6^{a}	0.58 ± 0.05^{a}	0.12 ± 0.00^{ab}	3412 ± 58^{a}
W	395 ± 27^{a}	195 ± 56^{a}	590 ± 78^{a}	76 ± 19^{a}	0.49 ± 0.12^{a}	0.13 ± 0.02^{a}	2767 ± 207^{b}
N	175 ± 13^{b}	100 ± 7^{b}	275 ± 20^{b}	24 ± 2^{b}	0.57 ± 0.01^{a}	0.09 ± 0.00^{b}	1192 ± 78^{c}
WN	175 ± 20^{b}	103 ± 9^{b}	278 ± 28^{b}	31 ± 5^{b}	0.60 ± 0.02^{a}	0.11 ± 0.02^{ab}	1110 ± 145^{c}
Significance of three-way ANOVA (P value)							
N	**0.003**	**0.020**	**0.006**	**0.022**	0.550	0.403	**0.008**
W	0.292	0.985	0.536	0.252	0.050	**0.007**	**0.010**
Y	0.222	**0.013**	0.422	0.265	**<0.001**	0.079	0.862
$N \times W$	0.484	0.070	0.157	0.087	**0.012**	**0.034**	**0.011**
$N \times Y$	0.923	0.098	0.380	0.335	0.913	**0.014**	0.504
$W \times Y$	0.792	0.116	0.300	0.927	**0.014**	0.652	0.774
$N \times W \times Y$	0.520	0.806	0.614	0.557	0.261	0.479	0.730

Results from three-way ANOVA were shown in the lower panels of the table, and significant P values were shown in bold. CK, control; N, N addition; W, increased precipitation; WN, N addition plus increased precipitation. GP, GN, bacteria and fungi shown in the table refer to the PLFA content (nmol g^{-1} C) of GP bacteria, GN bacteria, bacteria and fungi, respectively. GN/GP and F/B are the ratios of GN to GP bacterial PLFA and fungi to bacteria PLFA, respectively. Different letters denote significant differences at $P < 0.05$ (Duncan's multiple-range test).

NRTI of microbial communities using the six carbon categories (i.e. amines, amino acids, carbohydrates, carboxylic acids, polymers, and phenolic compounds) with and without water addition are shown in Fig. 1A. All C source categories tested were near or above the line of $y = x$. The distances to the 1:1 line, which represents the strength of water impact on responses of microbial carbon uses to nitrogen, increased with year from 2006 to 2008 (Fig. 1B). WRTI of microbial communities showed similar trends to NRTI (Fig. 1C). Accumulated effects of nitrogen on responses of microbial C utilization to water were also found during the experiment period (Fig. 1D).

Correlations of microbial community composition and C utilization potentials with soil and plant parameters

The results of RDA were shown in Fig. 2, and the first principal component (RDA1) and the second principal component (RDA2) accounted for 73.0 and 6.8% of total variance, respectively. According to the result of RDA, microbial PLFA composition under N addition but not under water addition separated from the control (Fig. 2), which coincided with the results of ANOVA analysis on microbial groups. Soil microbial PLFA composition was correlated with TN ($r = 0.58$, $P = 0.003$), DIN ($r = 0.86$, $P < 0.001$), soil pH ($r = 0.86$, $P < 0.001$), FL ($r = 0.45$, $P = 0.034$), FG ($r = 0.47$, $P = 0.023$), FF ($r = 0.49$, $P = 0.016$) and CG ($r = 0.65$, $P < 0.001$) (Fig. 2 and Table 4). Similarly, soil microbial C utilization potentials were related to SM ($r = 0.49$, $P = 0.003$), pH ($r = 0.55$, $P < 0.001$), DIN ($r = 0.59$, $P < 0.001$), FF ($r = 0.42$, $P = 0.013$), FG ($r = 0.38$, $P = 0.030$) and CG ($r = 0.54$, $P = 0.001$) (Table 4).

DISCUSSION

Effects of water addition on the PLFA concentrations of the major soil microbial groups

Soil water availability is crucial for growth and survival of soil microbes and considered to be able to trigger the variability of microbial community composition (Bell *et al*. 2008; Clark *et al*. 2009; McCulley *et al*. 2008; Williams and Rice 2007). However, impacts of water addition on the PLFA concentrations of GP, GN, bacteria and fungi were insignificant regardless of dramatic increase in water availability across 2006 and 2007 (Fig. 2 and Table 2), which indicate that water addition may not alter the biomass of specific microbial groups within a few years in the grassland ecosystem. The results of RDA analysis further confirmed the above argument (Fig. 2). In a field experiment carried out from 2006 to 2009 in the same area, non-responsive microbial PLFA composition to water addition was observed (Zhang *et al*., unpublished data). The results also suggest a possible occurrence of the resistance or resilience of microbial biomass to the future scenarios of precipitation shifts in semiarid grasslands of northern China (Zhang and Han 2008). A pronounced response of microbial community composition may depend on accumulative effects of water regimes, as

Table 3: effects of water and N addition on microbial utilization potential of six C source types and the AWCD across the 3 years from 2006 to 2008 (mean ± SE, $n = 4$)

	Amines	Amino acids	Carbohydrates	Carboxylic acids	Polymers	Phenolic compound	AWCD
2006							
CK	0.29 ± 0.03^{a}	0.43 ± 0.05^{b}	0.47 ± 0.14^{a}	0.37 ± 0.07^{b}	0.58 ± 0.06^{a}	0.38 ± 0.06^{b}	0.44 ± 0.08^{b}
W	0.20 ± 0.01^{b}	0.48 ± 0.04^{b}	0.57 ± 0.15^{a}	0.43 ± 0.08^{b}	0.64 ± 0.06^{a}	0.42 ± 0.05^{b}	0.50 ± 0.08^{ab}
N	0.29 ± 0.01^{a}	0.49 ± 0.01^{b}	0.66 ± 0.05^{a}	0.55 ± 0.02^{ab}	0.62 ± 0.02^{a}	0.55 ± 0.03^{ab}	0.57 ± 0.02^{ab}
WN	0.30 ± 0.04^{a}	0.61 ± 0.03^{a}	0.79 ± 0.07^{a}	0.67 ± 0.05^{a}	0.70 ± 0.04^{a}	0.63 ± 0.07^{a}	0.67 ± 0.04^{a}
2007							
CK	0.36 ± 0.09^{a}	0.46 ± 0.08^{a}	0.36 ± 0.08^{b}	0.41 ± 0.08^{b}	0.49 ± 0.09^{a}	0.28 ± 0.11^{a}	0.40 ± 0.08^{b}
W	0.15 ± 0.07^{a}	0.41 ± 0.06^{a}	0.40 ± 0.11^{b}	0.35 ± 0.04^{b}	0.48 ± 0.03^{a}	0.19 ± 0.02^{a}	0.37 ± 0.06^{b}
N	0.13 ± 0.02^{a}	0.43 ± 0.04^{a}	0.57 ± 0.05^{b}	0.39 ± 0.04^{b}	0.54 ± 0.03^{a}	0.15 ± 0.01^{a}	0.44 ± 0.03^{b}
WN	0.26 ± 0.09^{a}	0.50 ± 0.07^{a}	0.91 ± 0.10^{a}	0.62 ± 0.04^{a}	0.67 ± 0.09^{a}	0.24 ± 0.07^{a}	0.65 ± 0.06^{a}
2008							
CK	0.37 ± 0.04^{b}	0.50 ± 0.05^{b}	0.38 ± 0.09^{b}	0.42 ± 0.05^{b}	0.63 ± 0.05^{b}	0.46 ± 0.08^{b}	0.45 ± 0.06^{b}
W	0.29 ± 0.04^{b}	0.43 ± 0.03^{b}	0.34 ± 0.05^{b}	0.39 ± 0.04^{b}	0.51 ± 0.04^{b}	0.30 ± 0.09^{bc}	0.38 ± 0.04^{b}
N	0.24 ± 0.07^{b}	0.37 ± 0.05^{b}	0.39 ± 0.10^{b}	0.34 ± 0.05^{b}	0.40 ± 0.06^{b}	0.19 ± 0.03^{c}	0.35 ± 0.06^{b}
WN	0.74 ± 0.02^{a}	0.92 ± 0.08^{a}	1.16 ± 0.22^{a}	0.84 ± 0.14^{a}	1.15 ± 0.14^{a}	0.76 ± 0.07^{a}	0.99 ± 0.13^{a}
Significance of three-way ANOVA (P value)							
N	0.114	0.060	**0.005**	**0.004**	**0.021**	0.120	**0.009**
W	0.213	**0.001**	**0.001**	**0.001**	**0.001**	**0.020**	**<0.001**
Y	**<0.001**	**0.026**	0.616	0.334	**0.037**	**<0.001**	0.154
$N \times W$	**<0.001**	**<0.001**	**0.003**	**0.001**	**<0.001**	**<0.001**	**<0.001**
$N \times Y$	**0.048**	0.122	0.328	0.637	0.281	**0.045**	0.467
$W \times Y$	**0.006**	**0.010**	0.216	0.195	**0.021**	0.064	0.053
$N \times W \times Y$	**0.024**	**0.001**	**0.032**	0.052	**<0.001**	**0.001**	**0.004**

Results from three-way ANOVA were shown in the lower panels of the table, and significant P values were shown in bold. CK, control; N, N addition; W, increased precipitation; WN, N addition plus increased precipitation. Different letters denote significant differences at $P < 0.05$ (Duncan's multiple-range test).

observed in a field experiment with 7 years precipitation manipulation (Williams and Rice 2007). Little changes in soil bacteria were observed during the first 5 years of a precipitation experiment in a grassland ecosystem, but differences emerged among treatments during 6th and 7th years (Cruz-Martínez *et al*. 2009). In our study, the relative stable contents of bacteria and fungi in different water treatments cannot exclude a possibility of accumulative effects of water manipulation on microbial community composition in the grassland ecosystem.

Negative effects of N addition on the PLFA concentrations of microbial groups and a potential combined effect of water and N addition on microbial PLFA composition

Significant responses of microbial biomass and community composition to N addition have been found in most of the N application studies (Allison and Martiny 2008; Rousk *et al*. 2011; Treseder 2008). In our study, the reductions in the PLFA concentrations of GP, GN, total bacteria and fungi and consequential the decreasing trend of GN/GP and F/B in response to N addition were observed across 2006 and 2007 (Table 2). This indicates that N addition would decrease the bacterial and fungal biomass (PLFA) and alter soil microbial PLFA composition. Out results are consistent with observations in the150-year 'ParkGrass' UK grassland experiment (Rousk *et al*. 2011), in which high N application (14.4 g N m^{-2} $year^{-1}$) induced decrease in microbial biomass markers (bacterial, fungal and total PLFA). In some other experiments based on PLFA analysis (Bradley *et al*. 2006) and molecular data (de Vries *et al*. 2007), the reductions in microbial biomass were also found under N addition. Our results of RDA analysis also indicate that the variability of microbial PLFA composition could be induced by N addition in the semiarid grassland (Fig. 2).

Bacteria and fungi usually show different sensitivities to N addition, and decreases in F/B ratios were observed in many researches (e.g. de Vries *et al*. 2006; Rousk *et al*. 2011). However, dose effects were also found in these studies, i.e. significant decreases in F/B were found only in higher nitrogen treatments, and no change or marginal changes were found in lower N treatments. In our study, N addition with10 g N m^{-2} $year^{-1}$ caused a slight reduction in F/B, which indicates there were no selective pressures on soil bacteria and fungi. The amounts of N addition may partly interpret changes in F/B in our experiment.

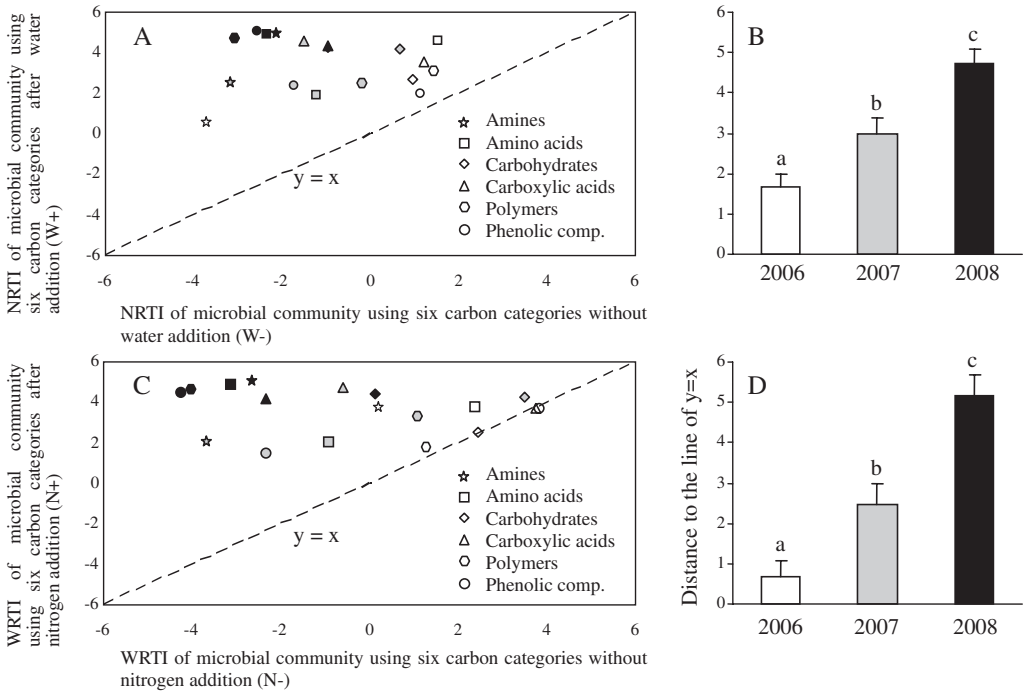

Figure 1: (**A**) NRTI under water-limited (W−) and -improved conditions (W+) and (**C**) WRTI under nitrogen-limited (N−) and -improved conditions (N+) of the microbial community. Six carbon categories were used on the Biolog plates. Yellow, blue and red showed the years 2006, 2007 and 2008, respectively. Distances of each carbon category to no interactive effects between nitrogen and water (the dash line, $y = x$) are shown for water treatments and nitrogen treatments in **B** and **D**, respectively. Different letters denote significant differences at $P < 0.05$.

It has been seldom documented whether and how water and N application had an interactive effect on soil microbial community composition, although combined effects of them on microbial enzyme activities and C utilization have been reported in some ecological researches (Grizzle *et al*. 2009; Stursova *et al*. 2006). In our study, combined water and N effects on GN/GP and F/B across the 2 years, suggest a potential interaction between water and N in affecting microbial PLFA composition. High water availability may relieve the declined trend of GN/GP and F/B under N addition.

N addition may induce great changes in soil environments (e.g. soil pH, Zhang *et al*. 2008a) and plant communities (Bassin *et al*. 2007; van den Berg *et al*. 2011). According to RDA (Fig. 2), RDA1 that accounted for most variance of microbial PLFA composition, reflected soil and plant changes with altered N availability caused by N addition. The results suggest a continuity of microbe–soil–plant in response to N addition. Besides by the pathway of altering soil pH, N addition may indirectly influenced microbial PLFA composition through modifying plant communities. Plant can compete with microorganisms for soil N (Kaye and Hart 1997). N addition may destroy the competitive balance between microbes and plants through stimulating plant growth and consequential more N absorption of the plant community in our study site (Yang *et al*. personal communication). The quality of plant C input into soils was also suggested as a determinant of microbial composition (Henriksen and Breland 1999). The changes in plant community, especially the decease of legumes and increase of grasses accompanying with N addition (see Fig. 3 and Table 1 in Yang *et al*. 2011a), may cause the decrease in the quality of plant C and will in turn affect the composition of soil microbial community.

Interactions between water and N addition affecting soil microbial C utilization potentials

Water and nitrogen are considered as two main limited resources in the semiarid grassland ecosystem, controlling plant growth (Bai *et al*. 2010; Yang *et al*. 2011a) and also microbial activities (Zhang *et al*. 2008a; Zhang *et al.* unpublished data). However, how the combination of nitrogen and water affect microbial community is still unknown. Soil microbial C utilization potentials were responsive to water and N variability in the present study, especially to combined water and N manipulation (Fig. 1 and Table 3), suggesting conjunct roles of water and N in regulating microbial C utilization potentials in the semiarid grassland. In natural ecosystems, multiple factors may affect the composition and function of microbial communities and the interactions among factors may modify the response of microbial communities to individual factors.

If there are two important resources in an ecosystem such as water and nitrogen in the grassland ecosystem, improvement of one resource may have positive, negative or neutral effects on the responses of microbial community to another resource. Our results showed that water and nitrogen affect the C source

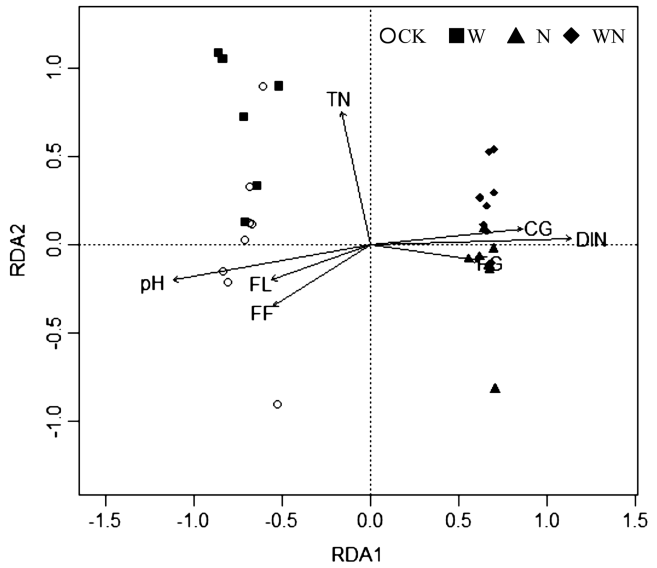

Figure 2: RDA of soil microbial community composition as measures of PLFA profiles. RDA1 and RDA2 account for 73.0 and 6.8% of total variance. Site scores were shown in the figure. Hollow circle, solid square, solid triangle and solid diamond represent the control (CK), increased precipitation (W), N addition (N) and increased precipitation plus N addition (WN). Soil and plant parameters that significantly correlated with microbial community composition were shown.

Table 4: correlations between the PLFA or CLPP and the properties: SM, soil pH, DIN, the frequency and coverage of legume (FL and CL), grass (FG and CG) and forb (FF and CF)

	CLPP		PLFA	
	r	*P* value	*r*	*P* value
Soil variables				
SOC	0.23	0.297	0.19	0.585
TN	0.26	0.206	0.58	0.003
DIN	0.59	<0.001	0.86	<0.001
pH	0.55	<0.001	0.86	<0.001
SM	0.49	0.003	0.20	0.570
Plant variables				
FL	0.33	0.078	0.45	0.034
FG	0.38	0.030	0.47	0.023
FF	0.42	0.013	0.49	0.016
CL	0.31	0.100	0.36	0.134
CG	0.54	0.001	0.65	<0.001
CF	0.30	0.117	0.35	0.147

P values were based on 9999 permutations. PLFA correlations are based upon the data from 2006 to 2007. CLPP correlations are based upon the data from 2006 to 2008.

utilization of soil microbial communities through bilateral positive interactions (Fig. 1 and Table 3). That is, the C source utilization of soil microbial communities are more sensitive to water addition when in conjunction with N addition and also more sensitive to N addition when in conjunction with water addition. We should also notice that most values of response *t* index under water- and nitrogen-limited conditions were near zero or even negative (see the *X* values in Fig. 1A and C), which indicates that N did not have a positive effect on C source utilization under water-limited conditions and water also did not have a positive effect under N-limited conditions. Similar synergistic effects between precipitation and nitrogen were also found on microbial enzyme activities (Stursova *et al*. 2006) and microbial C utilization potentials (Grizzle *et al*. 2009).

While several hypotheses such as 'Liebig's law of the minimum' and 'multiple limitation hypothesis' have been useful to describe responses of species and community to multiple resources (e.g. Rubio *et al*. 2003), most studies concerned on up-ground parts of ecosystem and the mechanisms controlling microbial community responses to multiple resources are still poorly understood. Our results showed multiple limitation hypothesis might be more suitable to explain the response mechanism of C source utilization of microbial community to water and nitrogen. Dual limitation factors of N and water rather than one 'minimal' limitation factor may control the C source utilization of microbial communities in the grassland ecosystem that we studied and synergistic effects may exist between N and water in regulation of microbial C utilization.

CONCLUSIONS

In conclusion, the PLFA concentrations of the major microbial groups was not responsive to water addition but significantly affected by N addition accompanying with reductions in the PLFA biomass markers of GP and GN bacteria, total bacteria and fungi and total PLFA of microbial communities. Moreover, effects of N addition on GN/GP and F/B interacted with water addition. Water addition counteracted the reductions in GN/GP and F/B caused by N addition. The findings suggest that water and N manipulations likely combined together to influence microbial PLFA composition in the semiarid grassland. The utilization potentials of six C categories and AWCD were commonly influenced by water plus N addition. The results showed bilateral positive interactions between water and nitrogen affecting microbial C utilization potentials, which implied soil microbial communities would be enhanced in soil C resource utilization when both N and water conditions are sufficient. Correlations between microbial community composition or C utilization potentials and soil and plant parameters suggest the continuity of microbe–soil–plant in response to water and N addition.

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