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Rate-specific responses of prokaryotic diversity and structure to nitrogen deposition in the *Leymus chinensis* steppe

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ABSTRACT

Serious nitrogen (N) deposition in terrestrial ecosystems causes soil acidification and changes the structure and function of the microbial community. However, it is unclear how these changes are dependent on N deposition rates, other factors induced by N (e.g., pH), and their interactions. In this study, we investigated the responses of soil prokaryotic community structure and stability after a 13-year N addition in the semi-arid *Leymus chinensis* steppe in Inner Mongolia, China. Our results demonstrated that the prokaryotic community structure changed at the low N addition rate of 1.75 g N m^{-2} yr^{-1}; however, dramatic changes in microbial abundance, respiratory quotient, and prokaryotic diversity occurred at N addition rates of more than 5.25 g N m^{-2} yr^{-1} when the soil pH dropped below 6.0. The two patterns indicated the difference in driving forces for different microbial properties. The N-driven and pH-driven processes are likely the most important mechanisms determining the responses of bacterial community to N. Some copiotrophic/oligotrophic bacteria, e.g., Proteobacteria and Acidobacteria, changed their relative abundances with the N addition continuously even at a low rate, indicating that they were more sensitive to N directly. Some bacterial groups significantly changed their relative abundance at a high N addition rate when pH dropped below 6.0, e.g., Verrucomicrobia and Armatimonadetes, indicating that they were more sensitive to pH below 6.0. N addition altered the prokaryotic community structure through enrichment of copiotrophic bacteria (species adjustment) at low N addition rates and through enrichment of nitrophilous taxa and significant loss of diversity at high N rates. The results also demonstrated that a high N addition diminished the stability of the prokaryotic community structure and activity through reduction in species diversity and bacterial interaction. Overall, this study supported the hypothesis that the responses of prokaryota to N were dependent on deposition rates, and N-driven and pH-driven processes were the important mechanisms to control the shift of the prokaryotic community.

1. Introduction

Industrial pollution and agricultural practices have resulted in a substantial increase of nitrogen (N) deposition in terrestrial ecosystems since the industrial revolution (Galloway et al., 2008; Liu

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et al., 2013). Since N is a limiting nutrient to plants in most terrestrial ecosystems (Vitousek et al., 2002), N deposition often has multiple effects, including changes in aboveground primary productivity, biodiversity, species composition, and ecosystem functioning (Galloway et al., 2003; Bai et al., 2010; Robson et al., 2010; Isbell et al., 2013). Soil microbiota are recognized as key players to sustaining ecosystem functions and services, and thus elucidating the feedbacks of the soil microbiome to N deposition is fundamental to understanding the consequences of global changes on ecosystem processes regulated by soil biota. It is shown that the average N deposition rate in China has increased from 1.3 g N m^{-2} yr^{-1} to 2.1 g N m^{-2} yr^{-1} between the 1980s and 2000s (Liu et al., 2013). However, due to the complexity of soil microbial communities, common and/or ecosystem-specific responses of microbial community structure and function to different N deposition rates remain poorly understood (Bardgett et al., 2008; Ramirez et al., 2012).

N deposition can exert direct effects on soil microbial physiology and indirect effects by changing plants and soil properties (Lauber et al., 2009a; Fierer et al., 2012). Ramirez et al. (2010) show consistent effects of N fertilization on soil bacterial communities in contrasting systems – grassland and agricultural fields – and bacterial communities are more structured by N and/or soil C availability than by shifts in the plant community or soil pH associated with the elevated N inputs. Long-term fertilization results in a significant change in the microbial community structure and function linked to changes in C and N availability and shifts in aboveground plant communities in the Arctic tundra soil (Campbell et al., 2010). Previous studies have shown that N addition to a mature community may disrupt microbial community coexistence, leading to a decrease of microbial diversity (Campbell et al., 2010; Ramirez et al., 2010, 2012). However, there are fewer quantitative studies to investigate the relationships between changes in bacterial community structure, diversity and other microbial properties and N addition rates. It is unclear to what extent the observed shifts in community structure and function are dependent on the N deposition rates, other factors induced by N (e.g., pH), and their interactions. It has been a common view that there exists an N input limit, above which ecosystem functions would be seriously affected (Bai et al., 2010), although explicit tests are lacking for microbial communities.

It has been shown that both plant aboveground biomass and dissolved organic carbon increase with increasing N input across various ecosystems (Liu and Greaver, 2010; Liu et al., 2014). Consequently, microbial activities are expected to increase with the increase in C supplied by plants. However, many studies demonstrate the reductions in soil microbial biomass and respiration under N enrichment (Treseder, 2008; Liu and Greaver, 2010; Ramirez et al., 2012; Liu et al., 2014). Particularly, soil microbial biomass C and N, and soil microbial respiration are stimulated by low levels of N inputs, but are suppressed by high levels of N inputs (Treseder, 2008; Liu et al., 2014). It is likely that the mechanisms of N deposition controlling microbial community shifts are different under low and high N input.

Bacterial diversity is higher in neutral soils and lower in acidic soils (Fierer and Jackson, 2006). N inputs can lead to soil acidification and a consequent reduction in buffering capacity and accumulation of toxins, which negatively impact soil microbial activity and diversity (Lauber et al., 2009b; Liu et al., 2014). It is reasonable to hypothesize that the responses of different prokaryotic groups (e.g., N sensitive community and pH sensitive community) to N would be largely dependent on deposition rates. N sensitive members prevail in the shift of a community even at a low rate, while those more sensitive to pH do not change significantly. After soil pH drops below a certain threshold at a high N deposition rate, both pH sensitive and N sensitive communities may shift, and one of two mechanisms or their interactions may predominate in controlling microbial community structure and diversity. Thus, the N threshold significantly altering prokaryotic community structure and diversity may be different.

The steppe ecosystem in Inner Mongolia lies in the east part of Eurasian steppe, which extends over 8000 km across northeastern China, Mongolia, Russia, Ukraine, and Hungary. This region suffers N deposition, mainly ammonium (NH_4^+) in bulk deposition, in recent several decades caused by human activities and industrial pollution (Liu et al., 2013). We conducted an intensive study on the response of soil prokaryotic communities to different N deposition rates after a 13-year N addition in a semi-arid *Leymus chinensis* steppe, located in Inner Mongolia, China. Specifically, we focused on testing the following hypotheses: H1: since the sensitivities of prokaryotic community structure and diversity to N and associated factors (e.g., pH) induced by N may be different, the thresholds of N deposition rates significantly altering prokaryotic community structure and diversity may differ; H2: at a low N rate, the shift of prokaryotic community may be mainly driven by N, while at a high N rate, it may be mainly driven by N or soil pH or other environmental factors associated with the elevated nitrogen inputs; H3: microbial interaction networks will become more simplified with increased N due to a decrease in diversity. By addressing these three issues together, this study provided novel insights into the threshold and mechanisms of N deposition effects on prokaryotic community structure and diversity and microbial interactions by contrasting pH and N gradients.

2. Materials and methods

2.1. Study site description

The long-term N deposition experiment was conducted at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS), Inner Mongolia, China. The background N deposition rate in the Inner Mongolia steppe is less than 0.3 g N m^{-2} yr^{-1} (Bobbink et al., 2010; Lan and Bai, 2012). The experimental site (43°33′13″N, 116°40′31″E) is a nature *Leymus chinensis* steppe, which is a typical steppe in this region, dominated by *Leymus chinensis*, a widely distributed perennial C_3 rhizome grass in the Eurasian steppe region. The site has been fenced against grazing by large animals since 1979. The mean annual temperature is 0.3 °C, with mean monthly temperatures ranging from −21.6 °C in January to 19.0 °C in July. The mean annual precipitation is 346.1 mm with 60–80% falling during the growing season from May to August. The soil is classified as dark chestnut or loamy sand in texture. A detailed site description was given previously (Bai et al., 2010).

2.2. Experimental design and soil sampling

The N deposition experiment was established in the autumn of 1999. An experimental plot was 5 × 5 m in size and arranged in a randomized block design and separated by a 1-m buffer zone. Each block included a natural control (no nutrient addition) and six levels of N deposition rates: 0, 1.75, 5.25, 10.5, 17.5, and 28 g N m^{-2} yr^{-1} with pelletized NH_4NO_3 fertilizer. These rates corresponded to low and high N application rates in grassland management. All treatments were laid out at six replicates. To ensure that N was the only limiting nutrient, for all treatments except the control, we also supplied the same amounts of P (10 g P_2O_5 m^{-2} yr^{-1}), S (0.2 mg m^{-2} yr^{-1}) and trace elements (Zn, 190 μg m^{-2} yr^{-1}, Mn, 160 μg m^{-2} yr^{-1} and B, 31 μg m^{-2} yr^{-1}) based on local soil census data. Nutrients were uniformly applied to each plot with manual broadcasting in the mid-growing season (1–5

July) every year, coinciding with high temperatures and precipitation.

Soil samples were collected in the middle of July 2012. Each sample was a mixture of 10 individual soil cores at the depth of 0–10 cm. Soil samples were transported to the lab on ice and stored at −20 °C for genomic DNA extraction. Part of each soil sample was sieved with a 2-mm mesh to remove visible grass roots and stones, stored at 4 °C, and used for soil property measurements.

2.3. Soil property analysis

Soil pH was measured in the soil-water slurry (1:5, soil:water, w:w) using a pH meter. NH_4^+–N was quantified with the indophenol blue method, total organic carbon (TOC) with the dichromate digestion method, total nitrogen (TN) with the Kjeldahl method, and soil microbial C (C_{mic}) and N (N_{mic}) were determined by the chloroform fumigation incubation method (Weaver et al., 1994). Soil basal respiration was measured according to the method described by Bauer (Bauer et al., 1991). The respiratory quotient, Q_{CO_2}, was calculated based on Meyer et al. (1996). The abundance of bacteria and archaea in the soil was measured using quantitative real-time PCR (qRT-PCR) (see "Supplementary method").

2.4. DNA extraction and MiSeq sequencing of 16S rRNA gene amplicons

Soil genomic DNA was extracted using a method previously described (Lueders et al., 2003), and its quality was checked using a NanoDrop Spectrophotometer. Extracted DNA was diluted to 10 ng/μL and stored at −20 °C for downstream use.

Two-round PCR amplification was conducted with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) designed to be universal for bacteria and archaea (Caporaso et al., 2011, 2012). In the first-round PCR, each 25 μl reaction volume contained 1 μl of each 10 μM primer, 10 ng of template DNA, and 0.5 units of Accuprime high-fidelity Taq (Invitrogen, USA). The PCR procedure was: 94 °C for 1 min; 10 and 20 cycles were used for the 1st and 2nd round, respectively, at 94 °C for 20 s, 53 °C for 25 s, and 68 °C for 45 s; 68 °C for 10 min. Each sample was amplified with three technical replicates, and then they were mixed in one tube, purified with a gel extraction kit (QIAGEN, USA) and used as a template for the second-round PCR. In the second PCR reaction, the reaction condition was the same as that in the first-round PCR except that barcoded primers were used. After two-round amplification, PCR products from three technical replicates were quantified with electrophoresis and mixed in one tube. All samples were pooled together with equal molar amounts from each sample and purified through the QIAGEN gel extraction kit. The purified library was diluted, denatured, re-diluted, mixed with PhiX, as described in the Illumina library preparation protocols, and then applied to an Illumina Miseq system for sequencing with the Reagent Kit v2 2 × 250 bp.

2.5. Sequence data analysis

The sequence data were processed using QIIME Pipeline–Version 1.7.0 (http://qiime.org/tutorials/tutorial.html). All sequence reads were trimmed and assigned to each sample based on their barcodes. Multiple steps were required to trim the sequences, such as removal of sequences less than 220 bp. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. The aligned 16S rRNA gene sequences were used for a chimera check using the Uchime algorithm (Edgar et al., 2011). Each sample was rarefied to the same number of reads (9930 sequences) for both alpha-diversity (phylogenetic distance whole tree, chao1 estimator of richness, observed species and Shannon's diversity index) and beta-diversity (PCoA, UniFrac) analyses, for which the rarefaction curves were generated from the observed species. Taxonomy was assigned using the Ribosomal Database Project classifier (Wang et al., 2007). The original sequence data are available at the European Nucleotide Archive by accession PRJEB6148 (http://www.ebi.ac.uk/ena/data/view/PRJEB6148).

2.6. Statistical analysis

The overall structural change of soil microbial communities were evaluated by principal coordinates analysis (PCoA) in Fast UniFrac (http://bmf.colorado.edu/fastunifrac/). The statistical significance among datasets was assessed by PerMANOVA using the weighted PCoA scores in PAST (http://folk.uio.no/ohammer/past/). The Mantel test was applied to evaluate the correlations among microbial communities with environmental variables using PASSaGE (http://www.passagesoftware.net/). Environmental variables with the highest Pearson's correlation coefficients with microbial communities were selected using the BioEnv procedure, and variance partitioning analysis (VPA) based on redundancy analysis procedure was performed to quantify the relative contributions of environmental variables using the varpart procedure in the R package Vegan (http://cran.r-project.org/web/packages/vegan/index.html). The untransformed data of microbial relative abundance at the OTU level were used as input data. Differences in relative abundances of taxonomic units between samples were tested by one-way-analysis of variance (ANOVA). The linear or non-linear correlations between microbial diversity, species abundance and soil properties, and environmental factors were analyzed using SPSS 17.0 software.

To evaluate the effect of chronic N deposition on the stability of soil ecosystems, we used the coefficient of variation of microbial biomass, soil respiration, the quantity of microbes, and microbial diversity as indices of soil microbial ecosystem stability. The higher variation means less stability. The coefficient of variation was calculated as previously described (Bai et al., 2004, 2010).

2.7. Network analysis of prokaryotic communities

The artificial neural network (ANN) method was used to establish a model between the relative abundance of main taxa and environmental variables using BANJO v2.2.0 software (http://www.cs.duke.edu/~amink/software/banjo/) (Smith et al., 2006). The relative abundances of main phyla, which significantly correlated with environmental factors ($p < 0.01$), were merged into a single input matrix in the ANN analysis, and all the soil property data were normalized. The predicted results were derived using Eureqa v0.99 beta software (Schmidt and Lipson, 2009).

To understand the interactions among different species within a community and their responses to environmental changes, the phylogenetic molecular ecological networks (pMENs) (Deng et al., 2012) were constructed following the molecular ecological network analyses pipeline (MENAP) (http://ieg2.ou.edu/MENA/main.cgi) with default parameters based on OTU data calculated from 16S rRNA gene sequencing data. Compared to other network construction methods, this approach is remarkable in that the network is automatically defined and robust to noise, thus providing excellent solutions to several common issues associated with high-throughput metagenomic data (Deng et al., 2012). The pMENs construction requires at least 8 replicate measurements; thus, we merged the data from natural grassland control and 0 g N m^{-2} yr^{-1} N addition treatment into a no-N treatment dataset (a total of 12 samples), data from 1.75 and 5.25 g N m^{-2} yr^{-1}

treatments into a low N dataset (a total of 12 samples), and those from 10.5, 17.5, and 28 g N m^{-2} yr^{-1} into a high N dataset (a total of 18 samples). Only the OTUs detected in more than half of the replicates were kept for network construction. Modules were detected by fast greedy modularity optimization. Modules with a total of 9 joint submodules with more than 6 nodes were used for eigengene network analysis. Cytoscape 3.0.2 software was used to visualize the network graphs (Saito et al., 2012).

3. Results

3.1. Soil microbial and chemical properties

All the measured soil microbial and chemical properties did not show significant differences except soil microbial C (C_{mic}), C/N ratio, and pH between the control and the treatment without N but supplied with other nutrients. However, N addition increased the extractable NH_4^+ concentration (from 1.1 to 267 mg kg^{-1}). Soil pH (from 7.93 to 5.34) decreased to below 6 at N addition rates higher than 5.25 g N m^{-2} yr^{-1}, then it kept stable at high N addition rates (Table 1). Soil C_{mic} and microbial N (N_{mic}) decreased significantly with N addition. Especially, when N deposition rate was higher than 5.25 g N m^{-2} yr^{-1}, N addition decreased soil C_{mic} by more than 89% and N_{mic} by more than 60%. N addition tended to suppress respiration and increase respiratory quotient Q_{CO_2} at high N rates, indicating more stress for microbial communities at high N.

3.2. Effects of N deposition on the prokaryotic community diversity and structure

In total, 849,878 high quality and chimera-free reads were obtained by MiSeq sequencing of 16S rRNA gene amplicons with 11,622 to 27,660 reads per sample. Both sequencing and qRT-PCR data showed that the relative abundance of bacteria was more than 98.4% and 99.3%, while archaea had less than 1.5% and 0.7%, respectively. Observed numbers of OTUs and microbial alpha-diversity indices based on the phylogenetic distance whole tree, Chao1 richness, and Shannon's diversity were negatively correlated to the N addition rate ($p < 0.05$); however, these parameters were not significantly decreased at low N addition (0–5.25 g N m^{-2} yr^{-1}) until the N addition rate was higher than 5.25 g N m^{-2} yr^{-1} (Table 2). The abundance of bacterial and archaeal cells quantified by qRT-PCR reduced significantly when the N rate was higher than 5.25 g N m^{-2} yr^{-1}, while the ratio of bacteria to archaea increased significantly, indicating that archaeal communities were more sensitive to high N than were bacterial communities (Table 1).

Table 2
Prokaryotic diversity indices at 97% sequence similarity of 16S rRNA gene sequence calculated based on 9930 sequences for each sample.

N deposition rate (g N m^{-2} yr^{-1})	Phylogenetic distance whole tree	Chao1 estimator of richness	Observed species	Shannon's diversity index
Control	136.9 ± 1.4a	4427 ± 66a	2137 ± 28a	9.30 ± 0.03a
0	138.8 ± 1.7a	4569 ± 94a	2154 ± 35a	9.30 ± 0.05a
1.75	134.9 ± 1.7a	4277 ± 70a	2095 ± 32a	9.25 ± 0.07a
5.25	133.7 ± 1.7a	4177 ± 89a	2056 ± 34a	9.17 ± 0.05a
10.5	117.8 ± 2.1b	3378 ± 94b	1728 ± 37b	8.78 ± 0.06b
17.5	100.2 ± 6.3c	2838 ± 259c	1434 ± 107c	8.20 ± 0.16c
28	79.8 ± 4.3d	2096 ± 130d	1046 ± 62d	7.19 ± 0.14d

To understand the effect of N deposition on the overall prokaryotic community structure, principal coordinate analysis (PCoA) was used to reveal their differences. The results showed that the prokaryotic community structure was significantly different (permANOVA tests, $p < 0.01$) between the control and the treatment samples, and among treatment samples with different N addition rates (Fig. 1) except the samples between natural control and zero N addition treatment ($p = 0.174$), and the samples between 1.75 and 5.25 g N m^{-2} yr^{-1} treatment ($p = 0.158$). The prokaryotic community structure changed significantly even at 1.75 g N m^{-2} yr^{-1} compared to that without N addition. When the N addition rate was more than 5.25 g N m^{-2} yr^{-1}, the prokaryotic community structure shifted to different directions (Fig. 1).

Different N addition rates appeared to be the main factor in the first principle coordinate axis (PCo1), which contributed 68.6% of total variation (Fig. 1A), and such a pattern was confirmed by hierarchical clustering based on the UniFrac distance matrix (Fig. 1B). The distance between prokaryotic community structures in natural control and low N treatment (0–5.25 g N m^{-2} yr^{-1}) was small, but it became larger with higher N addition rates (5.25 g N m^{-2} yr^{-1} and higher), indicating higher variation of microbial community structure under high N. The higher variation means less stability in the structure and activity of a microbial system. This was further corroborated by the higher coefficient of variation (CV) of prokaryotic diversity index, microbial biomass C and soil respiration (R) over six replicates at high N addition rates (Fig. S1).

Table 1
Soil and microbial properties at different N deposition rates.

	Control	0[a]	1.75[a]	5.25[a]	10.5[a]	17.5[a]	28[a]
pH	7.93 ± 0.14a	7.19 ± 0.12b	6.46 ± 0.07c	6.22 ± 0.12c	5.64 ± 0.14d	5.61 ± 0.38d	5.34 ± 0.09d
TN (%)	0.23 ± 0.01bc	0.23 ± 0.01c	0.23 ± 0.01bc	0.26 ± 0.01a	0.23 ± 0.01bc	0.24 ± 0.01abc	0.25 ± 0.01ab
TOC (%)	2.35 ± 0.12b	2.5 ± 0.08ab	2.34 ± 0.06b	2.64 ± 0.1a	2.39 ± 0.08ab	2.46 ± 0.06ab	2.46 ± 0.09ab
C/N	10.1 ± 0.21c	10.94 ± 0.1a	10.12 ± 0.33bc	10.13 ± 0.11bc	10.36 ± 0.27abc	10.44 ± 0.26ab	9.68 ± 0.12c
NH_4^+–N (mg kg^{-1} dry soil)	3.31 ± 2.19d	1.1 ± 0.19d	2.25 ± 1.1d	1.38 ± 0.26d	73.21 ± 11.07c	137.58 ± 25.57b	267.1 ± 37.21a
C_{mic} (mg kg^{-1} dry soil)	1126.51 ± 51.88b	1293.06 ± 26.8a	789.92 ± 42.55c	697.51 ± 52.99c	142.6 ± 16.23d	149.56 ± 63.73d	137.69 ± 21.75d
N_{mic} (mg kg^{-1} dry soil)	145.38 ± 6.93a	144.85 ± 3.63a	103.56 ± 6.12b	90.51 ± 7.55b	57.77 ± 13.98c	32.22 ± 16.32cd	28.71 ± 7.06d
C_{mic}/N_{mic}	7.80 ± 0.41a	8.96 ± 0.3a	7.71 ± 0.47a	7.76 ± 0.3a	2.12 ± 0.54b	7.01 ± 2.42a	6.77 ± 1.72a
R (mg CO_2–C kg^{-1} dry soil d^{-1})	37.29 ± 0.87a	28.21 ± 1.51ab	33.63 ± 1.54a	35.74 ± 3.38a	31.06 ± 2.62ab	29.52 ± 6.24ab	21.66 ± 4.43b
Q_{CO_2} (mg CO_2–C g^{-1} C_{mic} d^{-1})	33.54 ± 2.09c	21.9 ± 1.39c	42.78 ± 1.52c	53.34 ± 7.11c	206.69 ± 20.21a	138.77 ± 56.62b	114.38 ± 16.98b
Bacteria (cells g^{-1} dry soil, $\times 10^8$)	269 ± 51a	192 ± 41ab	263 ± 22a	239 ± 27a	131 ± 21bc	92 ± 14c	60 ± 10c
Archaea (cells g^{-1} dry soil, $\times 10^6$)	109 ± 8a	100 ± 17a	104 ± 10a	95 ± 10a	35 ± 5b	24 ± 4b	10 ± 2b
Bacteria/Archaea	253.82 ± 51.7c	185.19 ± 13.36c	260.43 ± 20.8c	252.16 ± 6.57c	383.72 ± 47.39b	398.88 ± 38.95b	637.54 ± 44.11a
Plant biomass (g m^{-2})	204.79 ± 18.93d	204.72 ± 18.12d	339.54 ± 15.84cd	455.93 ± 46.66bc	537.28 ± 62.34ab	604.27 ± 71.64a	603.16 ± 51.58a
Plant richness	8 ± 0.73a	7.5 ± 1.15ab	7 ± 0.82ab	7 ± 0.93ab	5.67 ± 0.67abc	5.17 ± 0.6bc	3.83 ± 0.7c

C_{mic}: soil microbial biomass C; N_{mic}: soil microbial biomass N; Q_{CO_2}: respiratory quotient; R: respiration rate.
[a] Deposition rate (g N m^{-2} yr^{-1}). Soil samples were collected at the depth of 0–10 cm. Values with different letters in a row mean significant difference at $p = 0.05$. Values are means of six replicates ± SE.

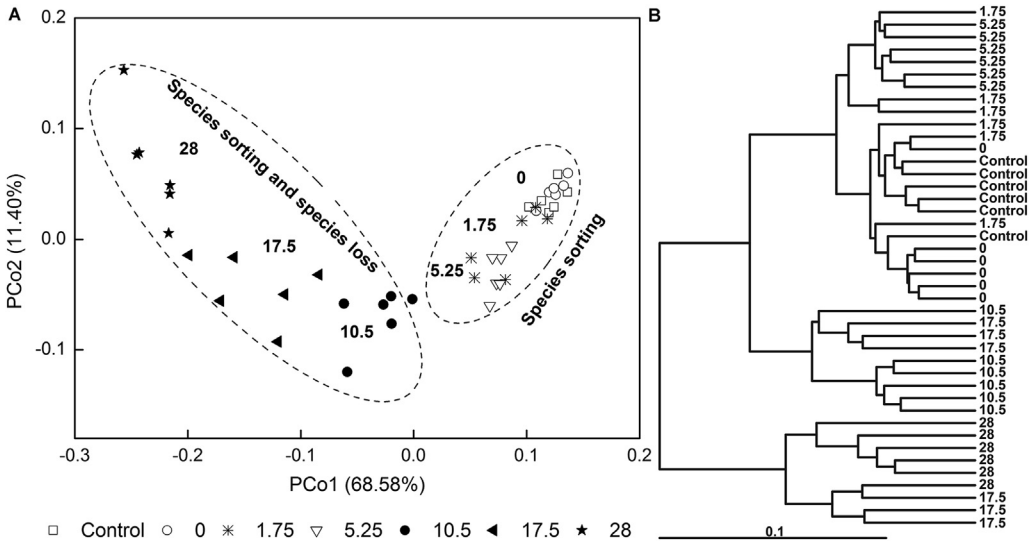


Fig. 1. Overall structure changes of soil prokaryote. (A) PCoA score plot based on weighted UniFrac metrics; (B) Clustering of soil prokaryote based on the Unifrac distances calculated in Fast UniFrac.

3.3. Effects of N deposition on the phylogenetic composition of soil prokaryotic community

Overall, the N deposition increased the relative abundance of Proteobacteria, Bacteroidetes, Firmicutes, Armatimonadetes, while decreased the relative abundance of Acidobacteria, Verrucomicrobia, Chloroflexi, Gemmatimonadetes, Nitrospirae, and Planctomycetes at the phylum level (Fig. 2 and Table S1). However, two patterns of the relative abundance vs N addition rate were observed at the phylum level and lower taxon ranks. Some bacterial groups decreased or increased continuously with increasing N addition rates, e.g., Proteobacteria and Acidobacteria. Some other bacterial groups did not change significantly at low N rates (less than 5.25 g N m^{-2} yr^{-1}), but significantly decreased or increased

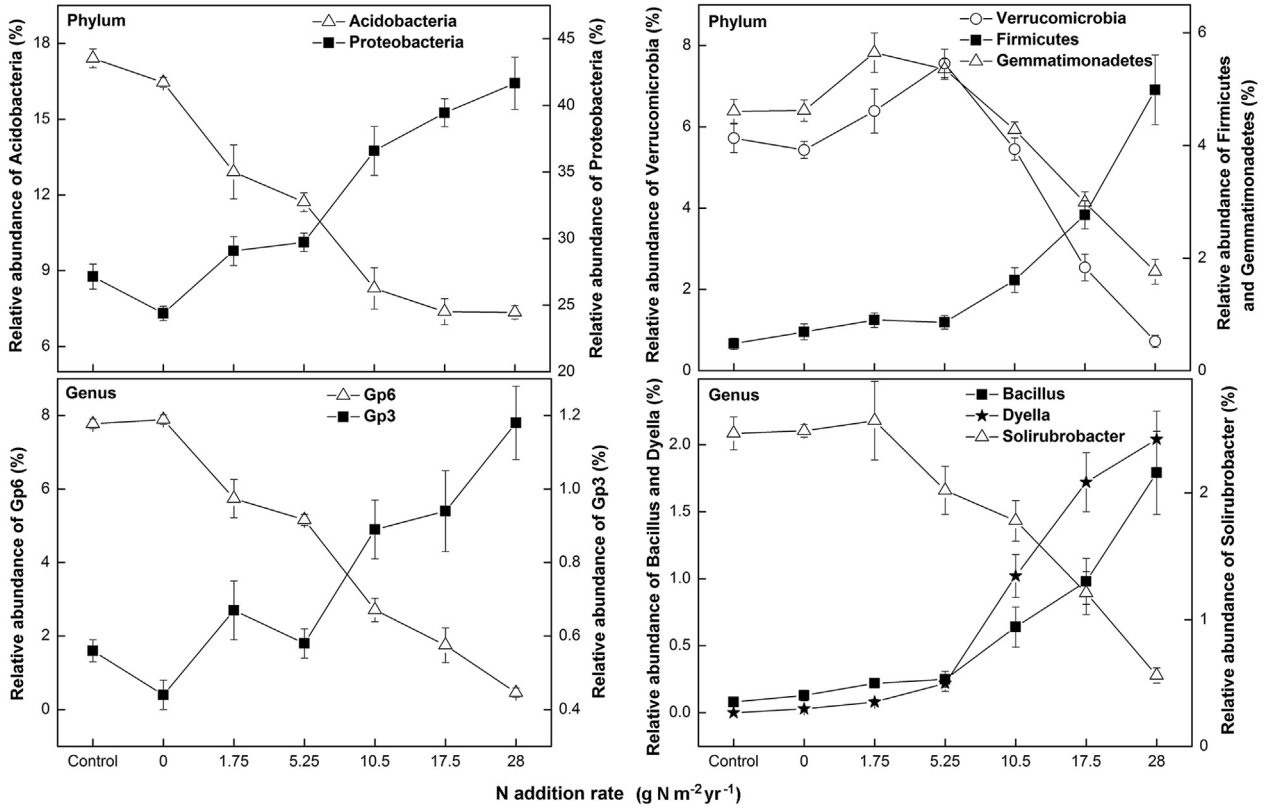


Fig. 2. The changes of the relative abundance of dominant bacteria with N addition rates at the phylum and genus levels.

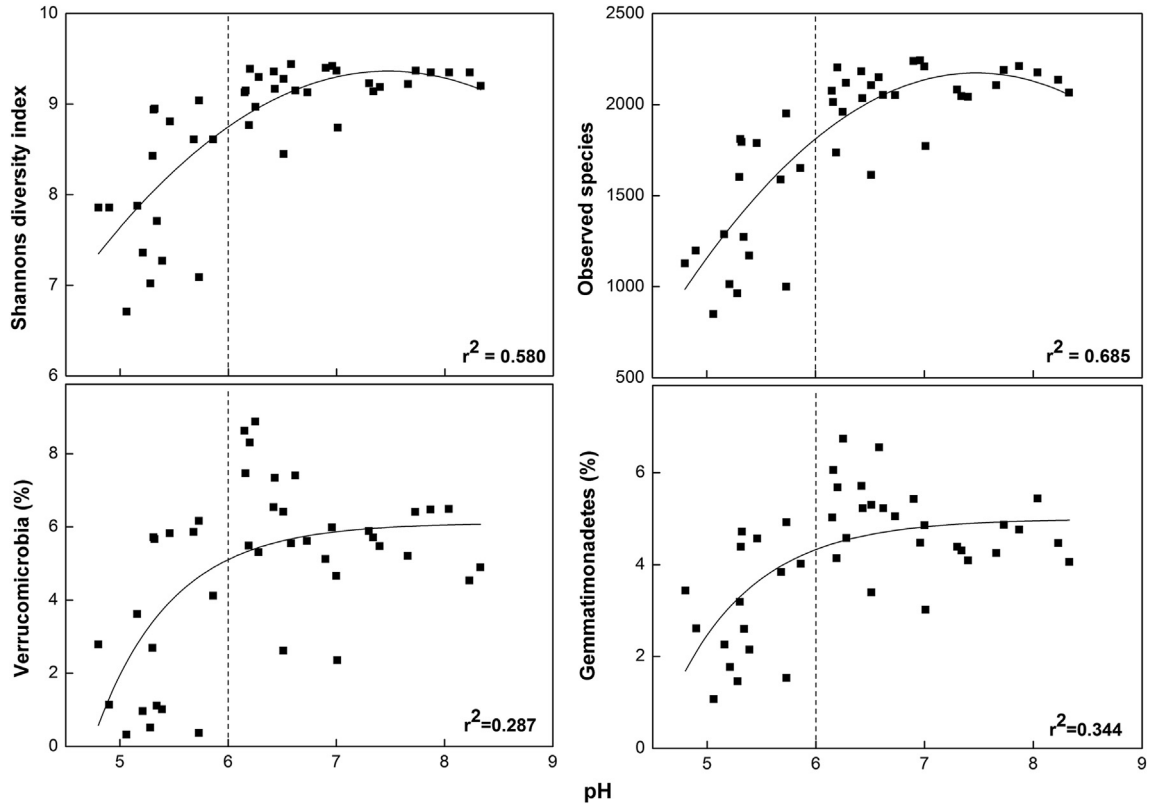


Fig. 3. The changes of Shannon index, observed OTUs, and the relative abundances of Verrucomicrobia and Gemmatimonadetes under different soil pH ($n = 42$).

their relative abundance when N addition rates were higher than 5.25 g N m^{-2} yr^{-1}, e.g., Firmicutes, Verrucomicrobia, and Gemmatimonadetes. The pattern was similar to that of the Shannon index vs N addition rates. Such a significant change corresponded to the soil pH dropped below 6.0 induced by a high N rate (Fig. 3).

The increase of Proteobacteria with N addition rates was mainly driven by increased abundances of dominant OTUs, such as OTU378 (genus *Dyella*) and OTU4940 (Proteobacteria), OTU17762 (Sphingomonadaceae) (Table S1). Notably, OTU378 and OTU4940 occurred at very high abundance at high N addition rates, though they were not detected in the natural steppe, indicating their nitrophilous nature or adaptation to low pH. However, we also found that some OTUs responded negatively to N deposition, such as OTU10729 (Sphingomonas). The increase in the relative abundance of phylum Bacteroidetes was mainly driven by OTU5867 (Chitinophagaceae), which occurred only at the addition of N.

The relative abundances of phylum Acidobacteria decreased significantly with the N addition rate, which was mainly driven by the changes of OTU7432 (genus Gp4), OTU18444, 18432 and 6420 (Gp6), and OTU16626 (Gp16). However, genus *Granulicella* and Gp3 showed positive responses to N deposition. The decrease of phylum Verrucomicrobia was mainly driven by the family Spartobacteriaceae (67–89% of total Verrucomicrobial reads).

Based on our sequence data, archaea were mainly found in the phylum Crenarchaeota (99.4% of total archaeal reads), and its abundance decreased with the N addition rate. The abundance of Euryarchaeota and other archaeal phyla was only 0.56%. The phylum Crenarchaeota was dominated by class Thaumarchaeota, in which the family Nitrososphaeraceae and the genus Candidatus *Nitrososphaera* decreased with N addition rates (Fig. S2).

3.4. Relationships between prokaryotic community structure and soil properties

To understand the relationships between the prokaryotic community structure, diversity, biomass and soil properties, their correlations were calculated using the Mantel test. N input, soil NH_4^+–N and pH followed by plant biomass and richness provided the highest Pearson's correlation ($p < 0.01$) with prokaryotic communities (OTUs), microbial diversity and biomass selected by the Mantel test. Variance partitioning analysis (VPA) was performed to quantify the relative contributions of different environmental parameters to the changes in the prokaryotic composition (at OTU level), microbial diversity and biomass by the *varpart* procedure. For prokaryotic community structure, N deposition rate alone explained 6.88%, soil NH_4^+–N 1.43% and pH 3.93% of the total variation, respectively, leaving 31.30% of the variation unexplained, while the above factors together explained 25.12%. For microbial diversity, N input rate explained 2.92%, soil NH_4^+–N 2.57% and pH 1.16% of the total variation, respectively, leaving 8.27% of the variation unexplained, while the above factors together explained 53.13%. For microbial biomass, the N input rate explained 7.51%, soil NH_4^+–N 0.66% and pH 7.59% of the total variation, respectively, leaving 22.98% of the variation unexplained, while the above factors together explained 42.13%.

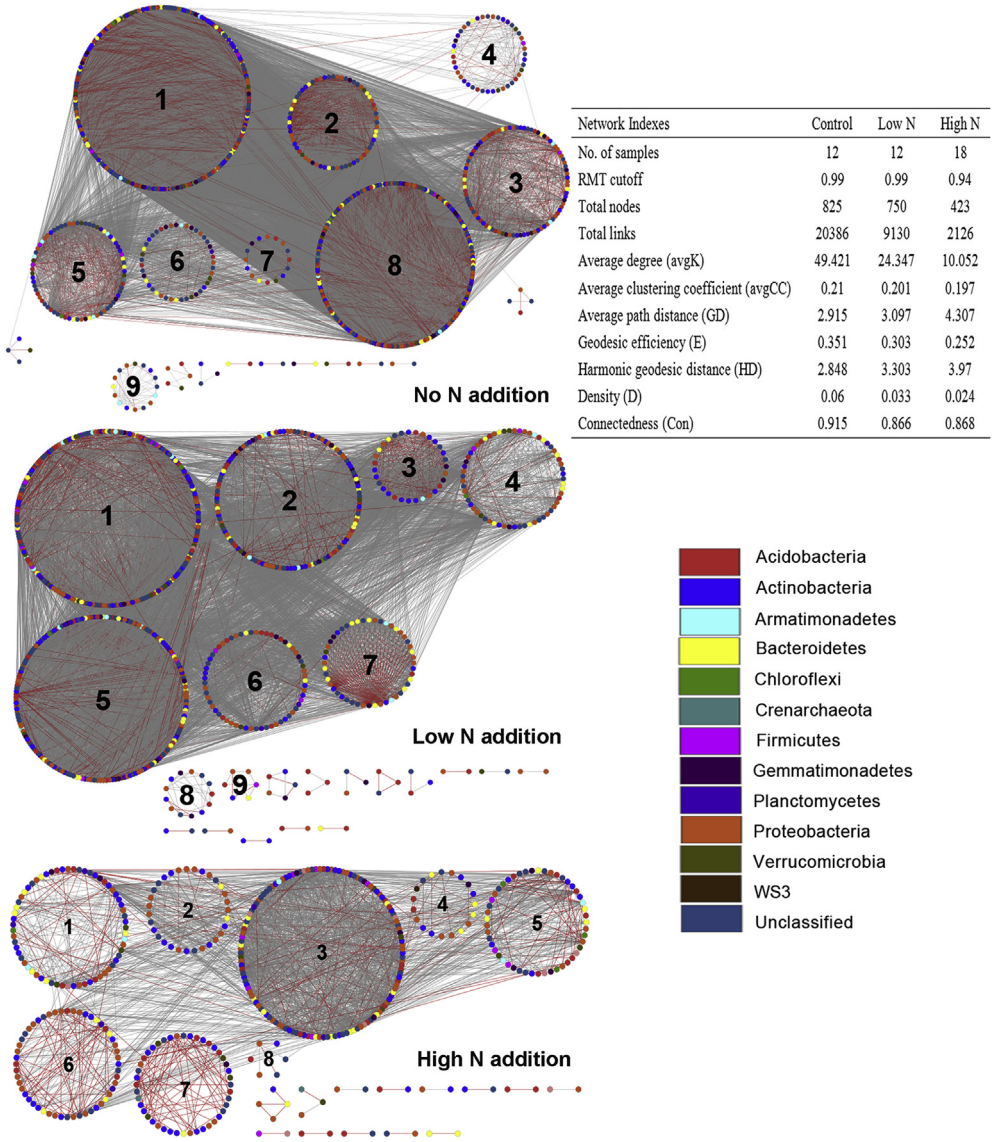
The relative abundance of prokaryotic microbiota (at genus levels) significantly correlated to N addition rates (50 genera), soil NH_4^+–N (50), C_{mic} (46), N_{mic} (45), pH (44) ($p < 0.01$, Table S2). Among these genera, 40 of them were significantly correlated to all above parameters, such as Acidobacteria Gp6, Gp4, Gp16, Gp7, Gp3, Gp17, Gp10, and Gp5. They were sensitive to N deposition and/or to ecological condition changes induced by the N input. The genus

Solirubrobacter was significantly correlated with TN, but no genus was significantly correlated with TOC at the $p = 0.01$ level. In addition, there was a significant correlation ($p < 0.01$) between environmental factors (except TOC and TN) and microbial biomass (Table S3).

3.5. Network analysis of prokaryotic communities

Artificial neural network analysis was used to model the relationships between microbial properties, the relative abundance of dominant bacteria and environmental factors. The model showed a close relationship and good correlations between dominant bacterial groups and environmental factors except the phyla Actinobacteria (Table S4). N input, NH_4^+ and pH were most selected in these models. The predicted microbial biomass and the relative abundance of dominant bacterial groups using artificial neural network fitted well with observed data (Fig. S3).

We also constructed phylogenetic molecular ecological networks (pMEN) to understand the interaction of prokaryotic communities in response to N deposition, with samples from no-N treatment and N addition treatments (Fig. 4). A module is a group of OTUs which may have similar ecological niches and are well connected among themselves but are less linked with OTUs belonging to other modules. Each node signifies an OTU. The number of total nodes, total links, average connectivity (avgK), density, and connectedness decreased with N addition rates, indicating a relatively simpler network under N deposition. The average path distance (GD) increased and Geodesic efficiency (E) decreased with N addition rate, indicating that the nodes in the network were more separated in the N addition treatments than that in the no-N treatment. This was further supported by the correlations between module-based eigengenes and environmental factors which were used to detect the modules' response to environmental changes (Fig. S4). There were 2, 2, and 1 modules which showed a positive correlation ($p < 0.05$) with NH_4^+–N and TN (these variables represent N level), while 0, 1, and 6 modules showed negative correlations at no-N, low, and high N treatments, respectively. This indicated that the prokaryotic community structure was



Network Indexes	Control	Low N	High N
No. of samples	12	12	18
RMT cutoff	0.99	0.99	0.94
Total nodes	825	750	423
Total links	20386	9130	2126
Average degree (avgK)	49.421	24.347	10.052
Average clustering coefficient (avgCC)	0.21	0.201	0.197
Average path distance (GD)	2.915	3.097	4.307
Geodesic efficiency (E)	0.351	0.303	0.252
Harmonic geodesic distance (HD)	2.848	3.303	3.97
Density (D)	0.06	0.033	0.024
Connectedness (Con)	0.915	0.866	0.868

Fig. 4. The Molecular Ecological Networks (MEN) of prokaryotic communities in no-N, low N and high N deposition rate. Number in the circles is module number. Red line means positive correlation, and gray line means negative correlation. Higher average connectivity (AvgK) means a more complex network. A smaller average path distance (GD) means all the nodes in the network are closer. Geodesic efficiency (E) is opposite to GD, which a higher E means that the nodes are closer.

differentiated. A group of nitrophilous species (module 1 at high N rate) was selected and separated from others with an increase in N addition rates.

4. Discussion

Based on the long-term N deposition experiment in this study, we observed that the responses of microbial properties (microbial biomass, abundance, respiratory quotient), prokaryotic diversity, and community structure to N deposition were dependant on deposition rates. Two patterns of microbial properties vs N addition rates were observed: (i) some microbial properties changed significantly even at low N addition rates (less than 5.25 g N m^{-2} yr^{-1}). These properties changed continuously with increasing N addition rates, e.g., microbial biomass and the relative abundance of Proteobacteria, Bacteroidetes, Acidobacteria, and Nitrospirae. This indicated that N was most likely the dominant factor influencing these properties. (ii) Some microbial properties did not change significantly at low N rates, but changed significantly when the N addition rate was higher than 5.25 g N m^{-2} yr^{-1}, e.g. diversity index, respiratory quotient, and the relative abundance of Firmicutes, Verrucomicrobia, Armatimonadetes, and Gemmatimonadetes. The occurrence of these two patterns reflected the sensitivity of various microbial properties to different driving factors, and this implicated that the mechanisms of N deposition controlling the prokaryotic community structure shift were different at low and high N rates.

4.1. The thresholds of N deposition rates significantly altering prokaryotic community structure and diversity are different

Prokaryotic community structure and microbial biomass C and N changed significantly even at a low N addition rate (1.75 g N m^{-2} yr^{-1}). However, prokaryotic diversity decreased significantly only after an N addition rate of higher than 5.25 g N m^{-2} yr^{-1} in the *Leymus chinensis* steppe system. Under such a situation, soil pH induced by N addition decreased to be below 6.0. Interestingly, the critical threshold for N-induced plant species loss and plant structure changes in the same experimental site is estimated to be below 1.75 g N m^{-2} yr^{-1}, and the changes in aboveground biomass, species richness, and plant functional group composition saturate at a N addition rate of 10.5 g N m^{-2} yr^{-1} (Bai et al., 2010), and plant properties are not sensitive to pH (Lan and Bai, 2012). Although the stimulation of plant growth by N input will increase aboveground litter inputs to soil and belowground C input by increasing root growth and root exudates (Liu and Greaver, 2010), negative effects of N deposition on microbial biomass and activities are demonstrated in this study and many other studies (Treseder, 2008; Liu et al., 2014). This indicates that plant factors are less important in controlling microbial community under high N (Wei et al., 2013).

VPA analysis and Mantel test indicated that N input, NH_4^+, and soil pH were most important to influence prokaryotic community structure and diversity. Previous research shows that there is a strong negative relationship between pH and bacterial diversity across a wide range of soil at pH below 6.5 (Lauber et al., 2009a). Soil acidification and consequent reduction in buffering capacity and accumulation of toxins bring a negative impact on soil microbial activity and diversity (Lauber et al., 2009b; Liu et al., 2014). Such negative impacts are exacerbated with increasing an amount and duration of N addition (Treseder, 2008). In this study, respiratory quotient increased significantly when the N addition rate was higher than 5.25 g N m^{-2} yr^{-1} and pH was lower than 6.0, implicating that environmental stress on microbial community became higher at a high N addition rate. N deposition-induced changes in pH may be an important mechanism in controlling prokaryotic diversity. This mechanism differs from those controlling phylogenetic and taxonomic composition (Ramirez et al., 2010; Wei et al., 2013). Our research further demonstrated that pH 6.0 was likely a key threshold point to alter prokaryotic diversity.

4.2. The dominant mechanisms controlling the shifts of prokaryotic communities are different at low and high N rates

The shifts in community assembly induced by N deposition may also be reflected in the trait differences of the dominant taxa. Similar to some other studies, the changes in bacterial community structure with N addition supported the copiotrophic hypothesis, which predicted that N addition increased the relative abundance of those bacteria with a fast growth rate, relying on more labile C sources, and decreased those with a slow growth rate and that thrive under poor nutrient conditions. Our results confirmed the increase in predicted copiotrophic groups (Proteobacteria and Bacteriodetes) (Goldfarb et al., 2011; Ramirez et al., 2012), and a decrease in predicted oligotrophic groups (Acidobacteria and Nitrospirae) (Jones et al., 2009; Bergmann et al., 2011) with N addition. The Acidobacteria shift pattern with N addition rate was in contrast to the general trend that it would be more abundant at a low pH (high N addition rate). These copiotrophic/oligotrophic bacteria decreased or increased their relative abundances with N addition continuously even at low rate, indicating that they were more sensitive to N than other factors, e.g., pH. However, some bacterial groups only significantly changed their relative abundances at high N addition rates when pH dropped below 6.0, indicating that they were possibly more sensitive to pH.

Our observation demonstrated that the loss of bacterial diversity was nonrandom, since N addition enriched copiotrophic bacteria. Prokaryotic structure significantly changed even at low a N addition rate (Fig. 1), and this agreed with the response of the plant community to N deposition in grassland (Galloway et al., 2003; Bai et al., 2012; Isbell et al., 2013). However, prokaryotic diversity loss is not significant at a low N rate. The facts support the hypothesis that the niche-selection induced by N deposition shifts bacterial community structure mainly through species sorting (Bárcenas-Moreno et al., 2009) at a low N deposition rate. N deposition selects those species genetically better adapted to a certain niche created by N, e.g., nitrophilous bacteria, which outcompete other less well-adapted species, e.g., oligotrophic bacteria. At higher N addition rates (>5.25 g N m^{-2} yr^{-1}), the decrease in pH (<6.0) resulted in significant diversity loss, and this provided an additional mechanism to shift the prokaryotic community structure.

The above patterns of the relative abundance of some bacterial group shifts vs. N deposition rates were also observed at the genus level. The relative abundances of genera *Dyella*, *Bacillus*, and *Solirubrobacter* significantly shifted only when N addition rate was more than 5.25 g N m^{-2} yr^{-1}. The shift patterns of these bacterial groups with pH were similar to the relationship between diversity and pH, indicating that these bacteria were also likely driven by both pH changes induced by N deposition and N deposition itself. An N-deposition-induced pH change was an important mechanism to change bacterial diversity nonrandomly, which was corroborated by the significant correlations between pH and microbial diversity and the relative abundance of some phyla. However, the effect of soil pH reduction on plant species loss was relatively minor in magnitude compared with the direct effect of chronic N input (Galloway et al., 2003).

Nitrogen is a strong ecological filter influencing prokaryotic community structure and diversity. The diversity and species richness loss likely result from niche-selection filtering out species from the native pool that could not tolerate such environmental

changes (Chase, 2007) induced by N deposition. Since environmental factors play important roles determining community assembly, the relative abundance of dominant bacterial groups and microbial biomass were predicted well using an artificial neutral network model. Several variables, including N input rate, pH, NH_4^+–N, TOC, and TN, were used successfully in predicting microbial biomass and the relative abundance of important bacterial groups, especially N addition rate and soil NH_4^+–N. They are important factors in affecting microbial biomass and the relative abundance of main bacterial groups. Soil pH strongly affected the microbial biomass and some other bacterial groups, such as Chloroflexi, Firmicutes, Planctomycetes, and Nitrospirae.

4.3. High N deposition simplifies the microbial interaction network and decreases the stability of a prokaryotic community

Our results showed that high N deposition decreased the stability of a microbial system, and this was reflected in a high coefficient of variations of microbial parameters among different replicates at a high N addition rate (17.5 and 28 g N m^{-2} yr^{-1}), including prokaryotic diversity indices, abundance of bacteria and archaea, soil microbial biomass, and soil respiration. The decrease in stability was likely attributed to diversity loss and community structure simplification caused by an N enrichment effect. A high N deposition rate drives a mature microbial community structure away from its native equilibrium state, which may be easy to be influenced by other biotic and abiotic factors. Prokaryotic diversity loss may reduce the redundancy of species interaction and the complexity of species co-occurrence network at high N deposition rates. On the other hand, the interactions of niche partitioning with extreme changes in key abiotic variables such as N deposition or pH would drive more deterministic processes through species interaction and niche partitioning (Chase, 2007; Chase and Myers, 2011). These processes are predicted to produce segregation in terms of species co-occurrence or even aggregation, resulting in significant variation in the influence of high N deposition rates on microbial properties. This was partially supported by the pMEN results that the nodes and modular in the network were more separated, which implicated from higher average path distance (GD), less modules, lower average connectivity, and total nodes, and from more differentiated modules in high N addition treatments (Fig. 4).

In summary, this study demonstrated that prokaryotic community structure was altered at a low N deposition rate of 1.75 g N m^{-2} yr^{-1}; however, dramatic changes in respiratory quotient and prokaryotic diversity occurred at N addition rates higher than 5.25 g N m^{-2} yr^{-1} when pH dropped below 6.0 in *Leymus chinensis* steppe. The two patterns indicated the differences in dominant mechanisms controlling the shift of the prokaryotic community at low and high N rates. The N-driven shift predominated at low N rates; however, pH-driven and N-driven shifts together likely dominated at high N rates. These patterns enable us to identify those microbes more sensitive to N itself (N sensitive community) or more sensitive to pH changes (pH sensitive community). The N deposition and induced pH changes determine the relative contributions of different mechanisms depending on N deposition rates. This study also highlights that N-induced shifts in the prokaryotic community structure and diversity loss may strongly impact the microbial community stability. Further research requires integrating local and regional perspectives to better understand how N deposition processes at broader scales influence the microbial community structure and ecosystem function and stability.

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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.2014.09.009.

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