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Long-time precipitation reduction and N deposition would alter the soil N dynamic by influencing soil bacterial communities and functional groups

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ABSTRACT

The effect of precipitation reduction and N addition on soil bacterial communities and functions, in turn, impact soil N cycling. Seasonal changes could modify the effects of precipitation reduction and N addition on bacterial communities and functions by changing soil environments and properties. Understanding soil microbial communities and the seasonal response of functions to precipitation reduction and N addition may be important in the accurate prediction of changes in the soil N dynamic. Thus, a long-term field manipulation experiment of N addition and throughfall exclusion was established to investigate soil bacterial communities' response to N addition and precipitation reduction in a temperate forest. We examined soil bacterial communities (Illumina Sequencing) under different treatments during the winter, freezing-thawing cycle periods and the growing season. The bacterial functional groups were predicted by the FAPROTAX database. The results showed that N addition, precipitation reduction, the combined effect of N addition and precipitation reduction, and seasonal changes significantly altered the soil bacterial community composition. Interestingly, by combining the result of previous study that N addition increased the N_2O flux in this experimental system (Geng et al., 2017), the loss of soil N was increased by decreasing denitrification and increasing nitrification bacteria under N addition; ammonification bacteria significantly increased and N-fixation bacteria significantly decreased with precipitation reduction compared with that of the control. For seasonal changes, aromatic-degradation, cellulolytic and ureolytic bacteria were lowest during the freezing-thawing cycle periods (FTCs), which indicated that FTCs might inhibit biodegradation. Nitrification and nitrite-oxidation bacteria were higher in N addition, precipitation reduction and FTCs than in the control treatments or other seasons. The interaction of treatments and seasons also significantly changed the soil bacterial communities and functions.

These results highlighted that N addition, precipitation reduction, season changes and their interaction might directly alter the bacterial community and indirectly alter the dynamic of soil N.

Key Words: N addition, precipitation reduction, season changes, soil bacterial functional groups

INTRODUCTION

Soil bacterial communities are an important part of the soil ecosystem and drive organic matter decomposition and soil biogeochemical processes that regulate soil carbon (C) and nitrogen (N) cycling (Bardgett and Wardle, 2010). Responses of bacterial communities to environmental change will be important in efforts to maintain ecosystem functions and to predict biogeochemical feedbacks to climate change (Evans and Wallenstein, 2014). Therefore, one of the important study directions is to understand how soil bacterial communities respond to environmental change, which is a particularly urgent and important need based on recent evidence of rapid global change.

Changes in global- and regional-scale precipitation patterns along with altered water availability have been observed in terrestrial ecosystems, and a significant decrease in the precipitation amount during the 21st century has been predicted in some areas (IPCC, 2013). Soil water influences N-mineralization mediated by bacteria, and plays a key role in the incellular metabolism and transportation of nutrients and serves as a medium for bacterial mobility (Drenovsky *et al*., 2004; Waring *et al*., 2018). Therefore, precipitation reduction can induce physiological stress and a change in the composition and functional characteristics of bacterial communities due to a decrease in water availability (Jurburg *et al*., 2018; Ochoa-Hueso *et al*., 2018). In general, precipitation reduction tends to decrease the diversity and abundance of bacterial communities (Fry *et al*., 2016; Ochoa-Hueso *et al*., 2018). However, some studies found no significant changes in the abundance and composition of bacterial communities with precipitation reduction (Griffiths and Philippot, 2013). These inconsistent results may be caused by background precipitation variability (e.g., seasonal variability), which could more strongly affect the bacterial communities than does the direct effect of experimental precipitation treatments (Cregger *et al*., 2012; Gutknecht *et al*., 2012). Moreover, seasonal variation in soil moisture may result in a microbial community that is acclimated to fluctuations in precipitation, thus resulting in a diminished response to precipitation manipulation (Clark *et al*., 2009; Griffiths and Philippot, 2013). Therefore, a better understanding of the responses of soil bacterial communities across seasons enables researchers to better predict bacteria responses to precipitation reduction.

As another widespread global change factor, global anthropogenic nitrogen (N) deposition has increased dramatically (Galloway *et al*., 2014). N is a fundamental element that regulates the soil bacterial community composition and function (Zhou *et al*., 2018), particularly in temperate forests, where N is generally considered to be a major limiting factor for microbial growth and development (Wang *et al*., 2012). Increasing N deposition can alleviate the N limitation of soil bacteria, which results in an increase in soil bacterial activity and a change in bacterial communities and functions (Li *et al*., 2016; Zhou *et al*., 2018). Previous studies have shown that increasing N deposition tends to stimulate copiotrophic bacteria (bacterial taxa that have higher N demands, such as Proteobacteria and Bacteroidetes) (Ramirez *et al*., 2012; Ho *et al*., 2017). Another study about the effects of N deposition on bacterial communities also found negative or neutral effects (Hesse *et al*., 2015; Zeng *et al*., 2016).

Although the responses of soil bacterial communities to simulated N deposition have been studied extensively, the effect of N deposition on bacterial communities remains unclear (Ramirez *et al*., 2012; Nguyen *et al*., 2018). In this study, we used multipoint measurements to find common responses of bacteria to N addition.

Moreover, soil water physically affects bacterial-mediated N processes, so the effects of increasing N deposition on soil bacterial communities may strongly depend on soil water regimes (Bi *et al*., 2011; Li *et al*., 2016; Shi *et al*., 2018). Previous studies have shown that sufficient water can enhance the effects of increasing N deposition on soil bacterial communities (Bi *et al*., 2011; Zhang *et al*., 2015), which suggests that decreased precipitation may alleviate the effects of N addition on soil bacterial communities. In addition, environments that have greater seasonal and temporal variation, particularly in temperate and boreal forests with cold winters and long spring freezing-thawing periods (FTCs), may regulate the effects of N addition and precipitation reduction on soil bacterial communities because a wide range of physiological tolerances may already exist within the community (Cruz-Martínez *et al*., 2009). Alternatively, long-time N addition and precipitation reduction also may increase the severity of this variation, thereby resulting in new dynamics of bacterial community composition (Waldrop and Firestone, 2006). Therefore, a better understanding of the responses of soil bacterial communities across seasons (especially FTCs) enables researchers to better predict bacteria responses to long-time and multiple climate changes.

In this study, to elucidate how soil bacterial communities and functional groups respond to increasing N deposition, precipitation reduction and their combined effects, we established a field experiment to manipulate precipitation reduction (-30% throughfall) and simulated N deposition (+5 g N m^{-2} yr^{-1}) in a temperate forest. Previous studies in this experimental system had examined the effects of these treatments on litter decomposition, the soil N_2O flux and plant N uptake, but had not examined its effects on the bacterial community (Geng *et al*., 2017; Zheng *et al*., 2017; Zhou *et al*., 2019). Therefore, soil samples were collected, and soil bacterial communities were measured under different treatments during the winter, the freezing-thawing cycle period and the growing season. We hypothesize that N addition would alter soil bacterial communities and functional groups by increasing the relative abundance of copiotrophic bacteria, and decreased precipitation would change bacterial communities and functional groups by decreasing the movement and diffusion of substrates. Seasonal water variability strongly regulates the response of soil bacterial communities to precipitation reduction, while soil water variability could also alter the effects of increasing N deposition on soil bacterial communities (Clark *et al*., 2009; Cruz-Martínez *et al*., 2009). Thus, we further hypothesize that the combined precipitation reduction and N addition effects and the interaction of treatments and seasonal changes would have a significantly different effect on soil bacterial communities compared with a single treatment.

MATERIALS AND METHODS

Site description

This study was conducted in Changbai Mountain, northeastern China (42°24’N, 128°06’E, and 738 m a.s.l.), which belongs to the temperate-continental climate. The mean annual temperature is 3.6 °C. The average annual precipitation is 760 mm, more than 80% of which falls between May and

August. The soil is classified as Eutric Cambisol according to the FAO classification (Nachtergaele *et al*., 2000). The slope is less than 30 degrees in the study site. The mean soil bulk density is 0.35 g cm^{-3} in 0-10 cm soil depth and 0.68 g cm^{-3} in 10-20 cm soil depth. The vegetation type is an old broad-leaved Korean pine mixed forest with an age exceeding 300 years (Zheng *et al*., 2017). The dominant four tree species are *Pinus koraiensis, Quercus mongolica, Tilia amurensis* and *Fraxinus mandshurica*. The mean canopy height and diameter at 1.3m breast height for these tree species are 26.5 m and 33.8 cm, respectively (Zheng *et al*., 2017). The tree density on the site is 435 trees hm^{-2}. The main shrub species include *Philadelphus schrenkii, Lonicera japonica, Euonymus alatus, Deutzia scabra*, and *Corylus mandshurica,* and the main herbaceous species include *A. cathayensis, Anemone raddeana, Funaria officinalis, Cyperus microiria, Brachybotrys paridiformis, Adonis vernalis,* and *Filipendula palmate*.

Precipitation rates have reduced in North China over the last 50 years (Wang *et al*., 2006). In the drought years of 1985, 1997, 1999, 2001, and 2003, the precipitation rate dropped to approximately 550 mm, which is approximately 30% less than the mean annual precipitation of 760 mm in the Changbai Mountains area in the last 30 years (these data were obtained from the Chinese Ecosystem Research Net (CERN)). In this study, snow cover lasted for 146 days, with a maximum thickness of 45 cm during the experimental periods of 2016/2017.

Experimental design

In this study, six 50 × 50 m plots surrounded by a > 20 m wide buffer strip were randomly established in an old broadleaf Korean pine mixed forest in the Changbai Mountains to examine the effect of precipitation reduction on soil bacterial communities in May 2009. Three plots were treated with 30% precipitation reduction, which was equivalent to the drought year's precipitation rate, and the other three plots were set as the control treatment. A high light transmittance (transparency 95%) polycarbonate V-shaped translucent panel was used to intercept throughfall, and the polycarbonate panels covered 30% of the plot area to intercept approximately 30% natural throughfall during the growing season (the Rainout Shelter Facility is showen in the supporting information, Figure S1). The panels were removed in winter (especially during the snowfall period) to allow for snow to fall onto the forest floor and to avoid effects of the uneven snow distribution on the soil system. The panels were fixed on an aluminum frame approximately 1 m above the soil surface to ensure normal air flow. In addition, in the study area, the average annual atmospheric N deposition was approximately 2.3 g N m^{-2} $year^{-1}$ (NO_3^- and NH_4^+ concentration, excluding N in snow) (Lü and Tian, 2007). To measure the effect of N addition on the soil bacterial communities, each of these six plots was split into two 25 × 50 m subplots using a PVC sheet inserted 50 cm deep into the ground to avoid nutrient mobilization between adjacent subplots. One of the subgroups of each plot received additional N, and the other did not. The selected N addition level (5 g N m^{-2} $year^{-1}$) was approximately two times the mean annual atmosphere N deposition for the Changbai Mountains area. In each N addition subplot, ammonium nitrate (NH_4NO_3) was weighed, mixed with 40 L deionized water, and sprayed monthly onto the forest floor using six equal applications at the beginning of each month over the entire growing season (May to October) with a backpack sprayer, beginning in May 2009. The control plots simultaneously received 40 L deionized water without N addition to avoid a difference in the moisture application.

This field experiment had been running for 8 years from May 2009 to October 2016, and the total N application rate was 40 g m^{-2}.

Soil sampling and measurement

Soil sampling schedule and corresponding climatic factors (e.g., soil temperature, air temperature, etc.) were showed in Table S1. The bacterial communities were measured in two extreme periods (winter and summer) and a strong fluctuation period (the spring freezing-thawing periods). We measured once in winter (coldest month) and once in a growing season, respectively. FTC was divided into three specified periods, the initial stage, mid-term and termination of FTC based on the snowpack thickness, soil temperature at 5cm depth (T_{5cm}) and air temperature (Ta), each of which had distinct biophysical characteristics. The initial stage of FTC (FTC-S) was defined as the period that started when the daily maximum Ta is above 0 °C (the start of snowmelt) and the Ta fluctuation made the water state of snow and soil alternation. The mid-term of FTC (FTC-M) was defined as the period that started when the daily maximum Ta was above 0 °C, the daily minimum T_{5cm} was below 0 °C and the snowpack was completely melted (the end of snowmelt). The termination of FTC (FTC-E) was defined as the period that ends when the daily minimum T_{5cm} was above 0 °C (the end of soil freezing at the 5 cm depth). During the five specified periods, six soil cores (diameter 5 cm) were randomly taken from the top layer (0-10 cm) of each subplot by using a handheld auger, and they were pooled into one composite sample of each subplot. All the visible extraneous materials (such as stones, roots, etc.) were removed by hand. Each composite soil sample was divided into two subsamples: the first one was sieved (2 mm) for the analysis of basic soil properties, and the second one was immediately flash-frozen in liquid N_2 and was stored in cooling dry-ice boxes for the transportation to the laboratory and then stored at -80 °C until it was processed for DNA extraction.

The soil properties of all samples were measured as was described in Yan et al., (2017). The soil pH was determined in a 1:2.5 (soil: water, dry weight/volume) solution using a pH meter (Sartorius PB-10, Gottingen, Germany). The soil total C (TC) and N (TN) were determined using an automated TC/TN analyzer (multi N/C 3100, Analytik Jena AG, Germany). The soil total phosphorus was measured by high-performance liquid chromatography after wet digestion with $HClO_4$ (Parkinson and Allen, 1975). Dissolved organic carbon (DOC) and dissolved total N (DTN) were extracted from soil using distilled water (1:10 w/v soil-to-solution ratio) (Jones and Willett, 2006)and determined by using a Multi N/C 3100 Analyzer (multi N/C 3100, Analytik Jena AG, Germany).

DNA extraction, PCR and sequencing

The total soil DNA was extracted from 0.25 g soil using the DNeasy PowerSoil Kit (Mobio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol and was stored at -20 °C until PCR amplification. The extracted DNA was confirmed by agarose gel electrophoresis. The ribosomal region (V3 and V4) of bacterial 16S rRNA genes were amplified with the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Zielinski *et al.*, 2006). The purification of PCR products was performed using the Agencourt AMPure XP (ThermoFisher Scientific, Finland) to Miseq according to the manufacturer's protocol. The purified samples were analyzed using the Fragment Analyzer (Advanced Analytical, USA) and

amplicons sequenced with an Illumina MiSeq platform (MiSeq v3.1 software) at the Majorbio Bio-Pharm Technology, Shanghai, China.

Bioinformatics

The sequence data (.fastq) was analyzed using mothur version 1.39.5 (https://mothur.org/wiki/Main_Page). The raw data files were contiged, and any sequences with ambiguous bases, 2 nucleotide mismatches or ambiguous characters in primer matching, and homopolymers longer than 8 bp and any without a minimum overlap of 50 bp were removed. We aligned the bacterial sequences to a SILVA reference alignment and removed erroneous reads. Then, the sequences were screened for chimeras using UCHIME (version 4.2.40) and the chimeric sequences were removed. Nonchimeric sequences were classified to bacterial taxa using the Naïve Bayesian Classifier. Any unclassified sequences were removed. Sequences were clustered to operational taxonomic units (OTU) at 97% similarity using the nearest neighbor-joining method. The data of OTUs were assigned to bacterial functional groups using the FAPROTAX database (Louca *et al*., 2016). Functional profiles were generated by associating individual organisms with metabolic functions of particular ecological relevance, such as photoautotrophy and nitrate respiration, using an annotation database based on the FAPROTAX database (Louca *et al*., 2016). Then, by comparing our data with database data, we explored the functional potential of the corresponding functional community.

The richness and diversity indices of bacterial communities were determined using mothur software. The observed OTU richness (Sobs), complement of Simpson's diversity and Simpson's evenness were calculated for each sample. The Bacterial Sørensen Dissimilarity Index (β_{SOR}), the Simpson Dissimilarity Index (βSIM), and the Nestedness-resultant Dissimilarity Index (β_{NES}) were also calculated for each sample using R software (version 3.2.5) (Baselga, 2010).

Statistical analysis

The homogeneity of variances was determined using Levene's test, and the normal distribution was analyzed using the Kolmogorov-Smirnov test. The experiment is a split-plot design. Therefore, the general linear mixed model (GLMM) with Tukey's multiple comparisons was used to evaluate the effects of the treatments and the sampling time on the soil properties, bacterial diversity indices and bacterial functional groups. The sampling time, N addition, precipitation reduction, N addition × precipitation reduction and treatments × sampling time were considered to be fixed factors, and blocks were used as random effects to determine the effects of N addition, precipitation reduction, season and their interaction on the soil properties, bacterial diversity indices and bacterial functional groups. Nonmetric multidimensional scaling (NMDS) ordinations were performed to determine the total bacterial communities. The permutational multivariate analysis of variance (PERMANOVA) was performed to evaluate the effects of the treatments and sampling time on bacterial communities. A linear discriminant analysis (LDA) effect size method (LEfSe) was performed to identify the significantly different abundant taxa of bacteria in all of the treatments or seasonal changes (http://huttenhower.sph. harvard.edu/galaxy/root? Tool_id = lefse_upload). When the anthropogenic treatments (N addition and precipitation reduction) were used as the classes of the LEfSe analysis, the

sampling times were used as subclasses of the LEfSe analysis, and vice versa. The threshold for the logarithmic LDA score was 2 for all that were evaluated in this study (Segata *et al.*, 2011). In addition to the LEfSe analysis, all statistical analyses were performed in R (version 3.2.5, R Development Core Team).

RESULTS

Simulated N deposition effects

Increasing the N deposition increased the soil total N and dissolved total N concentrations compared with those of the control, but the soil total N and dissolved N were not significantly different from those of the control (Table I). Increasing the N deposition significantly decreased the soil pH value and the soil total P concentration compared with those of the control (Table I). Increasing the N deposition tended to decrease the bacterial diversity and richness but was not significant compared with the control (Table II). Moreover, the Sørensen Dissimilarity Index and the Simpson Dissimilarity Index (βSIM) were lower in the increasing N deposition treatment than in the control.

TABLE I

Soil physicochemical properties under different treatments in 2017

	Total carbon(g/kg)	Total nitrogen(g/kg)	Total (g/kg)	phosphorus	pH	DTC(g/kg)	DTN(g/kg)
Control	98.42±4.69a	5.74±0.27a		0.22±0.01a	4.89±0.09a	0.64±0.03b	0.05±0.02a
-P	103.80±10.34a	5.52±0.55a		0.22±0.01a	4.68±0.14a	0.72±0.05a	0.08±0.02a
+N	97.80±7.48a	6.17±0.34a		0.20±0.01c	4.46±0.11b	0.63±0.10ab	0.06±0.01a
+N-P	99.91±9.10a	6.15±0.38a		0.21±0.01b	4.73±0.09a	0.76±0.05a	0.06±0.02a

Each value is the mean ± SE (n = 15). –P, the decreased precipitation treatments; +N, N addition treatments; +N-P, N addition and decreased precipitation treatments. DTC, soil dissolved total C; DTN, soil dissolved total N

TABLE II

The Bacterial Diversity Index (diversity, richness and evenness), the Sørensen Dissimilarity Index (β_{SOR}), the Simpson Dissimilarity Index (β_{SIM}), and the Nestedness-resultant Dissimilarity Index (β_{NES}) in different periods under different treatments in 2017

	Diversity	Richness	Evenness	β_{SOR}	β_{SIM}	β_{NES}
Treatments						
Control	167.10±10.15A	2148.00±51.84A	0.81±0.01AC	0.159	0.136	0.024
-P	215.95±10.98A	2275.13±33.50A	0.83±0.00A	0.162	0.138	0.025
+N	165.19±12.08A	2104.67±51.84A	0.81±0.01C	0.158	0.134	0.024
+N-P	180.99±11.55B	2249.33±53.96B	0.82±0.00B	0.161	0.147	0.014

Seasons						
W	195.75±13.45a	2188.75±51.39a	0.82±0.01a	0.128	0.113	0.015
FTC_S	181.39±15.11ab	2070.83±40.13b	0.82±0.01a	0.129	0.115	0.014
FTC_M	194.05±11.34a	2224.58±51.62a	0.82±0.00a	0.127	0.109	0.018
FTC_E	171.08±10.76ab	2167.08±42.08a	0.81±0.00ab	0.129	0.114	0.015
G	169.25±16.69b	2195.17±82.89a	0.81±0.01b	0.134	0.105	0.029

Each value is the mean±SE. ANOVA: Different lowercase letters indicate significant differences in different seasons; Different uppercase letters indicate significant differences among different treatments. –P, decreased precipitation treatments; +N, N addition treatments; +N-P, N addition and decreased precipitation treatments. W, winter season (January 2017); FTC_S, start of snowmelt (February 2017); FTC_M, end of snowmelt (March 2017); FTC_E, end of soil freezing at the 5 cm depth (April 2017); G, growing season (July 2017). (treatments, n = 15; seasons, n = 12)

There was a significant effect of increasing the N deposition on the bacterial community composition (NMDS, pairwise comparison; $p < 0.05$ by PERMANOVA) (Fig. 1). Increasing the N deposition decreased the relative abundance of the Proteobacteria phylum by 6.99% but increased the relative abundance of the Acidobacteria, Chloroflexi,Verrucomicrobia and Planctomycetes phyla by 2.25%, 12.05%, 12.88% and 24.23% ,respectively, compared with those of the control(Fig. 2). LEfSe is an algorithm for high-dimensional biomarker discovery and provides an explanation that identifies genomic features (genes, pathways, or taxa) that characterize the differences between different treatments or different periods. Based on the LEfSe analysis, at the genus level, the relative abundance of *Oligoflexus, Gemmata* and *Novosphingobium* significantly increased with increasing N deposition compared with the control (Fig. 3).

Fig.1

Fig. 2

Fig. 3

Increasing N deposition had different effects on the different bacterial functional groups (Fig. 4). The relative abundance of cellulolysis, nitrite_oxidation and nitrification bacteria significantly increased in increasing N deposition compared with the control, while the relative abundance ofdenitrification, nitrate_reduction, nitrate_respiration, nitrogen_fixation, nitrogen_respiration and ureolysis bacteria significantly decreased.

Fig. 4

Simulated precipitation reduction effects

The dissolved total Nconcentrations increased and the pH value decreased with precipitation reduction compared with the control (Table 1). The dissolved total C concentrations significantly

increased in precipitation reduction compared with control (Table 1). There was an increasing trend in all diversity indices with precipitation compared with the control (Table 2).

Precipitation reduction also significantly changed the bacterial communities ($p < 0.05$ by PERMANOVA) (Fig. 1). Precipitation reduction increased the relative abundance of Chloroflexi (17.34%), Bacteroidetes (24.79%) and Planctomycetes (18.68%) phyla compared with that of control, while precipitation reduction decreased the relative abundance of Verrucomicrobia (16.62%) and Nitrospirae phyla (21.35%) (Fig.2). The relative abundance of *Undibacterium,Shimazuella, Blastochloris, Alicyclobacillus, Cytophaga, Legionella,* and *Bdellovibrio* significantly increased with precipitation reduction compared with the control at the genus level(Fig. 3).

For the bacterial functional groups, there were significant increase in the relative abundance of ammonification, cellulolysis, nitrification and nitrite_oxidation bacteria with precipitation reduction compared with the control (Fig. 4). The relative abundance of nitrogen_fixation significantly decreased under precipitation reduction compared with the control (Fig. 4). Precipitation reduction did not significantly affect other bacterial functional groups.

Combined N deposition and precipitation reduction effects

The combined N deposition and precipitation reduction had a positive effect on the soil total N, dissolved total N concentrations and dissolved total C concentrations (Table 1). The combined effects significantly increased the bacterial diversity, richness and evenness indices compared with those of the control (Table 2). Likewise, the Sørensen Dissimilarity Index and the Simpson Dissimilarity Index increased with the combined N deposition and precipitation reduction treatment compared with that of the control, while the Nestedness-resultant Dissimilarity Index decreased under the combined treatment.

The combined effects also significantly changed the bacterial communities ($p < 0.05$ by PERMANOVA) (Fig. 1). The relative abundance of Actinobacteria, Acidobacteria, Chloroflexi, Bacteroidetes and Planctomycetes phyla increased by 11.94%, 4.78%, 11.71%, 9.21% and 19.51% with the combined effects compared with the control, respectively, while the relative abundance of the Proteobacteria phylum decreased by 12.35% (Fig. 2) . In addition, the combined N deposition and precipitation reduction significantly increased the relative abundance of *Acidobacteria, Gaiella, OM190, Latescibacteria, Syntrophaceae, Vulgatibacter, Catecholicum, Defluviicoccus, Blastocatellaceae* and *KD4-96* compared with those of the control at the genus level (Fig. 3).

There were significant increases in the relative abundance of ammonification, aromatic_degradation, cellulolysis and hydrocarbon_degradation bacteria in the combined effects compared with the control, while the relative abundance of nitrate_reduction bacteria significantly decreased under the combined effects (Fig. 4).

Seasonal effects

The sampling time had significant effects on all soil parameters ($p < 0.05$). The bacterial diversity was lowest in the growing season and highest in the winter (Table II). The bacterial richness also responded to seasonal changes, with highest value occurring in the FTC-M period and the lowest value occurring in the FTC-S period. The Nestedness-resultant Dissimilarity Index was highest in the

growing season and lowest in the FTC-S period. Moreover, the interaction of seasons and N addition, precipitation reduction or their combined effect had a significant effect on the bacterial diversity and richness (Table III).

TABLE III

Results of full factorial model tests of bacterial diversity and richness in the experimental periods for the effects of N addition (+N), decreased precipitation (-P), sampling date and their respective interactions

	Diversity		Richness		Evenness	
	F	P	F	P	F	P
-P*+N	5.122	0.024	9.001	0.003	6.389	0.017
season	28.539	<0.001	13.899	0.008	4.372	0.219
-P*season	11.115	0.025	46.009	<0.001	23.098	<0.001
+N*season	20.772	<0.001	30.995	<0.001	16.576	<0.001
+N*-P*season	16.865	<0.001	14.876	0.005	13.963	0.019

The seasonal changes significantly changed the bacterial communities (Fig. 1, Tables IV and V). The interaction of seasons and treatments also significantly changed the bacterial communities (Table IV). Specifically, at the genus level, the relative abundance of *PeM15* significantly increased in the FTC-E period; the relative abundance of *TK10, BIrii41, Phaselicystidaceae, Sphingorhabdus* and *Solirubrobacterales* significantly increased in the FTC-M period; the relative abundance of *Solibacter, Bryobacter* and *Methylobacter* significantly increased in the FTC-S period; the relative abundance of *H16, Gallionellaceae* and *Rhodoblastus* significantly increased in the growing season; and the relative abundance of *Gemmatimonas, Jatrophihabitans* and *Zixibacteria* significantly increased in the winter (Fig. 3).

TABLE IV

Bacterial community composition under different treatments in different seasons

	df	Sums of squares	Mean square	F Model	P-value
+N	1	0.164	0.164	1.379	**0.008**
-P	1	0.194	0.194	3.109	**0.004**
+N-P	1	0.202	0.202	3.259	**0.003**
Seasons	4	0.503	0.126	2.9372	**<0.001**
Interaction	12	1.492	0.124	2.9067	**<0.001**

Results of permutational multivariate analysis of variance (PERMANOVA); P-values less than 0.05 are highlighted in bold. -P, precipitation reduction; +N, N addition; +N-P, combined N addition and precipitation reduction; interaction, interaction of seasons and treatments.

TABLE V

Effects of seasons, treatments and their interaction on relative abundances of bacterial functional groups

	Seasons		Treatments		Interaction	
Bacterial functional groups	F value	P value	F value	P value	F value	P value
Ammonia_oxidation	9.908	0.042	2.982	0.394	69.873	<0.001
Ammonification	26.772	<0.001	10.745	0.013	27.674	0.006

Aromatic_degradation	18.515	0.001	11.555	0.009	36.417	<0.001
Cellulolysis	14.260	0.007	16.178	0.001	80.428	<0.001
Denitrification	4.405	0.354	0.725	0.867	47.245	<0.001
Human_pathogens	51.813	<0.001	50.246	<0.001	104.018	<0.001
Hydrocarbon_degradation	21.398	<0.001	28.187	<0.001	127.935	<0.001
Nitrate_reduction	1.049	0.790	3.760	0.439	55.412	<0.001
Nitrate_respiration	2.017	0.733	15.229	0.002	25.077	0.014
Nitrification	44.169	<0.001	31.906	<0.001	157.688	<0.001
Nitrite_oxidation	21.239	<0.001	23.797	<0.001	107.940	<0.001
Nitrogen_fixation	25.975	<0.001	39.014	<0.001	97.609	<0.001
Nitrogen_respiration	4.165	0.384	2.253	0.522	62.997	<0.001
Plant_pathogen	6.882	0.142	4.546	0.208	16.049	0.189
Ureolysis	10.352	0.035	3.232	0.357	28.712	0.004

Nitrate respiration means the process of respiration used by some anaerobic organisms, in which nitrate rather than molecular oxygen is used to oxidize organic molecules to obtain energy. Nitrogen respiration is the process of dissimilatory reduction of nitrogen compounds.

The denitrification, aromatic_degradation, cellulolytic and ureolytic bacteria initially decreased and then increased with the seasonal variation, with the lowest value occurring during the FTC-M period (Fig. 5). In contrast, nitrite_oxidation, nitrogen_fixation and nitrification bacteria increased first and then decreased with the seasonal variation (Fig. 5). The interaction of seasons and treatments significantly changed the bacterial functional group (Table V).

Fig. 5

DISCUSSION

Effects of increased N deposition

In the study, the simulated N deposition resulted in significant changes in the bacterial community composition, which is consistent with that of previous studies (Zeng *et al*., 2016; Chen *et al*., 2018). Shifts in the bacterial composition following N deposition were previously explained by the copiotrophic hypothesis, in which copiotrophic bacterial groups (r-selected, e.g., Proteobacteria) are more likely to increase under nutrient-rich conditions, while oligotrophic bacterial groups (K-selected, e.g., Acidobacteria) would likely decline (Fierer *et al*., 2007; Koyama *et al*., 2014). However, in the study, some copiotrophic organisms, such as Proteobacteria, decreased and some oligotrophic organisms, such as Acidobacteria, increased in abundance following N addition, which is contrary to the copiotrophic hypothesis. These results indicated that bacterial communities did not necessarily lead to a shift towards copiotrophic groups under the long-time N enrichment condition. Studies have found that short-time N addition might simulate copiotrophic groups, while long-time N deposition had a negative effect on the bacterial community based on its side effects (such as soil acidification) (Treseder, 2008; Ramirez *et al*., 2010; Yuan *et al*., 2016). The pH value was lower under N addition than that under control conditions in our study. The optimal growth of soil bacterial

communities has a narrow pH range, so the change in the pH value caused by N deposition may be an important factor affecting the bacterial community composition (Rousk *et al*., 2010; Zhang *et al*., 2017). Acidification makes the environment more restrictive, which may be the reason behind the change in the bacterial community. Previous studies showed that a low pH would lead to a decrease in the relative abundance of Proteobacteria and to an increase in the relative abundance of Acidobacteria (Ramirez *et al*., 2010; Zhang *et al*., 2017), thus indicating that the shifts in the bacterial community composition could be largely attributed to the decrease in soil pH under N addition in this study.

Changes in bacterial communities could result in altered rates and/or controls of corresponding functions. In this study, we found that increased N deposition significantly increased the relative abundance of nitrification bacteria. A previous study of this experimental system found that N addition increased the N_2O flux (Geng *et al*., 2017), which indicated that increased N_2O flux might be caused by an increase of nitrification bacteria. The change in nitrification bacteria that we observed in response to increasing N deposition may have resulted from the direct effects of NH_4NO_3 application to the soil. NH_4NO_3 fertilizer application would directly increase the concentration of soil NH_4^+, which may lead to an increase in the relative abundance of nitrification bacteria (Chen *et al*., 2013). However, nitrification produces hydrogen ions. Therefore, this increase in nitrification bacteria abundance may explain why we observed a decrease in the soil pH in response to increasing the N deposition. Subsequently, the denitrification rate generally decreases with the decreasing pH (Cavigelli *et al*., 2000), which may support our result showing that the relative abundance of denitrification bacteria significantly decreased with the increasing N deposition. Increases in the nitrification bacteria and decreases in the denitrification bacteria under N deposition would lead to soil nitrate accumulation. However, under N addition, the soil nitrate concentration did not increase significantly, and the plant nitrate uptake also did not increase significantly in the experimental system (Zhou *et al*., 2019). Although we did not have data on the response of nitrate leaching to N addition, previous studies have reported increases in nitrate leaching under N addition (Hoegberg *et al*., 2006; Fang *et al*., 2009). Thus, we speculate that the absence of an increase in the soil nitrate concentration under N addition could partly be attributed to the leachability of nitrates.

Effects of precipitation reduction

In this study, precipitation reduction significantly altered the bacterial community composition, which is consistent with previous studies that showed that bacterial communities changed significantly with throughfall exclusion (Bouskill *et al*., 2013; Felsmann *et al*., 2015). Under precipitation reduction, the soil water potential declines, the pore connectivity is reduced, and the solute concentrations increase and impart physiological and physical stresses on whole bacterial communities that can significantly alter bacterial communities (Chowdhury *et al*., 2011; Bouskill et al., 2013; Yao *et al*., 2017). A previous study in this experimental system found that precipitation reduction altered the nutrient uptake strategy (increasing NH_4^+ uptake and decreasing NO_3^-) (Zhou *et al*., 2019), which may lead to changes in root exudates and in turn affect bacterial communities. Nevertheless, based on bacterial characteristics with a high rate of growth and turnover, precipitation reduction may shift bacterial communities by selecting some taxa with tolerance to perturbation (Angel *et al*., 2010). In support of this, our results showed an increased relative abundance of some bacterial phyla (e.g., Planctomycetes) that could be considered to be tolerant to the decline in soil

moisture (Bouskill *et al*., 2013). The results are consistent with a previous study that showed elevated abundance of Planctomycetes under throughfall exclusion (Bouskill *et al*., 2013), which indicates that Planctomycetes are sensitive to changes in soil moisture and maintain adaptive traits that allow the group to increase competitiveness under precipitation reduction (Bacher *et al*., 2010). However, in this study, some bacterial phyla (e.g., Verrucomicrobia) showed the opposite response pattern due to being unable to adapt to precipitation reduction. These phyla, such as Verrucomicrobia, display a water-related opportunistic behavior; thus, the abundance may rapidly decline under precipitation reduction treatments (Barnard *et al*., 2013). Moreover, Verrucomicrobia performs a fast-response life-strategy, and Planctomycetes follow a resistant life-strategy (Ramirez *et al*., 2012), which may also explain why they respond differently to precipitation reduction. However, our study indicated that long-time precipitation reduction created a new dynamic for the bacterial community.

In this study, some N transformation bacteria (ammonification, nitrification and nitrite_oxidation bacteria) significantly increased under precipitation reduction, which indicated that water was an important factor influencing N cycling. Precipitation reduction may have a significantly positive effect on the relative abundance of nitrification and nitrite_oxidation bacteria by increasing the accumulation of ammonium ions, solute concentrations and proportion of oxygen-filled soil pores (Stursova *et al*., 2006; Szukics *et al*., 2012; Chen *et al*., 2013). Nitrification and nitrite_oxidation bacteria control the conversion of soil inorganic N to nitrate. Increases in their relative abundance may lead to soil nitrate accumulation, which would increase the risk of soil N leaching (Wang *et al*., 2019). Moreover, significant decreases in the relative abundance of nitrogen_fixation may be caused by nitrate accumulation and increased solute concentrations in the study. The result is consistent with a previous study that showed that the N-fixation rates would decrease in soils with high N availability (Reed *et al*., 2010).

Combined N deposition and precipitation reduction effects

Consistent with our expectation, there were significant combined effects of increasing N deposition and precipitation reduction on the soil bacterial community composition, which is consistent with a previous study (Nguyen *et al*., 2018). As mentioned above, soil water regulates diffusion and the mobility of nutrients. Thus, the effects of increasing N deposition on the bacterial community composition depend on the soil water availability (Zhang *et al*., 2015). The previous study showed that increased precipitation buffered the effects of N deposition on bacterial communities by increasing the diffusion and mobility of N availability (Li *et al*., 2016). In contrast, precipitation reduction may aggravate the effects of N deposition on bacterial communities. In this study, combined effects had a stronger effect on the relative abundance of the Proteobacteria phylum than did single N deposition, which supports the above hypothesis. However, in the study, precipitation reduction also relieved the effects of N deposition on some bacterial phyla (e.g., Acidobacteria), which indicated that precipitation reduction may reduce the effect of N deposition by increasing the absorption of plant NH_4^+ and decreasing the risk of soil pH reduction in the experimental system (Zhou *et al*., 2019). In summary, our study found that the combined effects of precipitation reduction and increasing N deposition on soil bacterial communities may not be general synergistic effects or antagonistic effects but should be multilatitudinal effects.

In addition, a previous study showed that most of the microbial functional groups significantly responded to N deposition only when there was a lack of water stress (Zhang *et al*., 2015), which is contrary to our result that showed that N deposition also significantly affected the N transformation of bacterial groups under precipitation reduction conditions. Zhou *et al*. (2019) showed that nitrate and ammonium concentration were significantly higher under the combined effects of N addition and precipitation reduction than under control conditions or with N addition alone in this experimental system in July 2017, which indicates that precipitation reduction reduced the loss of available N leaching under N deposition. Simultaneously, precipitation reduction can alter the soil physical structure and plant physiological properties. All these factors may lead to increased soil N loss by increasing the relative abundance of ammonification bacteria and decreasing the relative abundance of N-fixation bacteria (Fig. 4). These results indicated that the combined effects of precipitation reduction and N deposition played an essential role in the nutrient status and soil properties, which, in turn, influenced bacterial functional groups.

Seasonal effects

In this study, the bacterial diversity was lowest in the growing season and highest in the winter, which is consistent with the results of a previous study (Ladau *et al*., 2013). The growing season period of low substrate availability (plant-microbe competition) may be associated with bacterial diversity reduction (Moreau *et al*., 2015). Moreover, the Nestedness-resultant Dissimilarity Index was highest in the growing season and lowest in the FTC periods, which indicates that bacterial replacement is responsible for bacterial diversity in the growing season, and that bacterial turnover is responsible for bacterial diversity in the FTC periods. The result suggested that although spring FTC may kill a large number of bacteria, the spring FTC has less of an impact on the bacterial community structure. During the growing season the bacterial community is more affected by rhizodeposition, favoring copiotrophic organisms and reducing diversity, thereby increasing species replacement and altering the bacterial community structure (Preece and Penuelas, 2016). Furthermore, the plant rhizodeposition might explain the increase in aromatic compound related taxa during the growing season (Zhalnina *et al*., 2018).

In addition, a seasonal change also has a significant effect on the bacterial community composition, particularly an increase in Actinobacteria in winter and an increase in Proteobacteria in summer, in this study, which is consistent with the result of Kuffner *et al*. (2012). A previous study interpreted seasonal shifts of different phyla as reactions to fluctuations in the carbon substrate supply (Lipson, 2007). Strickland *et al*. (2009) also proved the effects of the substrate quality and availability on soil bacterial communities with an experiment. Thus, it is conceivable that the dynamics in the substrate supply may explain the seasonal shifts in the bacterial community composition in this study.

This shift in the bacterial community was characterized by increasing relative abundances of certain bacteria as well as decreasing relative abundances of certain bacteria, which led to altered bacterial functional groups (Fig. 5). In this study, we found that the relative abundance of cellulolytic and ureolytic bacteria was the lowest during the FTCs periods, which indicated that FTCs might lead to a reduction in biodegradation by reducing the abundance of related bacteria. Previous studies showed that soil FTCs could improve the litter composition (Zhu *et al*., 2013). We speculated that the acceleration of litter decomposition in FTCs may not be due to biodegradation but to physical effects

(mechanical fragmentation). Moreover, the physical effects of FTCs can breakdown soil aggregates, thereby increasing the availability of soil N (Song *et al*., 2017). FTCs can also promote some microbial cell destruction and stimulate the metabolism of surviving microbes via their internal release of C and N (Song *et al*., 2017). In this study, denitrification bacteria were the lowest, and nitrification bacteria were the highest in the FTC period compared with those in other periods (Fig. 4), which indicated that C and N are released by mechanical fragmentation, and cell destruction could promote the conversion of soil nitrate. Sanders-DeMott *et al*. (2018) reported that FTCs reduced the plant N uptake capacity and increased the soil solution ammonium, which could also lead to an increase in the soil nitrate transformation. However, FTCs are often accompanied by large amounts of soil moisture, so increasing nitrate transformation may increase the risk of soil N loss (Risk *et al*., 2013).

Environments that have greater seasonal and temporal variation, particularly in temperate forests with long and cold winters, may regulate the effects of increasing N deposition and precipitation reduction on soil bacterial communities because a wide range of physiological tolerances may already exist within the community (Cruz-Martínez *et al*., 2009). In this study, we found that seasonal changes and anthropogenic treatments had a significant interaction effect on bacterial communities. Alternatively, N addition and precipitation reduction may increase the seasonal variation severity of the bacterial community, thus resulting in new dynamics in the bacterial community composition (Waldrop and Firestone, 2006).

CONCLUSIONS

In summary, our study shows that seasonal changes, precipitation reduction and the N deposition pattern will likely significantly affect the composition of the soil bacterial community. Moreover, there were significant combined effects of increasing N deposition and precipitation reduction on the soil bacterial community composition. Because changes in the bacterial community composition would result in altered corresponding functions, N deposition and precipitation reduction might increase the loss of soil N by decreasing denitrification bacterial groups and increasing ammonification bacterial groups, respectively. The interaction of treatments and seasons also significantly changed the soil bacterial communities and functions, which indicates that the response of bacterial communities to N addition and precipitation reduction was complex and could be highly dependent upon the underlying seasonal variability.

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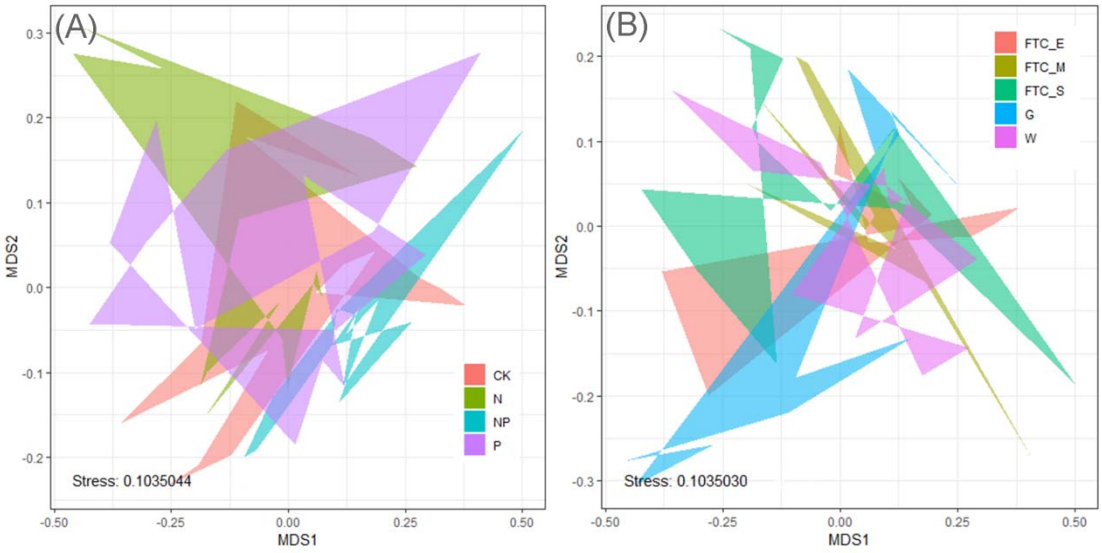

Fig.1 Nonmetric multidimensional scaling (NMDS) ordinations of bacterial community data by permutation-based analysis of variance illustrating significant separation of the bacterial community under different treatments (A) and seasonal changes (B). CK, control; P, precipitation reduction; N, N addition; NP, combined N addition and precipitation reduction. W, winter season (January 2017); FTC_S, start of snowmelt (February 2017); FTC_M, end of snowmelt (March 2017); FTC_E, end of soil freezing at 5 cm depth (April 2017); G, growing season (July 2017). Different colors represent different treatments or different seasons, and the more overlaps between different colors, the higher the similarity is. The stress value indicates the difference between the distance of points in 2-dimensional space and that of points in multidimensional space. (treatments, n=15; seasons, n=12)

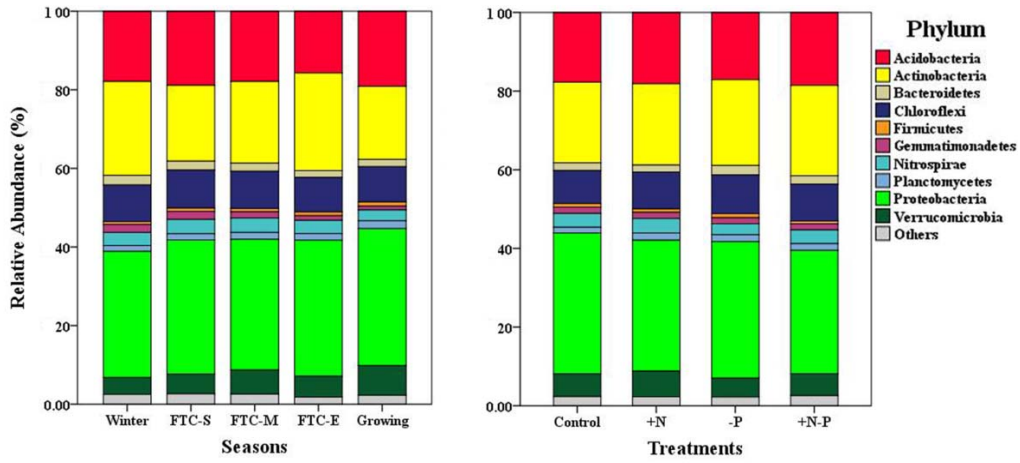

Fig. 2. Relative abundance of the main bacterial phyla in different seasons (A) under different treatments (B). -P, precipitation reduction; +N, N addition; +N-P , combined N addition and precipitation reduction. FTC_S, start of snowmelt (February 2017); FTC_M, end of snowmelt (March 2017); FTC_E, end of soil freezing at the 5 cm depth (April 2017); Growing, growing season (July 2017). (treatments, n=15; seasons, n=12)

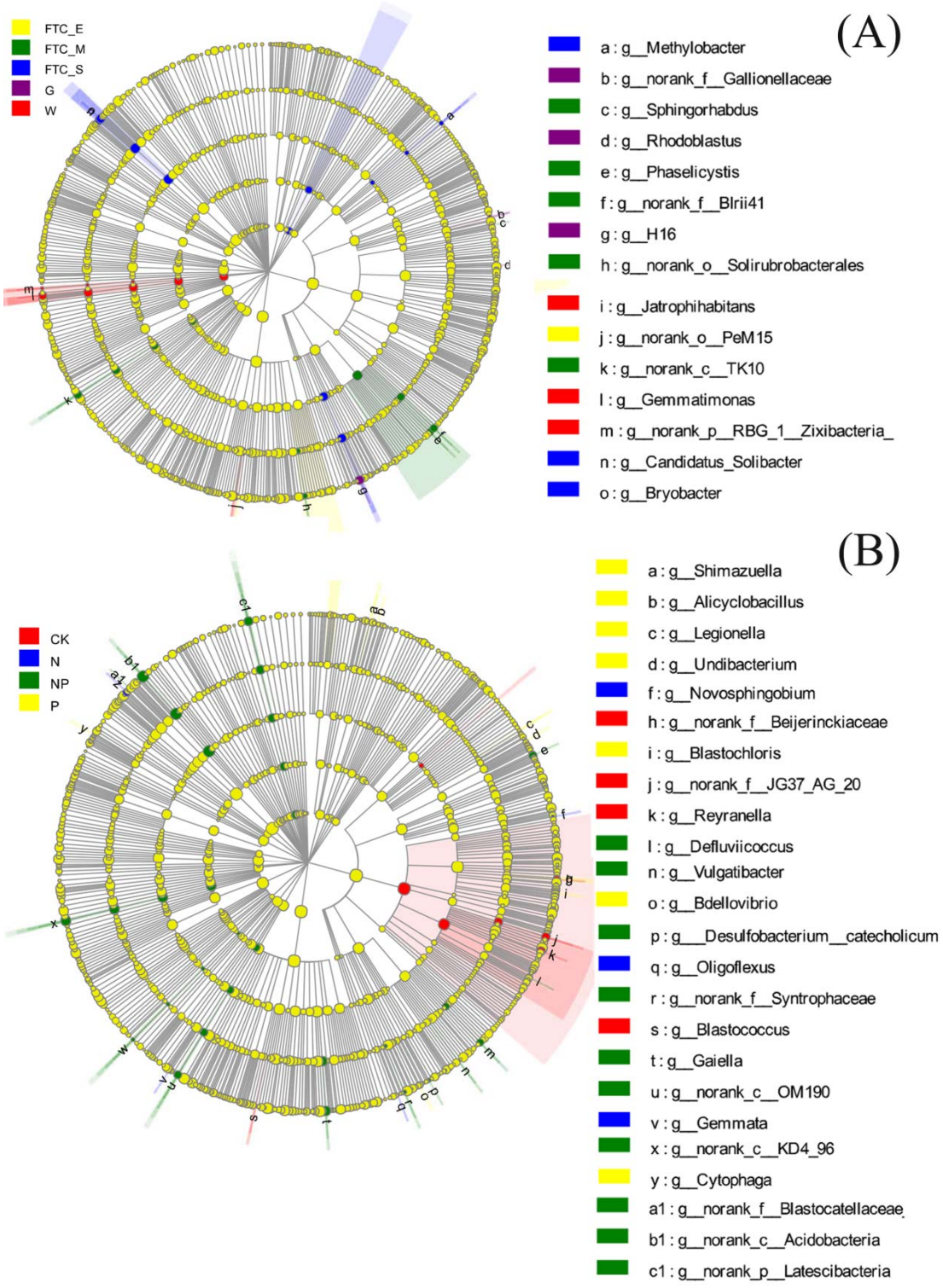

Fig.3. A linear discriminant analysis effect size method identifies the significantly different abundant taxa of bacteria in different seasons (A) and under different treatments (B). The taxa with significantly different abundances among seasons or treatments are represented by colored dots, and from the center outward, they represent the kingdom, phylum, class, order, family, and genus levels. The colored shadows represent trends of significantly different taxa. Only taxa that meet a linear discriminant analysis significance threshold of >2 are shown. P, precipitation reduction; N, N addition; NP, combined N addition and precipitation reduction. W, winter season (January 2017); FTC_S, start of snowmelt (February 2017); FTC_M, end of snowmelt (March 2017); FTC_N, end of soil freezing at 5 cm depth (April 2017); G, growing season (July 2017).

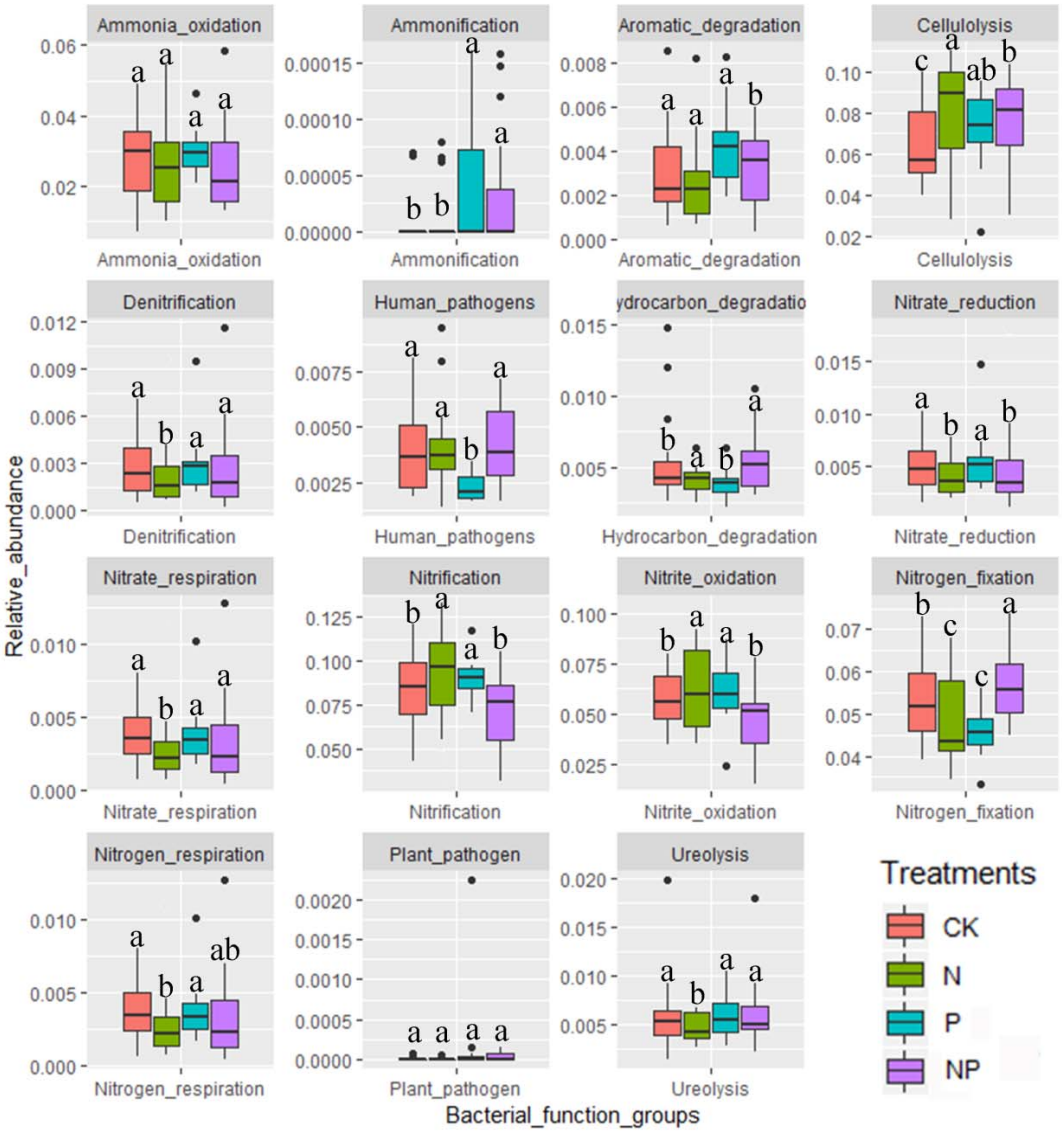

Fig. 4. Effects of N addition, precipitation reduction and their interaction on the relative abundances of bacterial functional groups (pathogenic bacteria, N input bacteria and N output bacteria were the focus in this study). Different lowercase letters indicate significant differences in different treatments ($p<0.05$). P, precipitation reduction; N, N addition; NP , combined N addition and precipitation reduction. Nitrate respiration means that the process of respiration used by some anaerobic organisms, in which nitrate rather than molecular oxygen is used to oxidize organic molecules to obtain energy. Nitrogen respiration is the process of a dissimilatory reduction of nitrogen compounds. These data are the combined data for all seasons (n=15)

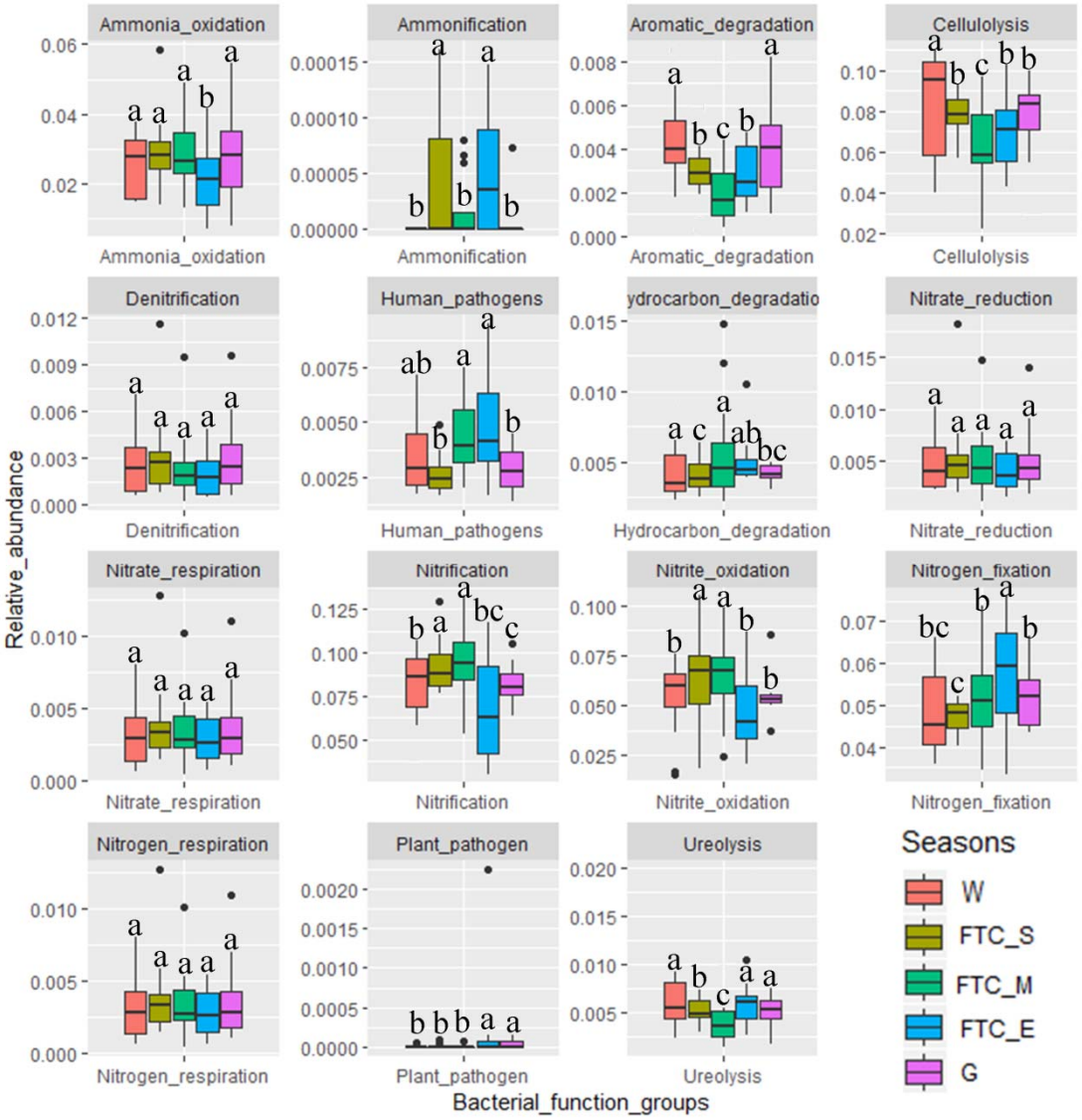

Fig.5. Effects of different seasons on the relative abundances of bacterial functional groups (pathogenic bacteria, N input bacteria and N output bacteria were focus in this study). Different lowercase letters indicate significant differences in different treatments ($p<0.05$).W, winter season (January 2017); FTC_S, start of snowmelt (February 2017); FTC_M , end of snowmelt (March 2017); FTC_E, end of soil freezing at the 5 cm depth (April 2017); G, growing season (July 2017). Nitrate respiration means the process of respiration used by some anaerobic organisms, in which nitrate rather than molecular oxygen is used to oxidize organic molecules to obtain energy. Nitrogen respiration is the process of dissimilatory reduction of nitrogen compounds. These data are the combined data for all treatments (n=12).