Applied Soil Ecology 108 (2016) 128–135

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Opposing effects of nitrogen and water addition on soil bacterial and fungal communities in the Inner Mongolia steppe: A field experiment

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ARTICLE INFO

Article history:
Received 28 September 2014
Received in revised form 2 August 2016
Accepted 3 August 2016
Available online xxx

Keywords:
Soil microbial community
Steppe
Irrigation
Fertilization
PLFA

ABSTRACT

Grasslands are important ecosystems and make up 40% of the terrestrial ecosystems worldwide. The Inner Mongolia steppe is the main grassland region of China, and nitrogen (N) and water availability are two important factors that limit the productivity of these grasslands. We tested how N and water addition influence the composition of the microbial community in the soil using PLFA, and soil physical and chemical properties in two semiarid grassland sites in Inner Mongolia during two consecutive years. In both sites, a split-plot design was employed with two water treatments (natural precipitation, stimulated wet year precipitation) and three N treatments (0 kg N ha^{-1}, 25 kg N ha^{-1}, 50 kg N ha^{-1}). Water addition greatly increased soil fungi and decreased bacteria while N had opposite effects. Water addition resulted in a significant increase in soil pH and electric conductivity. N addition did not lead to consistent changes in soil characteristics. Multivariate analysis showed that PLFA composition varied between all treatments but was mainly influenced by water addition. This study provides insight into how climatic changes such as alternations in rainfall and N deposition shape the soil microbial communities in Inner Mongolia steppes.

1. Introduction

Grasslands make up approximately 40% of the terrestrial land surface and grassland ecosystems are affected by human induced climatic changes (Wang and Zhou, 2012). Carbon (C) sequestration by vegetation and soil can be an important mean of mitigating increasing CO_2 concentrations in atmosphere (Batjes, 1998), and temperate grassland ecosystems may be an important C sink (Frank, 2002). Even though N is the major element in the atmosphere, N availability is one of the main factors that limits plant growth and most grassland ecosystems are N limited (Vitousek and Howarth, 1991; Bai et al., 2008). N-enrichment in grasslands often increases aboveground primary production and causes a decline in plant species richness (Tilman, 1987; Clark and Tilman, 2008; Pierik et al., 2011).

N-Enrichment can also affect the abundance of soil-dwelling organisms such as bacteria and fungi (Bardgett et al., 1999), and can result in changes in the activity or composition of soil microbes (Fierer et al., 2012). A meta-analysis of 82 N addition studies showed that on average microbial biomass declined by 15% by N fertilization and that the strength of the effects was positively related to the amount of N added and the duration of the experiment (Treseder, 2008). The effects of N addition are closely linked to carbon dynamics and mineralization in the soil (Sinsabaugh et al., 2005; Waldrop and Zak, 2006), which in turn influence soil microbes, as the soil microbial community is sensitive to soil carbon availability (Drenovsky et al., 2004).

Soil water plays an important role in the activity of the soil microbial community. It influences N-mineralization mediated by microbes (Araya et al., 2013). Also, soil water plays a key role in the transportation of nutrients, in cellular metabolism and serves as a medium for bacterial mobility (Drenovsky et al., 2004). Changes in soil water, therefore, can cause changes in the physiology and structure of the soil microbial community (Paul et al., 2003). A

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http://dx.doi.org/10.1016/j.apsoil.2016.08.008
0929-1393/

study in temperate grassland in the UK, for example, showed that changes in soil water content resulted in differences in soil bacterial and fungal phospholipid fatty acid (PLFA) patterns (Bardgett et al., 1999). Soil water availability can also impact substrate availability and soil properties, which in turn, impact the soil microbial composition and activity (Han et al., 2007). Soil microbes are important for below ground N availability, and many processes that are driven by soil microbes such as the decomposition of soil organic matter, mineralization and biological N fixation, are affected by N and water availability (Inglett et al., 2011). Hence, water and N availability interactively affect the soil microbial community and grassland ecosystem functioning.

In this study, we examined the effects of N and water addition on the soil microbial community of the Inner Mongolia steppe ecosystem, which is a preeminent part of the Eurasian continent (Chen et al., 2011). The net primary productivity of these steppe ecosystems is largely determined by N and water availability (Chen et al., 2011). We determined the microbial community and soil abiotic characteristics in a long-term field experiment with water and N addition treatments, with an identical set-up at two sites. Other studies conducted at the same site that examined the effects of N and water addition on these steppe ecosystems, have focused on net primary productivity (Li et al., 2011), plant species composition (Chen et al., 2011), and how tradeoffs between N and water-usage-efficiency affect dominant plant species of the semiarid steppe of Inner Mongolia (Gong et al., 2011). How the soil microbial community responds to water and N addition in the Inner Mongolia Steppe, and what part of the soil microbial community is influenced by these treatments has not received much attention so far (Bi et al., 2012). In this study, we use phospholipid fatty acid analysis (PLFA) to examine how three levels of N addition (0, 25 and, 50 kg N ha^{-1}) in plots with and without water addition influence soil microbial communities. PLFA analysis has been used in many studies as a reliable method to assess the structure of the soil microbial community (Green and Scow, 2000; Bardgett and Walker, 2004; Bååth and Anderson, 2003).

The objectives of our study were to determine how soil microbial communities change in response to N and water addition. We hypothesized that N will positively influence bacteria and have a negative effect on fungi, and that water addition will have the opposite effect. We also determined the effects of water and N addition on a range of abiotic soil properties, and analyzed how soil microbial community composition in the experimental plots was related to soil abiotic factors.

2. Materials and methods

2.1. Study area

The study was conducted at the Inner Mongolian Grassland Ecosystem Research Station (IMGERS), located in the Xilin River Basin (43°26′ – 44°9′N, 115°2′ – 117°2′E), Inner Mongolia, P.R. China. The average annual precipitation is 343 mm, 80% of which falls during the months May to September. The soil type is classified as Calcic Chernozems.

2.2. Experimental design

Two experimental sites fenced in 2005 were used in this study. The dominant plant species are perennial bunch grasses, perennial rhizome grasses, perennial forbs, and shrubs. The experimental design has been reported previously (Gong et al., 2011). Briefly, the experiment was set-up using a two-factorial split-plot design. The main plots have two water supply levels (natural precipitation and simulated wet year precipitation) divided into four blocks at each site. The subplots have three N addition levels (unfertilized control, 25 kg N ha^{-1} and 50 kg N ha^{-1}, N0, N25 and N50 respectively) with four replicates, one replicate of each N-level per block. Each subplot was 5×8 m in size. Main plots and subplots were separated by 3 m and 0.8 m walkways respectively. The same experimental design was established at two sites. Both experimental areas are 0.2 ha in size and have similar vegetation composition but the level of grazing prior to the start of the experiment was higher at Site 2 than at Site 1 (Li et al., 2011). The sites are 3 km apart. The experiment started in 2005, and annually since 2005 the vegetation was cut at 3 cm above the ground manually and the biomass removed.

2.3. N Application and irrigation

To make sure that each plot received the same amount of fertilizer, granular urea NH_2CONH_2 (1.5 mm diameter, Luxi Company, China) was mixed with air-dried and fine-sieved (<2 mm) soil particles at a ratio of 1:10 and then spread manually on May 15th each year.

The simulated wet year's amount of precipitation was 431 mm based on the long-term rainfall data (1982–2003) obtained from the meteorological station at IMGERS. To simulate the amount and distribution of the wet year precipitation from May to September, additional irrigation was applied at 10-day intervals using a pump-line injector system when wind was at a minimum (often at sunset). If the actual rainfall in a given 10-day interval during the experimental period was greater than the average in the same period of the predicted wet year simulation, no additional irrigation was applied during this 10-day interval and the amount of irrigation during the following 10-day interval would be adjusted accordingly.

2.4. Soil sampling

Soil samples were collected on August 18th 2011 and August 20th, 2012. In each plot, three cores were randomly taken with an auger (3.5 cm diameter) at 0–15 cm depth. Soil samples were mixed up evenly in a plastic bag in order to get a composite sample from each plot of about 400 g. Ten gram subsample of soil was immediately sieved (425 μm) and kept separately in an ice box filled with ice (Schnecker et al., 2012) and was transported to Nankai University, Tianjin, P.R. China within 1 day, and then stored at −20 °C for PLFA analysis. The other subsample of soil was stored at 4 °C. About 150 g subsample of soil was used to analyze soil total nitrogen (TN), soil total carbon (TC), electrical conductivity (EC), soil pH and soil moisture content.

2.5. Soil physicochemical measurements

Soil pH was measured in a 1:2 soil–distilled H_2O suspension with a glass electrode (Sartorius PB-10). Soil EC was determined using a Conductivity Meter (DDS-307A) using 4 g air-dried soil and 20 g deionized water. Soil total carbon (TC) of samples collected in 2011 was analyzed using the Potassium dichromate oxidation method, soil total nitrogen (TN) was analyzed using a Semi-micro kjeldahl method (Bao, 2000) and samples collected in 2012 were analyzed using an elemental analyzer (Elemantar Vario EL cube). Soil moisture was measured by oven-drying a 10 g subsample of soil at 105 °C until constant weight.

2.6. PLFA analysis

The biomass and composition of the soil microbial community were assessed by analyzing the PLFA composition of the soil, using the method outlined in Schutter and Dick (2000). Briefly, 50 ml

centrifuge tubes filled with 6 g field-moist soil were mixed with 15 ml of 0.2 M methanolic KOH. The mixture was vortexed every 10 min during one hour while being incubated at 37 °C. Hereafter, 3 ml of 1.0 M acetic acid was added to the tubes to neutralize the pH. Ten milliliter of hexane was then added to the tubes. After centrifugation at 4800g for 15 min, the hexane layer was transferred to a clear test tube, and evaporated under a stream of N_2. Finally, 0.5 ml of 1:1 hexane:methyl tertiary butyl ether was added to dissolve fatty acid methyl esters (FAMEs).

FAMEs were determined using an Agilent 7890-5975C gas chromatography mass spectrometer equipped with a HP-5MS column (length: 30 m × diameter: 0.25 mm × film: 0.25 μm). As external standard a supelco acterial acid methyl esters mix (catalog No. 47080-U) was used. For each sample the abundance of

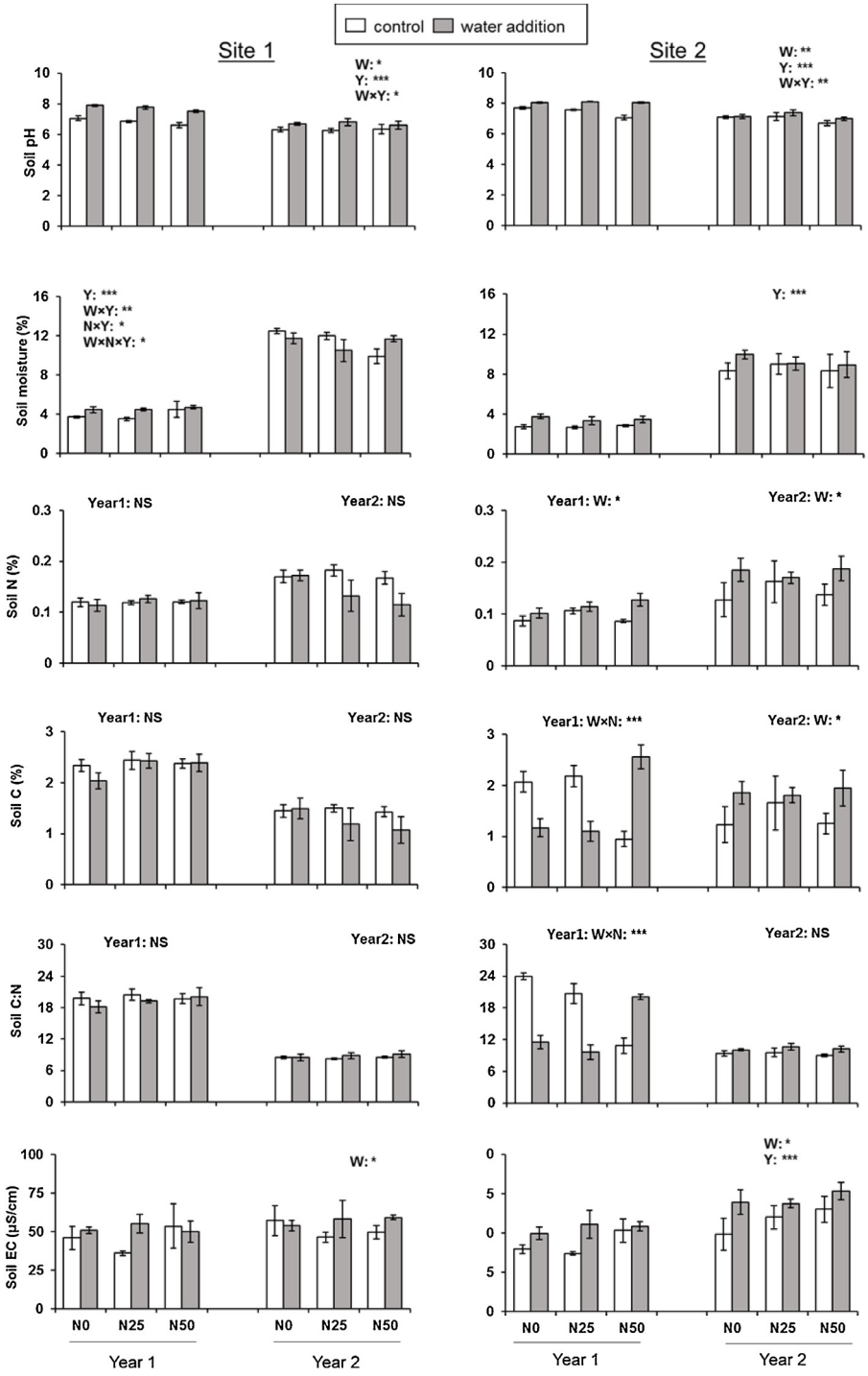

Fig. 1. Soil properties of the two experimental sites during the two sampling years. Means are shown ± 1 SE. N0, N25, N50 represent the three nitrogen treatments. White bars represent treatments without additional water, grey bars represent the water addition treatment. N – nitrogen; C – carbon. EC – electric conductivity. Significant effects based on a repeated measures split-plot ANOVA are also presented: Y: year effect, N: nitrogen effect; W: water effect. Data for N, C and C:N are analyzed separately for each year using ANOVA. Full statistical output is presented in Tables S1 and S2.

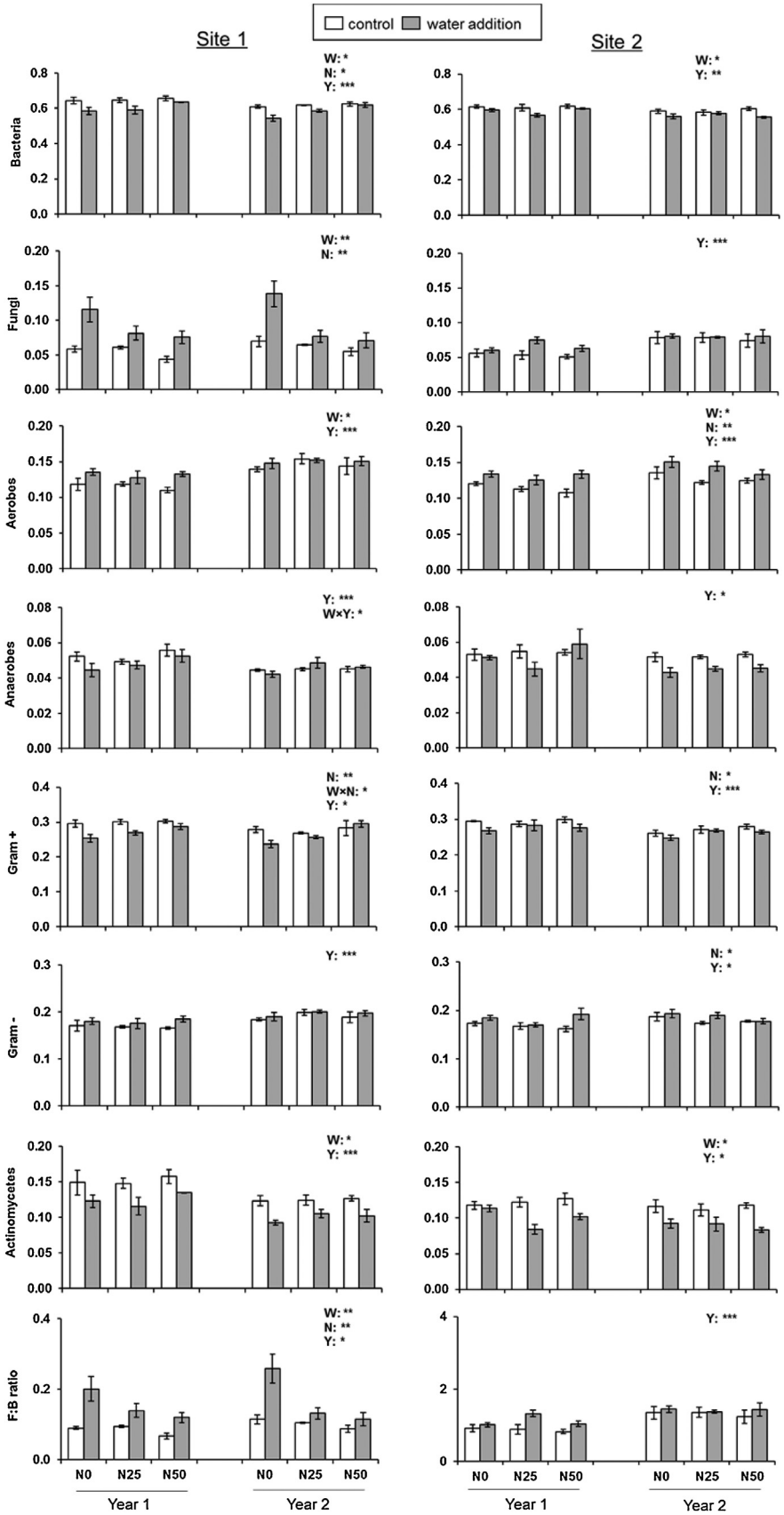

Fig. 2. Soil microbial community of the two experimental sites during the two sampling years. Shown are mean (±1 SE) proportions of PLFA allocated to different groups of soil microbes (see methods for more details). N0, N25, N50 represent the three nitrogen treatments. White bars represent treatments without additional water, grey bars represent the water addition treatment. Gram+ indicates gram positive bacteria; Gram- indicates gram negative bacteria; F:B ratio indicates fungi to bacteria ratio. Significant effects based on a repeated measures split-plot ANOVA are also presented: Y: year effect, N: nitrogen effect; W: water effect. Full statistical output is presented in Table S3.

individual fatty acid methyl-esters was expressed as percentage of the total PLFA of the sample.

Fatty acid nomenclature followed the description of Frostegård et al. (1991). The fatty acid 18:2ω6 was chosen to represent fungi (Frostegård and Bååth, 1996). 15:0, i15:0, a15:0, i17:0, a17:0, 17:0, i19:0, i16:0, 16:1ω7, 16:1ω9, 18:1ω7, cy17:0, cy19:0 were used as indicator of bacteria (Frostegård and Bååth, 1996). 16:1ω7, 16:1ω7t and 18:1ω7 were chosen to represent aerobic bacteria and cy17:0 and cy19:0 to represent anaerobic bacteria (Vestal and White, 1989). Mono-unsaturated fatty acids and cy19:0, cy17:0 were used to represent Gram negative bacteria (Zelles, 1997), i15:0, a15:0, i16:0, i17:0, a17:0 to represent Gram positive bacteria (Frostegård et al., 1991). 10Me18:0 and 10Me17:0 were used as an indicator of actinobacteria (Aliasgharzad et al., 2010).

2.7. Statistical analysis

The effects of N and water on soil microbial community and soil properties were analyzed using split-plot repeated measures ANOVA with water, N and year and the interactions as main factors and N nested in water nested in block as error structure (block/water/N). The analysis was done for each site separately. As the TC and TN was measured using different methods in the two years, TC, TN and C:N were analyzed separately for each year using ANOVA. These analyses were done in R (version 3.0.1, R Development Core Team, 2013). The relationship between PLFA composition, and soil characteristics (pH, TN, TC, moisture and C:N ratio) were analyzed using principal component analysis (PCA) and redundancy analysis (RDA). Year was also included as factor in these analyses. Significances in multivariate analyses were inferred by Monte Carlo permutation tests (999 permutations). To correct for multiple testing Bonferroni correction was used. The multivariate analysis was performed using CANOCO, version 5.03 (:Šmilauer and Lepš, 2014).

3. Results

3.1. Soil properties

At Site 1, water addition significantly increased soil pH and EC (Fig. 1). For soil pH this effect was stronger during the first sampling year resulting in a significant water × year interaction

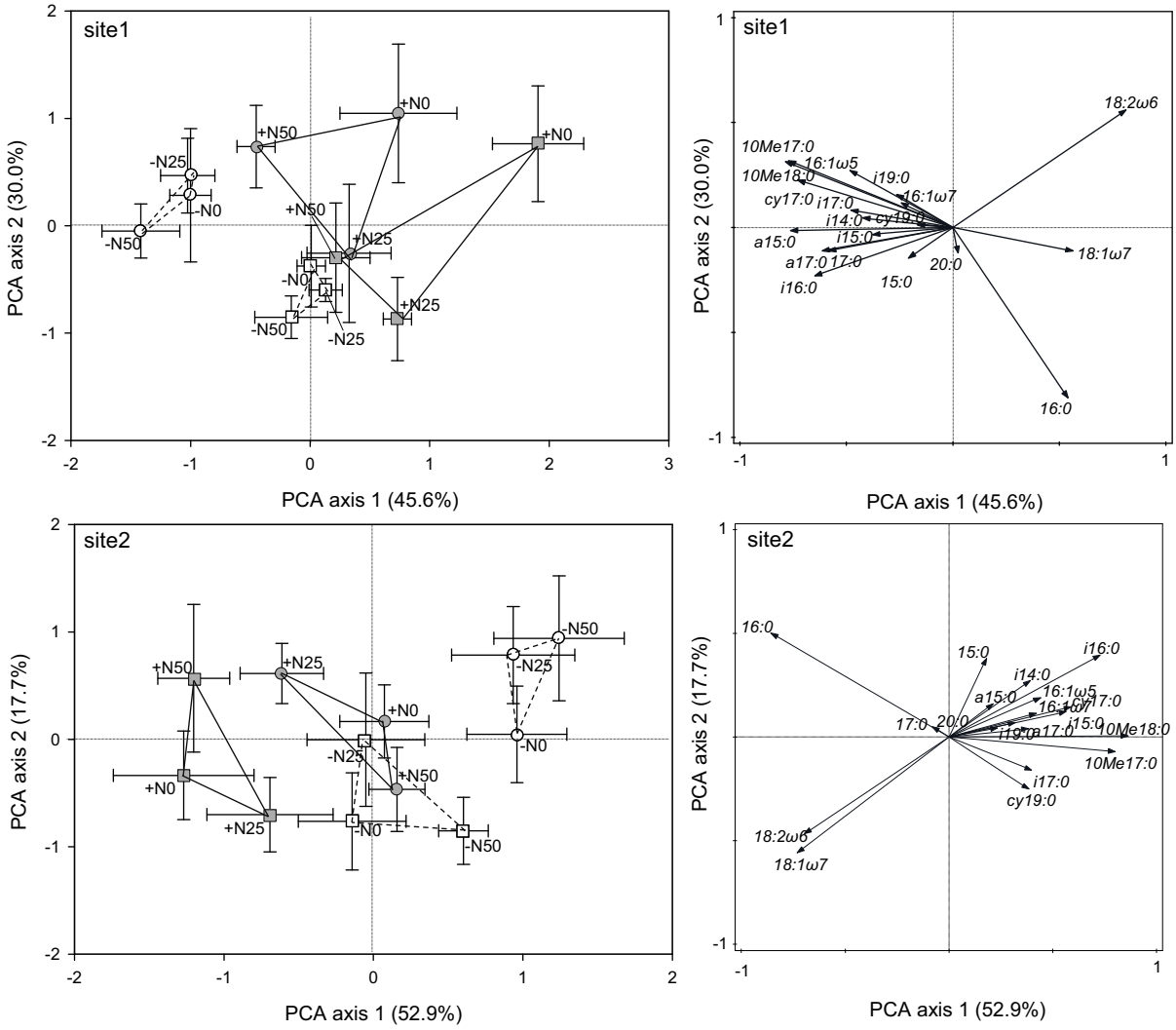

Fig. 3. Ordination plots showing the first and the second axes of a principal component analyses (PCA) of the soil PLFA profiles for Site 1 and Site 2. The mean scores (±SE) from each treatment/year combination for each site derived from the PCA are presented in the left panels. The PLFAs (denoted as arrows) are presented in the right two panels. In the left two panels the three nitrogen addition treatments (N0, N25, N50) within each water treatment (grey symbols indicate water addition treatment ("+" sign); white symbols indicate control "−" sign) are connected with lines. Squares indicate samples that were collected in year 1; circles indicate samples collected in year 2.

(Table S1). Soil pH, was lower in the second year than in the first year. In plots with no water addition nitrogen addition increased soil moisture during the first year while it caused a decrease during the second year. This resulted in a significant water × nitrogen × year interaction.

At Site 2, water addition significantly increased soil pH, soil N and soil EC. N addition caused a decrease in soil pH but the effects of water and N addition also varied between years (Fig. 1). In year 1, for the N0 and N25 treatments, water addition caused a decrease in soil C while the opposite was true for the N50 treatment.

3.2. Soil microbial community

At Site 1, water addition significantly decreased the relative proportion of PLFA for total bacteria and actinobacteria, and increased soil fungal PLFA, fungi:bacteria ratio and aerobes (Fig. 2; Table S3). N addition significantly increased total bacteria and gram positive bacteria, and decreased soil fungi and the fungi: bacteria ratio. All soil microbial groups were significantly influenced by year except fungi. The proportion of gram positive bacteria increased with N addition but only in plots which received additional water resulting in a significant interaction between water and N (Fig. 2; Table S3).

At Site2, water addition significantly increased the proportion of soil aerobes, and decreased the total proportion of soil bacteria and soil actinobacteria. N addition significantly increased gram positive and gram negative bacteria and decreased aerobes. Soil microbial groups also significantly differed between the two sampling years (Fig. 2; Table S3).

3.3. Analyses of soil microbial community composition

The first two axes of a PCA explained 75.6% and 70.6% of variation in PLFA composition at Site 1 and 2 respectively (Fig. 3). At both sites, bacterial PLFAs were negatively correlated with the fungal PLFA 18:2ω6. For both sites, the first axis corresponded most to the water treatment (grey vs white in Fig. 3) while the second axis separated the two years (squares vs circles symbols). Plots with different N treatments also separated from each other in the PCAs but not in a consistent manner (Fig. 3).

Together, soil properties accounted for 34.7% variation at Site 1 (RDA: pseudo-F = 3.6, P = 0.001) in PLFA composition. Among the soil characteristics, soil pH (RDA: pseudo-F = 8.4, P = 0.007; 13.0% explained variation) was the only significant explanatory variable for PLFA composition. At Site 2, soil properties explained 24.0% of the variation in PLFA composition however the overall model was not statistically significant (RDA: pseudo-F = 1.5, P = 0.065).

4. Discussion

This study shows that N and water addition can have opposite effects on soil bacterial and fungal communities. N addition significantly decreased the relative abundance of soil fungal PLFA, while water addition caused an increase. The opposite was true for bacterial PLFA that increased after N addition and decreased after long-term water addition. Water addition also caused an increase in soil pH and EC. Our study clearly shows that the effects of water and N addition are not simply additive. Moreover, an important result of this study is that the effects of water and N addition differed between the two sites, even though the experimental design was exactly the same and the sites were located only three kilometers apart. The multivariate analysis of the PLFA community showed that PLFA composition was explained mainly by soil pH and by the water treatment and not consistently by the N addition treatment.

4.1. Effects of water addition

Several studies have shown that soil water content is a major factor in influencing soil microbial composition and activity (Drenovsky et al., 2004; Guenet et al., 2012). A field study with seven years of continuous water addition in tall grass prairie soil, for example, showed that soil fungal biomass was enhanced and that the fungi to bacteria ratio increased (Williams and Rice, 2007). Similarly, in our study we observed that the proportion of fungal PLFA increased in plots that had been exposed to water addition for six years. One possible explanation for these results may be that fungi suffer from drying and rewetting stress which was reduced in plots in the water addition treatment (e.g. Denef et al., 2001). Alternatively, fungal enzymatic activity may also be influenced by soil moisture content (Hinojosa et al., 2004; Alarcón-Gutiérrez et al., 2010).

Several authors have argued that soil fungi are more sensitive to water stress than bacteria (Gordon et al., 2008). Most fungi are located in the outer part of soil particles, and this may explain why they are more susceptible to drying and rewetting than bacteria that are mainly located in the small pores between soil particles (Hattori, 1988). In our study the proportion of bacterial PLFA was negatively affected by water addition while the proportion of fungal PLFA was positively affected. As we do not have information about the absolute biomass of bacteria and fungi in the soil, we cannot confirm with our study that bacteria are less sensitive to water treatments than fungi. However, our results show that bacteria and fungi respond to water addition in opposite ways. Bacteria and fungi can respond in different ways to variation in abiotic conditions such as soil moisture and pH (Rousk et al., 2010; Fierer et al., 2003). This may explain the differences between bacteria and fungi that we observed in our study. However, the effects caused by water addition on the soil microbial community might also be indirect, e.g. via the effects of water on carbon input into the soil. In the semi-arid ecosystems that we studied, water addition for example, often leads to greater belowground plant productivity (Chen et al., 2011; Williams, 2007).

At both experimental sites, soil moisture levels were higher in plots that received additional water only during the first sampling year. In contrast, during the second year there were no differences in moisture content between control plots and plots with additional water. During this year, there was heavy rainfall for several days before we sampled the soil. In the experiment, additional irrigation is not applied if the actual rainfall in a given 10-day interval is greater than the average in the same period of the predicted wet year simulation. Therefore, no additional water was applied before sampling during this year. This can also explain why the soil moisture levels at both experimental sites were much higher during the second year. It is important to note that, although moisture levels were not different at the time of sampling, the microbial communities in the soil had been exposed to the water treatments for six years already. Indeed, the PCA plot showed that the long-term water treatment had altered the composition of the soil microbial community also in the second year. However, the PCA plots also shows that there were distinct differences between the two years, indicating that local variation in rainfall can greatly influence soil microbial communities.

4.2. Effects of N addition

In our study, in Site 1, N addition increased the proportion of bacterial PLFA and decreased the proportion of fungal PLFA. This decrease in fungal PLFA was particularly strong in plots with additional water. Similarly, De Vries et al. (2006) found an inverse relationship between fungal biomass and N application in pastures in the Netherlands, while a study conducted in forest soil reported

that N addition causes a reduction in soil microbial activity and the fungal-to-bacteria ratio (Bowden et al., 2004; Magill et al., 2004; Wallenstein et al., 2006). In conditions of increased soil N, the enzyme activity of fungi can be inhibited (Fog, 1988). This subsequently leads to a decrease in fungal biomass and may provide an explanation for the negative effect of N addition on the soil fungal community that we observed in our study.

N fertilization generally causes an increase in plant production. This can have a positive effect on the activity of the soil microbial community, in particular the bacterial community, in the short term. Hence, this may explain the positive effect of N addition on bacterial PLFA. However, N addition can also lead to a decrease in soil microbial community biomass and activity in systems that are C limited (Kuzyakov and Xu, 2013). Soil total N was not significantly influenced by N addition in either of the two sites. The levels of N addition were low (maximum 50 kg per ha) and it is likely that in these N-limited ecosystems the additional N that became available due to fertilization has been all used by the plants and only becomes available for the soil microbial community via plant residues.

N addition did not influence most soil physiochemical properties in the present study. Other work at the same experimental site has shown that N addition caused an increase in aboveground net primary productivity (Chen et al., 2011). However, root biomass did not proportionally increase with aboveground biomass (Chen et al., 2011). We propose that this can explain why total carbon in the soil did not increase consistently in plots with N fertilization (Chen et al., 2011). Heterotrophic microorganisms are thought to be C limited when the C:N ratio is below 30, and N limited when the C:N ratio is above 30 (Kaye and Hart, 1997). The C:N ratio in our study ranged from 7 to 24. These results suggest that soil micro-organisms in our study were C limited, and that changes in soil TC may greatly influence soil microbes. Shifts in C quality and quantity of plant litter entering the soil can be the result of changes in the composition of grassland vegetation caused by N addition (Clegg, 2006). In our study the vegetation was mowed and the biomass removed from the experimental sites every year, and this may have reduced the effects of N addition on the soil as we removed vegetation with greater ANPP under plots with N addition. This also means that there was more N and C output from the system in the N addition plots (Ruan et al., 2012). Alternatively, the rate of N application in our experiment may have been relatively low, even at the highest level, and hence it may not have caused clear changes in soil properties (Ruan et al., 2012).

We used PLFA as a method to qualify soil microbial communities. This technique has been used in many studies and can provide a broad overview of groups of soil bacteria. More recently, DNA and RNA sequencing techniques have provided the possibility to identify the (active) members of the microbial community in different soil samples (Barberán et al., 2012). Future studies should employ these molecular techniques to determine how the composition and functioning of the bacterial and fungal community in the soil is changed by N and water addition.

In summary, our study provides evidence that climatic changes will influence soil biotic communities in grasslands. These effects ultimately will influence the functioning and productivity of grasslands. However, our study also exemplifies that climatic changes can have different effects, even directionally. N addition significantly decreased the relative abundance of soil fungi and increased the proportion of bacteria, whereas water addition had opposite effects. The results further confirm the strong relationship between fungi and pH. Future studies should examine how these changes in composition influence the functioning of the soil food web including interactions between the soil microbial community and soil-dwelling invertebrates (such as nematodes and micro-arthropods) and the aboveground plant community.

Acknowledgements

We thank the National Basic Research Program of China (973 Program) (2007CB106802) and the National Nature Science Foundation of China (41071207). We also thank the Inner Mongolia Grassland Ecosystem Research Station of Botany Institute, Chinese Academy of Science, for providing working facilities, and two anonymous reviewers for very useful comments on a previous version of the manuscript. This is publication 6149 of the Netherlands Institute of Ecology (NIOO-KNAW).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.08.008.

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