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Precipitation modifies the effects of warming and nitrogen addition on soil microbial communities in northern Chinese grasslands

Naili Zhang [a], Shiqiang Wan [b], Jixun Guo [c], Guodong Han [d], Jessica Gutknecht [e], Bernhard Schmid [f], Liang Yu [a], Weixing Liu [a], Jie Bi [g], Zhen Wang [d], Keping Ma [a, *]

[a] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, The Chinese Academy of Sciences, Xiangshan, Beijing 100093, China
[b] Key Laboratory of Plant Stress Biology, College of Life Sciences, Henan University, Kaifeng, Henan 475004, China
[c] Institute of Grassland, Northeast Normal University, Changchun, Jilin 130024, China
[d] College of Ecology and Environmental Science, Inner Mongolia Agricultural University, Huhhot, Inner Mongolia 010018, China
[e] Department of Soil Ecology, Helmholtz Centre for Environmental Research – UFZ, D-06120 Halle (Saale), Germany
[f] Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland
[g] Inner Mongolia University of Science and Technology, Baotou, Inner Mongolia 014010, China

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ABSTRACT

Terrestrial ecosystems experience simultaneous shifts in multiple drivers of global change, which can interactively affect various resources. The concept that different resources co-limit plant productivity has been well studied. However, co-limitation of soil microbial communities by multiple resources has not been as thoroughly investigated. Specifically, it is not clearly understood how microbial communities respond to shifts in multiple interacting resources such as water, temperature, and nitrogen (N), in the context of global change. To test the effects of these various resources on soil microorganisms, we established a field experiment with temperature and N manipulation in three grasslands of northern China, where there is a decrease in precipitation from east to west across the region. We found that microbial responses to temperature depended upon seasonal water regimes in these temperate steppes. When there was sufficient water present, warming had positive effects on soil microorganisms, suggesting an interaction between water and increases in temperature enhanced local microbial communities. When drought or alternating wet–dry stress occurred, warming had detrimental effects on soil microbial communities. Our results also provide clear evidence for serial co-limitation of microorganisms by water and N at the functional group and community levels, where water is a primary limiting factor and N addition positively affects soil microorganisms only when water is sufficient. We predict that future microbial responses to changes in temperature and N availability could be seasonal or exist only in non-drought years, and will strongly rely on future precipitation regimes.

1. Introduction

Soil microorganisms require multiple essential resources (e.g., water, carbon (C), nitrogen (N), and phosphorus (P)) to produce energy and synthesize cellular macromolecules, and they depend on environmental factors such as temperature, soil moisture content, pH, and salinity (Atlas and Bartha, 1998). It is crucial to better understand how these resources and environmental constraints influence soil microbial communities in the context of global change. This is attributable to the fact that global change drivers simultaneously and interactively alter multiple resources (IPCC, 2007; Castro et al., 2010; Rousk et al., 2011) and potentially modify the microbial mediation of ecosystem C and nutrient cycling. Thus, the influence of resources and environmental constraints on microbial communities may have far-reaching effects on ecosystem feedback in relation to global change (de Vries et al., 2006, 2007; Allison et al., 2010; Feng et al., 2010; Dijkstra et al., 2011; Zhang et al., 2011).

Optimal foraging theory predicts that organisms should allocate energy in such a way that they are equally limited by different resources, in order to maximize net resource uptake per unit time

* Corresponding author. State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, The Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, China. Tel.: +86 10 62836223; fax: +86 10 82599518.
E-mail address: kpma@ibcas.ac.cn (K. Ma).

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(Bloom et al., 1985; Chapin et al., 1987). There have been various theories about how the simultaneous limitation of multiple resources or co-limitation occurs, including independent co-limitation at the level of individual organisms (Saito et al., 2008), populations, or communities (Harpole et al., 2011). These theories serve as a framework in which to investigate co-limitation by multiple resources, which is defined as an instance when organisms are successfully able to utilize one resource depending upon sufficient supply of another resource (Gleeson and Tilman, 1992; Saito et al., 2008; Harpole et al., 2011). The definition of co-limitation has developed from single resource limitation, as stated in Liebig's law of the minimum (Liebig, 1855). At the community level, Harpole et al. (2011) classified categories as independent, simultaneous, or serial co-limitation. The community-level co-limitation of microorganisms may simply reflect biochemical-level co-limitation only when all of the species in a community are co-limited by the same resources. However, community-level co-limitation by multiple resources is usually quite complicated to detect or interpret, because of species-specific physiological needs or adaptations (Schimel et al., 2007), and it can be especially difficult considering shifts in multiple nutrients and environmental constraints due to global change.

Water occupies 70–90% of the cell mass of microorganisms (Atlas and Bartha, 1998) and often co-limits soil microbial communities along with other factors. A body of evidence has demonstrated that warming may interact with water fluctuation to affect soil microbial communities in arid and semi-arid ecosystems (Allison and Treseder, 2008; Liu et al., 2009; Nielsen and Ball, 2015). Warming can reduce soil microbial biomass and activities in the cell by inducing water-stress on microorganisms (Zhang et al., 2005; Rinnan et al., 2007, 2009; Liu et al., 2009). Alternatively, warming may suppress microbial growth indirectly by reducing plant growth and consequently providing less nutrient and energy input into soils (Allison and Treseder, 2008; Hoeppner and Dukes, 2012). In contrast, improved water availability may negate the negative effect of warming; for instance, there is a counter-intuitive increase in soil moisture under warming conditions, which is driven indirectly by plant senescence (Zavaleta et al., 2003). As such, the dependence of warming effects upon water fluctuation can be predicted, especially in arid or semi-arid ecosystems.

Similarly, N effects on soil microbial communities may strongly depend upon water regimes (Herman et al., 1993; Grizzle et al., 2010; Bi et al., 2011). A previous study showed that the addition of water and N had a synergistic effect on the population of N_2-fixing bacteria (Herman et al., 1993). High amounts of water availability can enhance the responses of soil microbial communities to N deposition as well (Grizzle et al., 2010). These findings are not surprising because water physically influences microbially mediated N processes (Schimel et al., 1996). Moreover, N addition can exert a pronounced influence on soil microbial activities, but only under high water availability (Bi et al., 2011), suggesting a serial co-limitation of water and N. Additionally, microbial communities from different climate regimes, and therefore diverse evolutionary histories of adaptation, may respond differently to changes in either water or temperature fluctuations (Schimel et al., 2007; Balser and Wixon, 2009). We predicted that the serial community-level co-limitation of water and N generally exists in temperate grasslands, given that water is a predominantly limiting factor in temperate ecosystems (LeBauer and Treseder, 2008; Liu et al., 2009) and is usually linked to N availability or cycling rates (Schimel et al., 1996).

To test whether and how soil microbial communities are co-limited by multiple resources, which dramatically and simultaneously shift in the context of global change, we established an experiment in which we were able to manipulate warming and N addition continually since April 2006 in three temperate grasslands of northern China. The three temperate grasslands are along a decreasing natural precipitation gradient and include a meadow (440 mm annual rainfall), a semi-arid steppe (380 mm annual rainfall), and a desert steppe (313 mm annual rainfall). To further compare global change in natural weather regimes, we also carried out measurements during two years that had contrasting levels of precipitation. This allowed us to examine how both local climate regimes and natural fluctuations in weather from year to year alter microbial responses to temperature and N addition. We hypothesized that these global change drivers would alter multiple resources, among which (1) water and temperature could interactively influence soil microorganisms, (2) water and N could serially co-limit soil microorganisms at the community level, and (3) differences could exist in both microbial responses and in interactions between treatments among the three temperate steppes. We theorized that this would be due to water deficiency in the semi-arid and the desert steppes and the local adaption of microbial communities to the two ecosystems regularly affected by droughts.

2. Materials and methods

2.1. Field sites

We conducted field experiments in three temperate grasslands of northern China. Concurrent changes in temperature, precipitation, and N deposition were recorded in these temperate zones of northern China (IPCC, 2007; Liu et al., 2007, 2010; He et al., 2007; Zhang et al., 2008b). Three grasslands, including a meadow steppe, a semi-arid steppe, and a desert steppe, were included in this experiment. The meadow steppe is located in Changling County in the southwestern region of the Songnen Plain of Northeast China, and stands at the eastern edge of the Eurasian steppe (Fig. 1 and Table 1). The semi-arid and desert steppes are situated in Duolun County and the Siziwang Banner of Inner Mongolia, respectively (Fig. 1 and Table 1). All three steppes are in a continental temperate climate. There is a gradual decrease in the mean annual precipitation from the meadow to the semi-arid steppe, then finally to the desert steppe (Fig. 1 and Table 1). The soils in the meadow steppe, semi-arid steppe and desert steppe are characterized as Chernozem with high sodic and saline content, Haplic Calcisols and Kastanozem, respectively, according to the Food and Agriculture Organization (FAO) classification (Table 1). In comparison with the meadow and the desert steppe, the soil of the semi-arid steppe has higher concentrations of organic C and N, but a lower pH (Table 1). The dominant plant species in the meadow steppe are *Leymus chinensis*, *Puccinellia tenuiflora*, *Calamagrostis epigejos*, *Chloris virgata*, and *Suaeda glauca*. The vegetation of the semi-arid steppe is dominated by *Stipa krylovii*, *Artemisia frigida*, *Potentilla acaulis*, *Cleistogenes squarrosa*, *Allium bidentatum*, and *Agropyron cristatum*, while that of the desert steppe is dominated by *Stipa breviflora*, *A. frigida* and *Cleistogenes mutica*.

2.2. Experimental design

The experiment was established in April 2006 and lasted for 4 years. Three sites (a meadow, a semi-arid and a desert steppe), were used to set up plots in which the temperature or N concentrations could be manipulated. In the semi-arid steppe, we used a randomized block design with 6 treatments including: control (C), continuous warming per day (W, 24 h), N addition (N), warming plus N addition (WN), daytime warming (6:00 a.m.–6:00 p.m., 12 h) and nighttime warming (6:00 p.m.–6:00 a.m., 12 h) (Xia et al., 2009). All treatments were replicated 6 times. Thirty-six 3 × 4 m

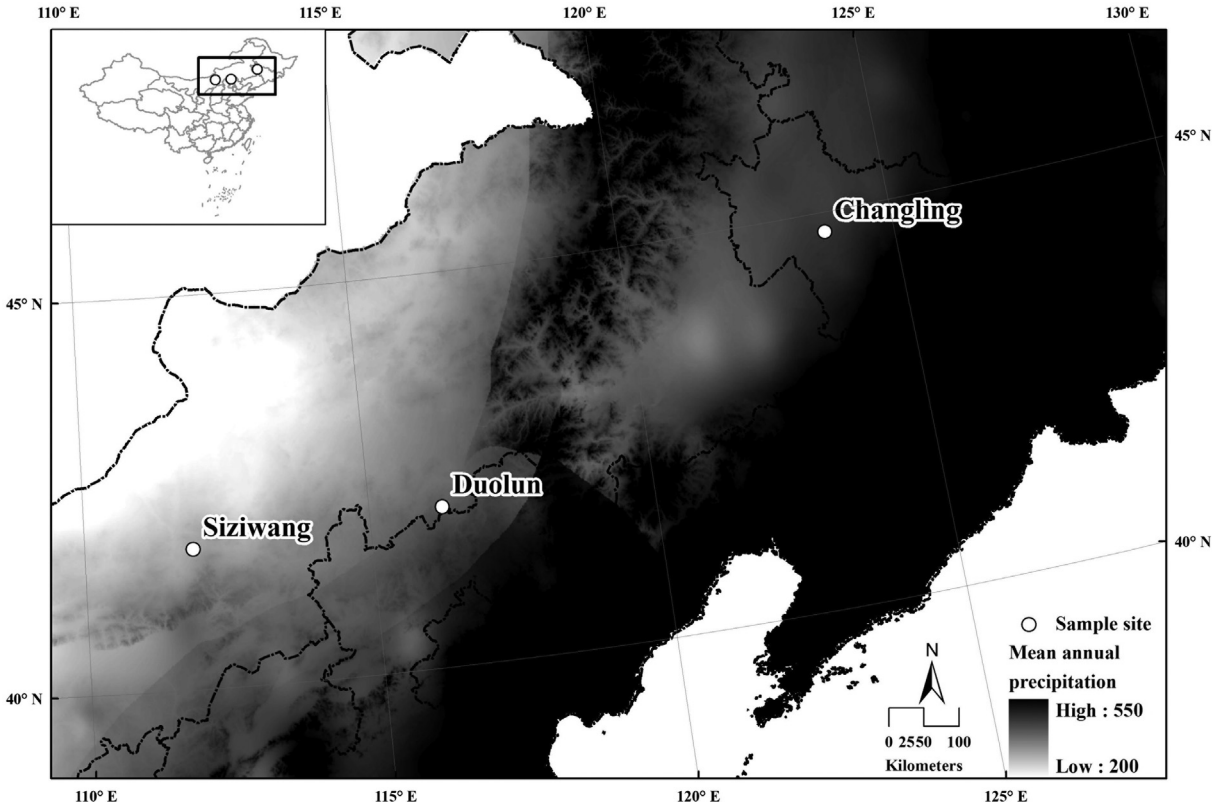

Fig. 1. The locations and long-term mean annual precipitation (mm) map of the three grasslands in northern China.

Table 1
Geographic, climate and soil parameters in the three temperate steppes. MAT = mean annual air temperature from 1959 to 2007, MAP = mean annual precipitation from 1959 to 2007. Seasonal precipitation in 2006 and 2007 refers to precipitation in the growing season from May to October of each year. The means of soil parameters (mean ± standard error, n = 12) in the control plots across 2006 and 2007 are shown in the table.

Sites	Meadow steppe	Semi-arid steppe	Desert steppe
Geographic coordinates	44°.45′ N, 123°.45′ E	42°.02′ N, 116°.17′ E	41°.47′ N, 111°.53′ E
Elevation (m a.s.l)	160	1324	1456
MAT (°C)	5.6	2.4	3.6
MAP (mm)	440	380	313
Seasonal precipitation in 2006 (mm)	348	404	193
Seasonal precipitation in 2007 (mm)	219	186	253
Soil type	Chernozem	Haplic Calcisols	Kastanozem
Soil organic C (g kg^{-1})	9.13 ± 0.76	14.13 ± 0.78	10.96 ± 0.65
Soil total N (g kg^{-1})	0.95 ± 0.09	1.45 ± 0.05	1.13 ± 0.07
Soil C/N ratio	10.04 ± 0.60	9.74 ± 0.51	9.27 ± 0.35
Soil pH	9.34 ± 0.18	7.21 ± 0.08	7.98 ± 0.04
Soil moisture (%)	14.41 ± 0.84	8.82 ± 1.14	5.60 ± 0.47

plots were arranged in a 6 × 6 matrix. The distance between two adjacent plots was 3 m. Among the six treatments, daytime and nighttime warming were not used in this study in order to maintain similar treatments to those in the other two sites.

At the other two sites, we used a design with continuous warming applied to blocks and N addition applied to plots. We established twelve 3 × 4 m blocks, and kept a 3 m distance between adjacent blocks. Six blocks were warmed with an infrared heater and the other six blocks were used as controls, which were not warmed. N addition was applied to 3 × 2 m plots within each block.

At all three experimental sites, one infrared heater (165 × 15 cm, MSR-2420, Kalglo Electronics Inc., Bethlehem, PA, USA) was suspended 2.25 m above the ground for each warmed plot. The heaters were set at outputs of approximately 1600 W radiation. In order to simulate the shading effects of the heater, 'dummy' heater installations with the same shape and size, were also set up in control plots. An additional continuous 4-year experiment provided strong evidence that plants became saturated at an N addition rate of 10.5 g N m^{-2} y^{-1} in temperate grasslands of Inner Mongolia (Bai et al., 2010). To ensure that all taxa were not limited by N concentration, N was added once a year at a rate of 10 g N m^{-2} y^{-1} using NH_4NO_3, in the early growing season of each experimental year.

2.3. Sampling and measurements

Three soil cores (2 cm in diameter and 15 cm in depth) were collected from each plot in the three sites at the peak of the growing season of August 2006 and 2007. Soil cores were mixed into one composite fresh sample per plot to avoid bias from spatial

heterogeneity. After removing plant roots and stones with a 2-mm mesh sieve, soil samples were immediately cooled with ice blocks and transported to the laboratory for further analysis.

Soil organic carbon was estimated by dichromate oxidation and titration method (Kalembasa and Jenkinson, 1973). Soil samples were digested using the Kjeldahl acid-digestion method, and then Kjeldahl digests were analyzed for ammonium concentration on an Alpkem autoanalyzer to determine total soil nitrogen (Kjektec System 1026 Distilling Unit, Sweden). Soil was dried at 105 °C for 48 h to measure water moisture. Soil pH was measured using a glass electrode (1:2.5 soil to water ratio, Thermo Orion T20, USA).

Soil microbial community composition was estimated using phospholipid fatty acid (PLFA) analysis. This method does not require the use of a pure microbial culture, and it can be used to assess the composition of phospholipid fatty acids in most natural populations in soil microbial communities (Amann et al., 1995). We extracted, fractionated, and quantified PLFAs from fresh soils following the procedure from Bossio and Scow (1998). Fresh soil samples equivalent to 8 g dry mass of soil were extracted for 2 h using a single phase mixture containing a chloroform, methanol and phosphate buffer (1:2:0.8 v/v/v). After the supernatant was decanted, soil was extracted again for 30 min. The supernatants obtained after these two extractions, along with additional phosphate buffer and $CHCl_3$, were decanted into a separation funnel. After the phases were allowed to separate overnight, the organic phase ($CHCl_3$ layer) was obtained and dried under N_2. After reconstitution, fatty acids were separated using a silica-bonded phase column and eluted first using 5 ml chloroform, 10 ml acetone, and then 5 ml methanol successively in order to separate polar lipids from neutral and glycolipids. Polar lipids were converted to fatty acid methyl esters via mild alkaline methanolysis. A 2 μl sample of each fatty acid methyl ester extract was injected and analyzed by an Agilent 6850N gas chromatograph with a flame ionization detector and an HP-1 Ultra 2 capillary column (Agilent Technologies, Inc., Santa Clara, CA, USA). Gas chromatography was performed as recommended by the MIDI standard protocol (Microbial ID. Inc., Newark, DE). Peak areas of each resulting fatty acid methyl ester were recorded on a chromatogram and identified by chromatographic retention time and comparison with peaks from a standard qualitative mix ranging from C9 to C30 using a microbial identification system (Microbial ID. Inc., Newark, DE). The mole percentage (mol%) of PLFA was expressed as the relative concentration of each PLFA in initial soil extracts. The PLFAs i15:0, a15:0, i16:0, a17:0, i17:0, 16:1ω7c, 18:1ω5c, cy17:0 and cy19:0 were used as biomarkers for bacteria (Frostegård and Bååth, 1996; Zak et al., 1996; Ringelberg et al., 1997; Zelles, 1997; Zogg et al., 1997). Terminally branched saturated PLFAs i15:0, a15:0, i16:0, i17:0 and a17:0 were used as indicators of Gram-positive bacteria (Gram+), while cy17:0, cy19:0 and16:1ω7c were considered as indicators of Gram-negative bacteria (Gram−) (Frostegård and Bååth, 1996; Zak et al., 1996; Ringelberg et al., 1997; Zelles, 1997; Zogg et al., 1997). The unsaturated PLFAs containing 18:1ω9c, 18:2ω6c and 18:3ω6 were used as biomarkers for fungi (Zak et al., 1996; Ringelberg et al., 1997; Zelles, 1997; Zogg et al., 1997; Madan et al., 2002; Pinkart et al., 2002). 16:1ω5 was used to represent arbuscular mycorrhizal fungi (AMF; Olsson et al., 1995).

Three of the six experimental replicates from the meadow and semi-arid steppe sites were randomly chosen to perform measurements of soil microbial C utilization. Soil microbial C utilization was measured using Biolog redox technology, which is a culture-based method for detecting soil microbial C source utilization, especially in the case of quick-growing, r-strategist species (Garland and Mills, 1991; Gomez et al., 2006; Mijangos et al., 2006). The procedure was performed according to Classen's description (Classen et al., 2003). After shaking for 30 min on a reciprocal shaker, 4 g of fresh soil were extracted with 36 ml of 50 mM K_2HPO_4 buffer. The supernatant was obtained after allowing the sample to settle for 30 min. The supernatant was then diluted 1:1000 with a sterile incubation solution in order to prepare the bacterial suspension. The sterile incubation solution consisted of 0.40% NaCl and 0.03% Pluronic F-68. 150 μl of bacterial suspension was then injected into each well of the EcoPlate. Each EcoPlate was put into a polyethylene bag to avoid desiccation and incubated in darkness at 25 °C. The optical density (OD) values were read at 595 nm every 24 h. A preliminary experiment demonstrated fungal detection after 96 h; therefore, the OD values at 96 h were used to assess bacterial C utilization in order to avoid bias induced by fungal growth. The net OD values were calculated as the substrate OD subtracted from the control well OD. If the result was negative or less than 0.06, the net OD was considered to be zero or below the detection limit of the system (Miguel et al., 2007). Average well-color development (AWCD) was used to reflect bacterial metabolic potential, and was determined as follows (Garland and Mills, 1991):

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

where x_i is the OD value in the substrate well, and c is the OD value measured in the control well.

2.4. Data analysis

Statistical software R 2.15.3 was used for conducting all statistical analyses (R Development Core Team, 2013). Since the warming effect was applied to plots within the semi-arid steppe and to blocks at the other two experimental sites, and the otherwise fully balanced design without missing values, we used the 'aov' function with Error () function, which allowed us to conveniently separate the contribution of warming to variation in the dependent variables between block and plot level. It must be noted that this type of design cannot be easily handled by other mixed-model approaches such as those implemented in the R-functions "lme" or "lmer." Using the "aov" function allowed us to test the warming effect and its interaction with the year at the semi-arid steppe at the block level, however this was tested at the plot level in the other two sites. The effect of N addition was always tested at the plot level.

We analyzed the phospholipid fatty acid composition of soil microbial communities combining data from all experimental plots of all sites using principal component analysis (PCA). The PCA was conducted using the "rda" function of the vegan package in R. To reduce noise for PCA, only 18 PLFAs with a mol% (moles individual lipid/moles total lipids) >0.5 were used. We also performed post-hoc permutations using the function "envfit" of the vegan package to detect associations of the microbial community composition as determined by phospholipid fatty acid profiles with environmental variables. The function "cor.test" of the stats package of R was used to perform Pearson's correlation test for associations between microbial functional groups and environmental variables.

We also analyzed the matrix of net OD values from the soil microbial C utilization analysis using PCA to test the responses of microbial C utilization profiles to warming and N addition, including data from all plots of meadow and semi-arid steppes. We also performed permutation tests for the associations between microbial C utilization profiles of r-strategist bacteria and environmental variables, similar to those performed with the lipid data.

3. Results

3.1. Soil parameters

There were similar differences in soil organic C and total N in the plots among the meadow, semi-arid, and desert steppe sites in both sampling years, except for the instances where it was otherwise noted (2006 and 2007; Tables 2 and S1). Soil organic C and total N were greater in the semi-arid steppe than in the meadow or desert steppes (Table S1). The ratio of soil C to N was, on average, the highest in the meadow steppe (Tables 2 and S1). Soil pH was the highest in the meadow steppe as well, followed by the desert steppe and then the semi-arid steppe (Tables 2 and S1). Soil moisture among the three experimental sites was significantly different, depending on the year. A larger difference in soil moisture between the meadow and semi-arid steppe was observed in 2006 compared to 2007 (Table S1).

The effect of N addition on soil organic C concentration was dependent upon the site (Table 2). The greatest increase in soil organic C, which was induced by N addition was observed in the meadow steppe (Table 3). N addition markedly reduced soil pH across the three temperate steppe sites, however the effect of N addition on soil pH was both dependent on the year and site sampled (Tables 2 and 3). We found that the negative effects of N addition on soil pH had the greatest influence in the meadow steppe in 2006 in comparison to the other two sites, but had the greatest impact overall in the semi-arid steppe in 2007 (Table 3). Moreover, the negative effects of N addition on soil pH were more evident in 2007 than 2006 in both the semi-arid and desert steppes (Table 3). Warming effects on soil pH were also dependent on the year of sampling (Table 2). There were large contrasting effects of warming on soil pH between 2006 and 2007 in the meadow steppe. However, we did not find main warming effects or interactive effects between warming and N addition on soil parameters in any of the three temperate steppe sites (Tables 2 and 3).

Table 3
Warming and nitrogen-induced relative changes (%) on soil physicochemical parameters during the two hydrologically contrasting years in the three temperate steppes. SOC = soil organic carbon, TN = total soil nitrogen, C/N = the ratio of soil organic carbon to nitrogen, SM = soil moisture, pH = soil pH. For statistical significance of effects, see Table 2.

Variance of source	Site/year	SOC	TN	C/N	SM	pH
Meadow steppe						
Warming effect	2006	2.04	18.29	−13.21	−7.57	2.85
	2007	11.18	−1.26	−0.66	−2.47	−4.70
Nitrogen effect	2006	26.47	19.65	6.10	−2.40	−4.39
	2007	43.51	25.88	24.24	−13.31	−4.19
Semi-arid steppe						
Warming effect	2006	−1.72	0.60	−6.20	−4.50	0.06
	2007	−2.99	−10.24	13.13	−3.24	0.17
Nitrogen effect	2006	−8.09	3.38	−7.01	−8.07	−0.40
	2007	−3.09	−7.36	0.56	−4.30	−5.06
Desert steppe						
Warming effect	2006	−4.23	−4.08	−0.18	−6.25	−0.47
	2007	−13.23	0.09	−13.02	−7.39	0.09
Nitrogen effect	2006	1.78	1.27	3.57	2.13	−0.56
	2007	−2.91	0.62	6.86	4.19	−1.29

3.2. Microbial community composition according to PLFA profiles

We focused on analyzing the first and second principal components (PC), which explained 40.2% and 25% of variance in phospholipid fatty acid composition, respectively. Although one principal component analysis (PCA) was performed, we display the PCs separately for each site in order to illustrate the differences in microbial responses to treatments at each site. The PCA biplot also displayed clear separation of warming, N addition, and warming plus N addition from the control in 2006 (Fig. 2, top), indicating that warming and N addition had a significant impact on microbial PLFA composition in this year in the meadow steppe. According to the loading scores, which quantify the contribution of each individual

Table 2
Results (F values) of a four-way ANOVA with multiple error strata on the effects of site, warming, N addition and year on soil physicochemical parameters as dependent variables. SOC = soil organic carbon, TN = total soil nitrogen, C/N = the ratio of soil organic carbon to nitrogen, SM = soil moisture, pH = soil pH. For each of these dependent variables we fit the following statistical model in R, e.g., aov (SOC ~ year × site × warm × nitrogen + Error (block/(plot × year))). This type of ANOVA allowed us to test the warming treatment, applied at block level at two sites and at block:plot level at one site, at the corresponding error strata in a single analysis. Degrees of freedom (d.f.) are the same for all dependent variables, mean squares (MS) and F-values (F) are different. Significance codes ˆ, *, **, and *** refer to $P < 0.1$, <0.05, <0.01 and <0.001, respectively.

Variance of source	d.f.	SOC		TN		C/N		SM		pH	
		MS	F	MS	F	MS	F	MS	F	MS	F
Block stratum:											
Site (S)	2	172.53	19.70***	0.69	7.82**	40.62	8.89**	954.60	137.35***	47.90	207.63***
Warming (W)	1	0.12	0.01	0.04	0.41	11.39	2.49	8.00	1.15	0.08	0.33
S × W	2	7.70	0.88	0.05	0.59	0.02	0.00	0.45	0.06	0.02	0.09
Block residuals	25	8.76		0.09		4.57		7.00		0.23	
Block:plot stratum:											
W	1	1.31	0.18	0.05	0.57	1.23	0.20	1.12	0.23	0.00	0.00
N addition (N)	1	28.13	3.95ˆ	0.20	2.07	11.08	1.81	8.09	1.66	1.79	9.26**
S × N	2	63.25	8.88**	0.25	2.58ˆ	10.97	1.79	4.87	1.00	0.32	1.67
W × N	1	9.28	1.30	0.01	0.13	14.86	2.43	7.28	1.49	0.01	0.07
S × W × N	2	13.80	1.94	0.14	1.44	11.41	1.86	11.62	2.38	0.01	0.03
Block:plot residuals	35	7.12		0.10		6.13		4.89		0.19	
Block:year stratum:											
Year (Y)	1	83.48	32.95***	2.12	23.55***	28.31	7.17*	8.40	1.89	0.12	0.88
Y × S	2	15.93	6.29**	0.26	2.89ˆ	12.24	3.10ˆ	356.10	79.93***	1.26	9.36**
Y × W	1	0.07	0.03	0.05	0.53	0.00	0.00	1.60	0.35	0.64	4.74*
Y × S × W	2	2.16	0.85	0.06	0.70	4.95	1.25	0.45	0.10	0.41	3.07ˆ
Block:year residuals	25	2.53		0.09		3.95		4.50		0.13	
Block:plot:year stratum (individual measurement units):											
Y × W	1	0.08	0.02	0.07	0.78	11.78	1.44	0.08	0.03	0.00	0.01
Y × N	1	2.18	0.45	0.02	0.27	9.54	1.17	2.43	1.04	0.15	5.79*
Y × S × N	2	2.16	0.45	0.02	0.24	2.15	0.26	2.08	0.90	0.10	3.68*
Y × W × N	1	0.98	0.20	0.00	0.05	3.86	0.47	2.45	1.06	0.02	0.72
Y × S × W × N	2	1.42	0.30	0.02	0.24	2.45	0.30	1.61	0.69	0.01	0.48
Unit residuals	35	4.82		0.09		8.16		2.33		0.03	

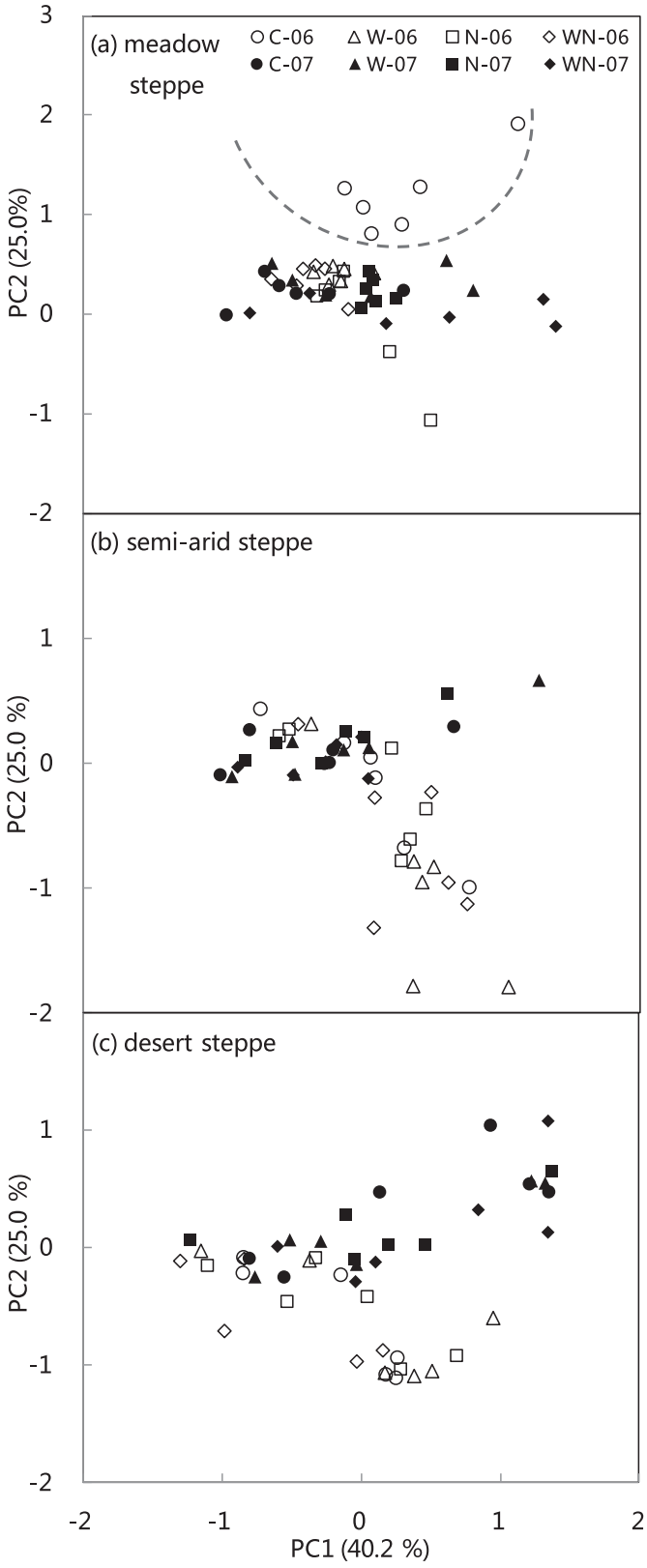

Fig. 2. Principal component analyses (PCA) of soil microbial community composition as indicated by PLFA profiles during the two hydrologically contrasting years in the three temperate steppes. Principal component (PC) 1 and PC2 accounted for 40.2% and 25% of the total variance, respectively. The PCA ordination biplot for all 24 surveyed subplots, separated for visual comparison by each steppe, showing control (C, open circle), warming (W, open triangle), nitrogen (N) addition (N, open square), warming plus N addition (WN, open diamond) in 2006, and control (solid circle), warming (solid triangle), N addition (solid square), warming plus N addition (solid diamond) in 2007.

fatty acid to the first two principal components, terminally branched saturated PLFAs i15:0, i16:0, a15:0, saturated PLFA16:0, unsaturated PLFAs 16:1ω5c, 16:1ω7c, 18:1ω9c and 18:2ω6c largely contributed to changes in soil microbial community composition in the meadow steppe (Table S3). Warming and N addition had no effects on microbial PLFA composition in the semi-arid or desert steppe, however samples still clustered according to the year (Fig. 2, middle and bottom).

3.3. Microbial functional groups according to specific PLFAs

The microbial functional groups included here, as determined by the presence of specific PLFAs, include total bacteria, Gram-negative (Gram−) and Gram-positive bacteria (Gram+), non-mycorrhizal fungi, and arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhizal fungi were markedly different among the three sites, with the largest proportion observed in the meadow steppe (Tables 4 and S2). Among the three sites, the proportion of non-mycorrhizal fungi was largest in the desert steppe site in 2006, but was largest in the semi-arid steppe site in 2007 (Tables 4 and S2). The trends of the fungal/bacterial ratio mirrored the trends of non-mycorrhizal fungi since there was no difference observed in the total amount of bacteria present in any of the three sites in either 2006 or 2007 (Table 4).

Although there were no main effects of warming or N addition on bacterial groups (i.e., total bacteria or Gram+), non-mycorrhizal fungi, or AMF across the three sites, we did observe interactions between the year and N addition as well as the year, site, and N addition affecting the relative proportion of microbial groups (Table 4). These results suggest that the effects of N addition were both dependent on the year of sampling and the ecosystem in question. The contrasting effects of N addition on microbial functional groups in 2006 and 2007 were especially noticeable in the meadow steppe, in respect to the amount of bacteria, Gram+, non-mycorrhizal fungi observed (Fig. 3, right side, uppermost panel). In the meadow steppe, N addition increased the proportions of total bacteria as well as Gram−, Gram+, the ratio of Gram−/Gram+ and non-mycorrhizal fungi in 2006, which had a wetter growing season (Fig. S1, upper panel). In contrast, N addition dramatically decreased the amounts of total bacteria, Gram+ bacteria, and non-mycorrhizal fungi, and the fungal/bacterial ratio in 2007, which had a drier growing season (Fig. S1, upper panel). N addition also proved to have contrasting effects on bacteria, Gram+, fungi, and AMF in 2006 and 2007 in the desert steppe (Fig. 3, right side, lowest panel). Similar to what was observed with N addition in the meadow steppe, warming increased the proportion of total bacteria, Gram−, Gram+, non-mycorrhizal fungi and AMF in 2006 while it reduced their proportions as well as the fungal/bacterial ratio in 2007 (Fig. 3, left side, uppermost panel). Warming had negative effects on the amount of microbial functional groups in the semi-arid steppe, and the warming effects were stronger in 2006 than 2007 (Fig. 3, left side, middle panel).

3.4. Microbial C utilization profiles and average metabolic potentials

A principal component analysis (Fig. 4) showed that sample scores between 2006 and 2007 were arranged in two distinct groups in the meadow steppe site and were marginally separated in the semi-arid steppe site. This implies that C utilization profiles of r-strategist bacteria experienced large inter-annual fluctuation in both the meadow and semi-arid steppe sites. According to the PCA analysis, we found that bacterial C utilization profiles in the plots affected by both warming and the addition of N were only clearly distinct from the control in 2006 in the meadow steppe site, while

Table 4
Results (F values) of a four-way ANOVA with multiple error strata on the effects of site, warming, N addition and year on main microbial groups and their ratios as dependent variables. Bacteria = bacterial PLFA, Gram− = Gram-negative bacterial PLFA, Gram+ = Gram-positive bacterial PLFA, Fungi = non-mycorrhizal fungal PLFA, AMF = arbuscular mycorrhizal fungal PLFA, Gram−/Gram+ = the ratio of Gram-negative to Gram-positive bacteria, F/B = the ratio of fungi to bacteria. For each of these dependent variables we fit the following statistical model in R, e.g.: aov (SOC ~ year*site*warm*nitrogen + Error (block/(plot*year))). This type of ANOVA allowed us to test the warming treatment, applied at block level at two sites and at block:plot level at one site, at the corresponding error strata in a single analysis. Degrees of freedom (d.f.) are the same for all dependent variables, mean squares (MS) and F-values (F) are different. Significance codes ^, *, **, and *** refer to $P < 0.1$, <0.05, <0.01 and <0.001, respectively.

Variance of source	d.f.	Bacteria		Gram−		Gram+		Fungi		AMF		Gram−/Gram+		F/B	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Block stratum:															
Site (S)	2	4.04	0.10	0.56	0.07	4.96	0.37	15.85	1.91	27.83	20.55***	0.04	1.81	0.03	4.13*
Warming (W)	1	0.84	0.02	0.00	0.00	1.21	0.09	0.61	0.07	0.18	0.14	0.02	1.15	0.00	0.67
S × W	2	1.40	0.04	0.33	0.04	0.45	0.03	0.47	0.06	0.28	0.21	0.00	0.00	0.00	0.22
Block residuals	25	39.06		7.99		13.44		8.32		1.35		0.02		0.01	
Block:plot stratum															
W	1	29.23	3.82^	3.66	1.39	10.34	3.40^	1.38	0.46	0.82	2.53	0.00	0.08	0.00	0.02
N addition (N)	1	1.00	0.13	10.33	3.93^	4.08	1.34	1.67	0.56	0.59	1.83	0.09	4.84*	0.01	0.99
S × N	2	1.21	0.16	0.31	0.12	1.49	0.49	2.93	0.98	0.19	0.57	0.01	0.34	0.01	1.36
W × N	1	1.98	0.26	0.53	0.20	4.96	1.63	2.61	0.87	0.42	1.30	0.02	0.93	0.00	0.07
S × W × N	2	0.85	0.11	0.58	0.22	1.57	0.52	2.68	0.90	0.90	2.77^	0.01	0.59	0.01	1.88
Block:plot residuals	35	7.65		2.63		3.05		2.98		0.32		0.02		0.01	
Block:year stratum:															
Year (Y)	1	74.05	2.13	7.38	1.23	115.37	7.57*	8.64	1.54	0.93	1.67	0.68	24.07***	0.10	23.23***
Y × S	2	37.65	1.09	21.36	3.57*	3.78	0.25	55.62	9.88**	1.56	2.81^	0.14	4.77*	0.08	18.92***
Y × W	1	90.71	2.61	6.14	1.03	44.21	2.90	19.46	3.46^	1.19	2.14	0.01	0.23	0.01	2.53
Y × S × W	2	12.50	0.36	2.74	0.46	2.05	0.13	6.56	1.16	0.38	0.69	0.00	0.17	0.01	1.38
Block:year residuals	25	34.70		5.98		15.24		5.63		0.56		0.03		0.00	
Block:plot:year stratum (individual measurement units):															
Y × W	1	17.74	1.98	0.97	0.34	10.03	3.41^	0.47	0.15	0.00	0.00	0.01	0.70	0.00	0.26
Y × N	1	69.97	7.82**	3.90	1.38	36.29	12.32**	27.03	8.29**	1.02	3.47^	0.01	0.49	0.02	2.99^
Y × S × N	2	32.07	3.58*	2.79	0.99	13.68	4.64*	9.33	2.86^	0.44	1.49	0.01	0.45	0.01	1.77
Y × W × N	1	3.54	0.40	7.82	2.76	0.82	0.28	0.19	0.06	0.04	0.12	0.05	3.39^	0.00	0.10
Y × S × W × N	2	27.01	3.02^	11.52	4.07*	4.12	1.40	3.27	1.00	0.33	1.12	0.02	1.57	0.01	1.33
Unit residuals	35	8.95		2.83		2.95		3.26		0.29		0.02		0.01	

other plots affected by warming and N addition were only marginally different from the control (Fig. 4, upper panel). However, we did not observe significant effects of warming, N addition, or any synergistic effects on bacterial C utilization profiles in the semi-arid steppe site (Fig. 4, lower panel).

The average metabolic potentials (AWCD) of r-strategist bacteria in the control plots in the meadow steppe were larger than those in the semi-arid in 2006 ($F = 123.6$ (1), $P < 0.001$), while there was no significant difference observed between the two sites in 2007. Bacterial AWCD was significantly different in 2006 and 2007 in both the meadow and semi-arid steppe sites. Bacterial AWCD across treatments was 59.8% ($F = 141.02$ (1), $P < 0.001$) higher in 2006 than that in 2007 in the meadow steppe site, but was 227% ($F = 30.08$ (1), $P < 0.001$) lower in 2006 than in 2007 in the semi-arid steppe. Bacterial AWCD declined with N addition by 15.7% ($F = 5.6$ (1), $P = 0.031$) in both years in the meadow steppe site, but was not altered by N addition in the semi-arid steppe site. There was no significant effect of warming or interactions between warming and N addition on bacterial AWCD in either the meadow or semi-arid steppe.

3.5. Correlations between the soil characteristics and microbial communities

According to the *post-hoc* permutation test, soil microbial community composition closely correlated with soil organic C concentrations ($r = 0.26$, $P = 0.005$) and N ($r = 0.24$, $P = 0.019$), soil moisture ($r = 0.65$, $P < 0.001$), and pH ($r = 0.56$, $P < 0.001$). Across all plots and both experimental years, Gram+ was marginally correlated with soil organic C/N ratio ($r = 0.14$, $P = 0.099$). The Gram− was marginally related to soil organic C ($r = 0.16$, $P = 0.059$) and N ($r = 0.16$, $P = 0.051$), while the ratio of Gram−/Gram+ was marginally related to soil organic C ($r = 0.15$, $P = 0.065$) but significantly correlated with soil total N ($r = 0.26$, $P = 0.002$). The mole percentage PLFA from the total bacteria population was only marginally correlated with soil moisture ($r = 0.14$, $P = 0.086$). The non-mycorrhizal fungal PLFA was positively correlated with soil organic C ($r = 0.19$, $P = 0.019$), but negatively correlated with pH ($r = -0.15$, $P = 0.076$). The fungal/bacterial ratio was significantly associated with organic C ($r = 0.22$, $P = 0.009$) and total N ($r = 0.19$, $P = 0.025$), and marginally correlated with soil pH ($r = -0.15$, $P = 0.082$). Arbuscular mycorrhizal fungi was markedly related to organic C ($r = 0.24$, $P = 0.004$), N ($r = 0.17$, $P = 0.042$), soil moisture ($r = 0.59$, $P < 0.001$) and soil pH ($r = 0.40$, $P < 0.001$).

According to the permutation test, soil microbial C utilization profiles were closely correlated with soil organic C ($r = 0.58$, $P = 0.002$) and total N ($r = 0.50$, $P = 0.002$), as well as the organic C/N ratio ($r = 0.37$, $P = 0.039$), soil moisture ($r = 0.82$, $P < 0.001$) and pH ($r = 0.80$, $P < 0.001$). Across all plots and both experimental years, the average metabolic potentials (AWCD) of r-strategist bacteria were significantly correlated with soil moisture ($r = 0.78$, $P < 0.001$) and pH ($r = 0.48$, $P < 0.001$), but not with soil organic C, total N, or C/N ratios.

4. Discussion

4.1. Annual precipitation modified the effects of warming on soil microbial communities

Liebig's law suggests that there is a single limiting factor that controls the growth of organisms (Liebig, 1855); expanding this concept is the theory of co-limitation, or the simultaneous limitation of more than one resource (Saito et al., 2008; Harpole et al., 2011; Ågren et al., 2012). In the present study, we found that annual precipitation had the ability to dramatically modify warming effects on soil microbial communities, especially soil fungi in

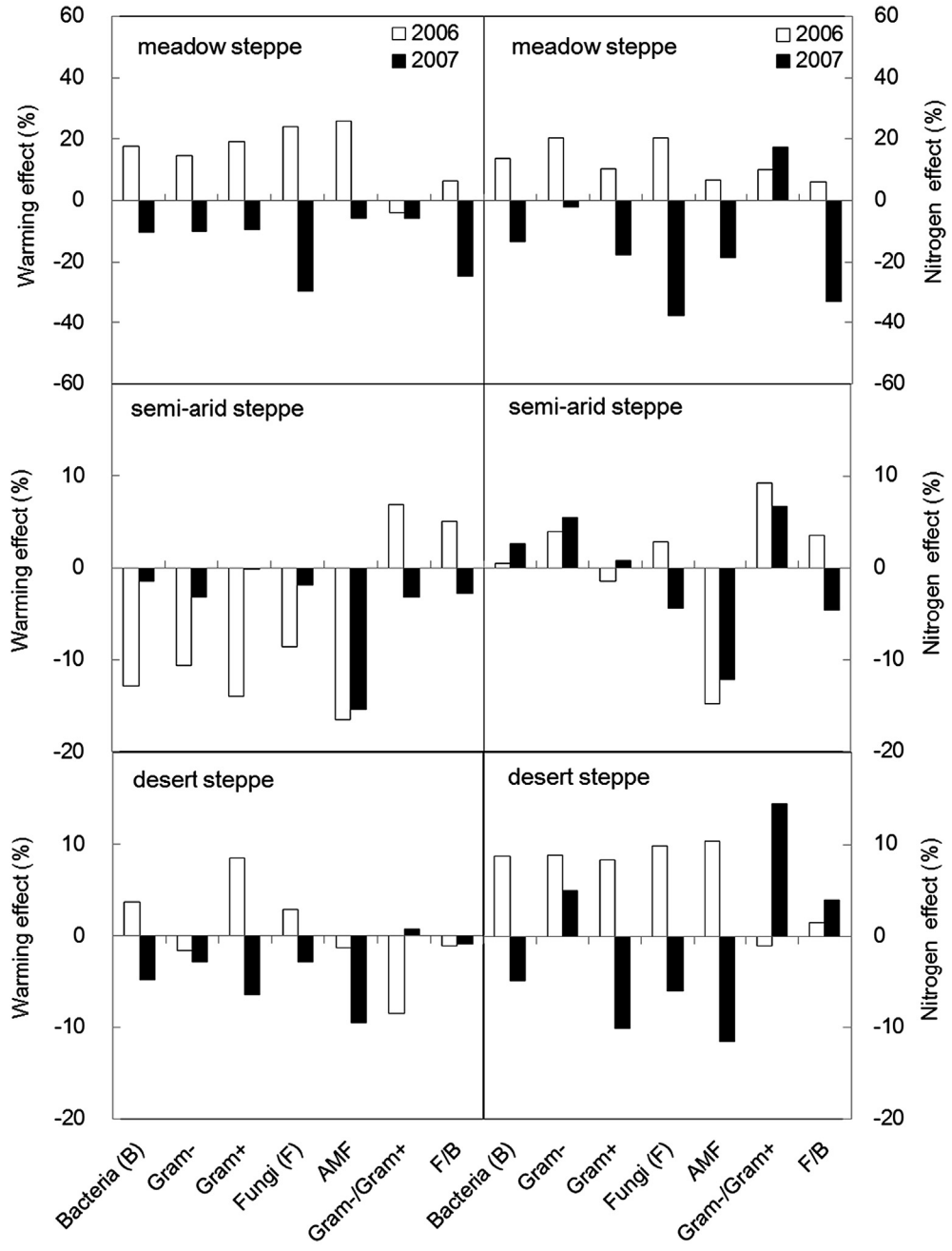

Fig. 3. Warming and nitrogen-induced relative changes (%) in the proportion (mole percentage PLFA) of the main functional groups of the microbial communities during the two hydrologically contrasting years in the three temperate steppe sites. Bacteria = bacterial PLFA, Gram− = Gram-negative bacterial PLFA, Gram+ = Gram-positive bacterial PLFA, Fungi = non-mycorrhizal fungal PLFA, AMF = arbuscular mycorrhizal fungal PLFA, Gram−/Gram+ = the ratio of Gram-negative to Gram-positive bacteria, F/B = the ratio of fungi to bacteria. For the statistical significance of the effects, see Table 4.

soils of the meadow steppe. Our results demonstrated that in the meadow steppe, microbial functional groups were stimulated by warming or N addition only when an abundance of water was available as was the case in 2006, but not in the drought-like environment of 2007. In this system co-limited by multiple factors, water appears to be the primary limiting resource while the effect of temperature was secondary and only influential in conditions of abundant water. This implies that the influence of the warming effect on the functional group level of microbial communities is dependent on the amount of available water (Harpole et al., 2011). The PCA results indicate that water dependence may also exist at the community level in the meadow steppe.

The importance of the effect of water limitation on soil microbial communities is easily understood. Water stress usually creates unfavorable growing conditions for soil microbial communities and inhibits activity in the majority of microorganisms, which may result in a lack of response of microbial communities to warming (Sheik et al., 2011). Alternatively, soil microbial phylotypes from historically more often water-stressed environments may be better adapted to a drought environment and may have notably inherent

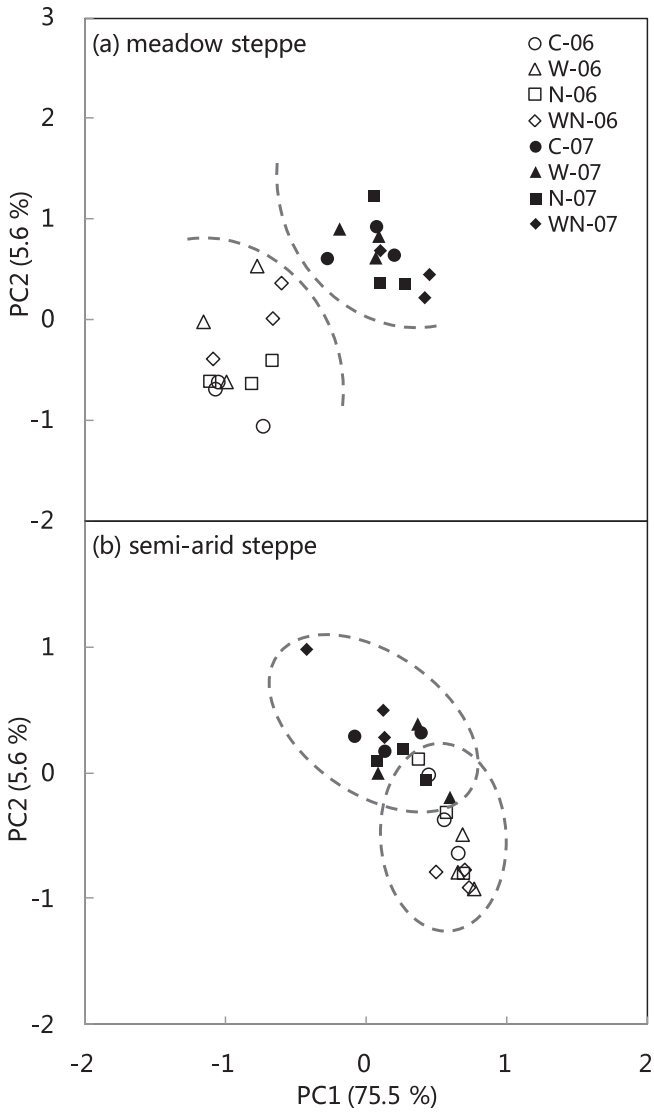

Fig. 4. Principal component analyses (PCA) of soil microbial carbon (C) utilization profiles during the two hydrologically contrasting years, in the meadow and semi-arid steppe sites. Principal component (PC) 1 and PC2 accounted for 75.5% and 5.6% of the total variance, respectively. The PCA ordination biplot, for all 24 surveyed subplots, is separated for visual comparison by each steppe, with the following symbols used for each site: control (C, open circle), warming (W, open triangle), N addition (N, open square), warming plus nitrogen (N) addition (WN, open diamond) in 2006, and control (solid circle), warming (solid triangle), N addition (solid square), warming plus N addition (solid diamond) in 2007.

resistance to water stress (Schimel et al., 2007; Bradford et al., 2008). This may explain why soil microbial groups did not show significant responses to warming in either study year (2006 or 2007) in the semi-arid and the desert steppe sites. However, warming would be expected to most likely stimulate microbial growth when water is sufficient. High temperatures that do not kill microorganisms usually lead to higher enzyme activities and rates of nutrient assimilation (Atlas and Bartha, 1998; Allison et al., 2010). Microbial populations surviving in conditions with sufficient amounts of water are more active in comparison with those under drought conditions (Sheik et al., 2011), and diverse microbial populations may exhibit variable temperature responses for different temperature growth ranges (Atlas and Bartha, 1998). This may explain why the effects of warming depended upon water availability not only at the functional group level but also at the community level in our study.

4.2. The effects of alternating wet–dry regimes and extreme drought on microbial responses to temperature change

Alternating wet–dry regimes or extreme drought both create formidable challenges and induce physiological stresses for microorganisms (Austin et al., 2004; Schimel et al., 2007; Zeglin et al., 2013). We found that the occurrence of an alternating wet–dry regime (during July and August of both 2006 and 2007, in the semi-arid steppe) or extreme drought (during August of 2007 in the meadow steppe) during sampling, led to negative responses to warming in most of the microbial functional groups. Warming can potentially reduce water and nutrient availability, or the supply of root exudates, all factors that work together to alter microbial population dynamics (Atlas and Bartha, 1998; Austin et al., 2004) and microbial community composition, since different microorganisms have variable responses or abilities to acclimate to water stress (Zhang et al., 2005; Rinnan et al., 2007; Schimel et al., 2007; Evans and Wallenstein, 2012). We found that warming exerted a stronger suppression on non-mycorrhizal fungi than bacteria under the conditions of extreme drought in the meadow steppe. This is inconsistent with the generally accepted view that bacteria are largely dependent upon water and thus more sensitive to water stress while fungi are drought-tolerant (Wardle, 2002; Hawkes et al., 2011; Yuste et al., 2011). We suggest that the high salinity and alkaline nature of the soils in the meadow steppe changes the responses of microbial communities to the drought stress, which was aggravated by warming since saline–alkaline soils are detrimental to fungal growth (Rousk et al., 2009; Djukic et al., 2010; Rousk et al., 2011). An alternative explanation could be that the majority of the bacteria in the saline–alkaline soils found in the meadow steppe were halotolerant or halophilic (Pan et al., 2012), and could better tolerate (e.g., by excluding the high and toxic sodium ion concentration from the cell interiors) or even require high salt concentrations (Atlas and Bartha, 1998). Thus, these bacteria may be more resistant to the drought regimes aggravated by warming than the fungi in the saline–alkaline soils of the meadow steppe.

4.3. Precipitation dependent N limitation of soil microbial communities

We added N at the rate of 10 g N m^{-2} y^{-1} to avoid N limitation for the majority of the taxa in the microbial communities, although the rate of N addition was greater than the natural N deposition found in the experimental areas (Lü and Tian, 2007). Our previous study demonstrated that N addition at rates $\leq$16 g N m^{-2} y^{-1} could stimulate microbial growth in the semi-arid grassland (Zhang et al., 2008a). Moreover, we also found that most of the microbial functional groups positively responded to N addition only when there was a lack of water stress. The results imply that N substantially limits microbial growth, but its effects depend upon the fluctuation in precipitation. In this case, water may be a primary limiting factor relative to N for microorganisms, suggesting a serial co-limitation of microbial communities by water and N in the temperate steppes of northern China.

Water is essential for nutrient diffusion and replenishment in soil. High water availability could contribute to the replenishment of added N to the soil solution and consequently increase available N for microbial use (Park et al., 2002), and accelerate microbial-mediated enzyme activities (Wang et al., 2014). In this case, the replenished N could enhance microbial growth by reducing N limitation. This could specifically explain why N addition along with sufficient amounts of water stimulated bacterial growth, especially since they are much more sensitive to water regimes than fungi (Wardle, 2002; Min et al., 2013). The accumulation of soil

organic C in instances where N was added lead to an increase in the relative abundance of fungi in 2006 in the meadow steppe site, which is consistent with the other previous observations (Cusack et al., 2011). However, a change in soil organic C due to N addition cannot explain the significant decrease in the relative abundance of fungi in 2007 in the meadow steppe. The drought in 2007 may have had limited C and nutrient availability, weakening N effects on available nutrients in the soil as well as abundance of fungi. Alternatively, drought could allow for the accumulation of nitrate or ammonium ions (Stursova et al., 2006), even possibly up to inhibitory levels for some extracellular enzymes, further suppressing the growth and activity of fungi (Donnison et al., 2000). Taken together, our results indicate that N effects on soil microbial communities are strongly dependent on precipitation in the temperate steppe sites of northern China.

4.4. Water dependence of treatment effects on bacterial C utilization profiles and average metabolic potentials

The elucidation of diverse aspects of microbial physiology is required for the effective integration of microbial and ecosystem ecology (Schimel et al., 2007; Balser and Wixon, 2009; Allison et al., 2010). Despite some limitations of culture-based Biolog redox technology, Biolog has been proven to be a useful fingerprinting method to assess the physiology of microbial C use (Balser and Wixon, 2009). We found that the bacterial C utilization profiles and the average metabolic potentials of r-strategist bacteria were largely correlated with soil moisture, consistent with the other observations (Bell et al., 2008). This knowledge can help explain why water fluctuation between sampling years caused significant inter-annual differences in bacterial C utilization profiles and average C metabolic potentials in both the meadow and semi-arid steppes. Microbial growth and metabolic activities require water (Young and Ritz, 2005; Liu et al., 2009) and depend upon favorable water conditions to function. High soil water availability can provide conditions for improving microbial growth and metabolic activities (Zak and Kling, 2006; Keeler et al., 2009; Liu et al., 2009), especially for bacteria, since they are particularly sensitive to the moisture content in soil (Degens et al., 2000; this study). The dependence on water may also explain why a much stronger treatment effect is observed on C utilization of bacterial communities in the wetter (2006) compared to the dryer year (2007) in the meadow steppe. In the semi-arid steppe, however, we found little change in the bacterial C utilization profiles and their average metabolic potentials in response to treatments. Bacterial adaptation to environments that historically have experienced drought conditions possibly explains this lack of fluctuation.

5. Conclusions

Soil microbial communities in the semi-arid and the desert steppe appear to have the ability to resist effects due to warming and changes in N compared to those in the meadow steppe. The adaptation of microorganisms inhabiting drought affected environments in both the semi-arid and desert steppes may be a possible explanation for the different responses of the microbial communities at these two sites. Our results further demonstrated that annual precipitation modified the warming effects at the functional group and community levels in the meadow steppe, as we had originally proposed in our first hypothesis. We suggest that warming has positive effects on soil microorganisms only when there is a sufficient amount of water in the environment. In addition, we found that alternating wet–dry regimes and extreme drought regimes modified microbial responses to changes in temperature. As we theorized in our second hypothesis, we found that water and N co-limited soil microorganisms. Furthermore, N addition increased the abundance of microbial functional groups and altered microbial community composition in the meadow steppe only under conditions of high water availability. We showed that there was either no change or negative influences of N addition on the community when water was the limiting condition. Overall, our results highlight that soil microbial communities in the temperate steppes of northern China are highly dependent on changes in precipitation in response to shifted temperature and N availability.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.06.022.

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