



# Soil Bacterial Community Responses to N Application and Warming in a Qinghai-Tibetan Plateau Alpine Steppe

Zhiyuan Mu<sup>1</sup>, Shikui Dong<sup>1,2\*</sup>, Yaoming Li<sup>2</sup>, Shuai Li<sup>3</sup>, Hao Shen<sup>1</sup>, Jing Zhang<sup>1</sup>, Yuhui Han<sup>1</sup>, Yudan Xu<sup>1</sup> and Zhenzhen Zhao<sup>4</sup>

<sup>1</sup> State Key Joint Laboratory of Environmental Simulation and Pollution Control, School of Environment, Beijing Normal University, Beijing, China, <sup>2</sup> School of Grassland, Beijing Forestry, Beijing, China, <sup>3</sup> College of Resource and Environment, Shanxi Agricultural University, Jinzhong, China, <sup>4</sup> School of Environmental and Chemical Engineering, Shanghai University, Shanghai, China

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### \*Correspondence:

Shikui Dong  
dongshikui@bjfu.edu.cn;  
dongshikui@sina.com

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Nitrogen deposition and climate warming can alter soil bacterial communities. However, the response of soil bacteria in an alpine steppe to these changes is largely unknown. In this study, a field experiment was performed on the northeastern Qinghai-Tibetan Plateau to determine the changes in soil bacterial communities of alpine steppes in response to nitrogen application and warming. The experiment consisted of four treatments, namely no-N application with no-warming (CK), N application (8 kg N ha<sup>-1</sup> year<sup>-1</sup>) with no-warming (N), warming with no-N application (W), and N application (8 kg N ha<sup>-1</sup> year<sup>-1</sup>) with warming (W&N). This study aimed to investigate (1) the changes in soil bacterial diversity and community structure under simulated nitrogen deposition and warming conditions, and (2) the key environmental factors responsible for these changes. Based on the results, soil bacterial diversity and community composition did not change significantly in the short term. Warming had a significant effect on overall bacterial composition, rare species composition, and individual bacterial taxa. Besides, the interaction between nitrogen application and warming had a significant effect on community  $\beta$ -diversity. Above-ground plant variables were highly correlated with bacterial community characteristics. Nitrogen application and warming did not significantly alter the distribution range of the bacterial community. Overall, this study suggests that soil bacterial communities can remain relatively stable at the level of simulated nitrogen application and warming and that short-term climatic changes may have no significant impacts on soil bacterial communities.

**Keywords:** Qinghai-Tibetan Plateau, soil bacteria, diversity, community composition, nitrogen application, warming

## INTRODUCTION

Ecosystems are profoundly affected by an ocean of human activities, including the usage of fossil fuels and changes in land use, resulting in an increased concentration of atmospheric carbon dioxide, increased deposition rates of nitrogen compounds, and increased average temperatures. Studies on the ecological responses to these global changes have shown that subsurface physical, chemical, and biological processes usually involve and are mediated by soil

microbes (Hu et al., 1999). For example, subsurface microbial-mediated processes can facilitate the fixation and release of nutrients that limit primary production and may influence the response of ecosystems to global changes in the long term.

The increase in surface temperature and nitrogen deposition rate is more pronounced at higher elevations (Bodelier and Laanbroek, 2004), affecting highly sensitive biogeochemical processes that depend on cold systems (Zhou et al., 2016). The Qinghai-Tibetan Plateau (the QTP), also known as the “Third Pole of the Earth,” is highly vulnerable to climate change and therefore considered a “pre-warming region” for the world (Wang et al., 2002). Nitrogen deposition in this region ranges from 8.7 to 13.8 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Lü and Tian, 2007), whereas warming is about three times the average global warming level of the past 50 years (Qiu, 2008). These more dramatic changes have significantly affected the biochemical processes of the alpine ecosystems on the QTP. Therefore, we speculated that the microbial community in alpine ecosystems of the QTP is highly sensitive and susceptible to climate change. Thus, the response of the soil microbial community to N deposition and warming in QTP grasslands could be used to predict future climate change, the carbon cycle, and ecosystem functions on the Tibetan Plateau.

The relationship between bacterial communities and environmental variables has been widely investigated. Although Boxman et al. (1998) and Frey et al. (2004) found insignificant effects of short-term N application on soil bacterial biomass, diversity and community composition, Xue et al. (2007) reported that short-term N application greatly increased the number of soil bacteria and thus changed bacterial community composition. This inconsistent result has been caused by the different nitrogen requirements of various bacterial species/taxa under a low soil nitrogen status, making it necessary to verify exactly how the soil bacterial community responds to short-term N addition. Also, N application has a certain acidifying effect on the soil, and some studies have shown that soil bacterial diversity, especially species richness, is significantly positively correlated with pH (Tripathi et al., 2012). This study also verified whether there is an indirect effect of such N application on the soil bacterial community. Meanwhile, Ratkowsky et al. (1982) found that temperature is an important factor for microbial growth. Also, warming likely affects soil bacterial communities indirectly by influencing water availability (Sheik et al., 2011; Waghmode et al., 2018) and above-ground vegetation (Chung et al., 2007). Previous studies have shown that warming significantly reduces the richness and diversity of bacterial communities (Waghmode et al., 2018) and substantially alters microbial community composition (DeAngelis et al., 2015). Other studies have reported a decrease in bacterial abundance and diversity in response to soil warming (Allison and Treseder, 2008; Castro et al., 2010; Hayden et al., 2012). In contrast, Zhang et al. (2005) showed that microbial community diversity increase with increasing temperature. Based on these contradictory findings, we aimed to further investigate how soil bacterial communities in alpine steppe on the QTP respond to warming treatments. Xiong et al. (2014) showed that short-term (15 months) warming does not significantly affect the  $\alpha$ -diversity of bacterial communities, but

it significantly shifted the soil bacterial community composition in the grasslands on the QTP. Xiong et al. (2016) found that the combined treatment of warming and nitrogen application significantly promotes soil microbial diversity and relative abundance, including Gram-positive bacteria and fungi, in a high-elevation alpine meadow. These observations highlight that microbial responses to N application and warming are complex and difficult to understand without knowing their potential interactive effects.

Soil bacteria represent a large fraction of the subsurface biomass and biome and regulate the biogeochemical cycling of organic matters. Therefore, the responses of soil bacterial diversity and composition to global climate change remain a major research interest. We performed field experiments to investigate how soil bacterial communities in an alpine steppe on the QTP respond to nitrogen deposition and warming. The experiment consisted of four treatments, including no-N application with no-warming (CK), N application (8 kg N ha<sup>-1</sup> year<sup>-1</sup>) with no-warming (N), warming with no-N application (W), and N application (8 kg N ha<sup>-1</sup> year<sup>-1</sup>) with warming (W&N). The experiments were conducted using ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) and open-top chambers (OTC) in an alpine steppe (AS), one of the major types of alpine grasslands at Tiebujia Town on the QTP (Zhang Y. et al., 2016), during the growing season. This study aimed to determine: (1) the changes in soil bacterial diversity and community composition at different gradients of controlled nitrogen deposition and warming; (2) the key environmental factors responsible for these changes?

## MATERIALS AND METHODS

### Study Sites

This study was conducted in Tiebujia Town of Gonghe County, Hainan Tibetan autonomous prefecture (37°02' N, 99°35' E, 3270 m ASL), Qinghai Province, China. The study site is located west of the Qinghai Lake, covered by the alpine steppe (AS). The mean annual temperature is approximately 0°C, with a yearly evaporation capacity of 1484 mm, and a mean annual precipitation of 380 mm (Zhao et al., 2017). The typical vegetation is alpine steppe, dominated by *Leymus secalinus*, *Stipa capillata*, *Poa crymophila*, and *Agropyron cristatum*. The soil is mostly composed of loam-clay (Zhao et al., 2017).

### Experimental Design

We applied a two-way factorial design (N application and warming) with four treatments: control treatment (CK), only N application (N), only warming (W), and a combined treatment of warming and N deposition (W&N). In early 2015, six plots in the grassland part of the alpine steppe (AS) were fenced. Each plot had an area of 10 m<sup>2</sup> (2 m × 5 m). We randomly selected three plots (replicates) for the control treatment and another three plots (replicates) for the nitrogen application treatment, with ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) of 8 kg N ha<sup>-1</sup> year<sup>-1</sup>. According to a previous study, 8 kg N ha<sup>-1</sup> year<sup>-1</sup> is the current level of annual N deposition in this area (Lü and Tian, 2007). Also, there

were six open-top chambers (OTCs), where the temperature was about 2°C higher than on the outside. Three OTCs (replicates) were randomly placed in the fenced plots for only warming treatments (W), and the other three OTCs (replicates) were randomly placed in the fenced plots for a comprehensive treatment of warming and N deposition (8 kg N ha<sup>-1</sup> year<sup>-1</sup>) (W&N). All the treatments had a similar land use history and topography. The N was applied in form of ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) in early May, early July, and early September of 2015–2017. The ammonium nitrate was first dissolved in deionized water and sprayed evenly in the sample plots using a spray bottle.

## Soil Sampling

Soil samples were collected in July, 2017, after 3 years of experimental N addition and warming. In each plot, three 3.5-cm diameter soil cores (0–20 cm in depth) were collected after fertilization in early July. The sample were mixed and sieved through a 2-mm mesh to remove roots and stones, then stored at –80°C until analysis.

## Soil and Vegetation Property Measurements

To measure nitrate nitrogen (NO<sub>3</sub>-N) and ammonium nitrogen (NH<sub>4</sub>-N), the soil was extracted with a 0.01 mol/L CaCl<sub>2</sub> solution at a soil-solution ratio of 1:2.5, shaken in a shaker at 180 rpm for 30 min and then removed and left to stand. Subsequently, the supernatant was filtered through a filter membrane, and NO<sub>3</sub>-N and NH<sub>4</sub>-N were determined using a flow injection auto-analyzer (AACE, Germany). For the measurement of available potassium (AK) and available phosphorus (AP), the soil was air-dried and ground, passed through a 100-mesh sieve, weighed with an accuracy of 0.1 g, and placed in a Teflon jar. We then added 5 mL of nitric acid, 2 mL of hydrofluoric acid, and 1 mL of perchloric acid. The jar was then placed in an automatic digestion apparatus and heated according to the following protocol: 120°C 1 h, 140°C 1 h, 160°C 1 h, 180°C 45 min. Next, the reflux funnel was removed, and the acid was droved to nearly dry. After cooling, the volume was brought to 25 mL with ultra-pure water, and AP and AK were determined using inductively coupled plasma spectrometers (ICP) (SPECTRO ARCOS EOP, Germany). For the measurement of total carbon (TC), the soil was passed through a 100-mesh sieve. About 0.2 mg of the sieved soil was taken and wrapped in a tin cup. TC was measured using an element analyzer (EA 3000, Italy). Soil pH was determined from the filtered supernatant using a pH meter (Mettler Toledo). Soil moisture (SM) was measured using the wet soil and dry soil weight. To obtain dry soil, a small portion of the wet soil from each sample was placed in an aluminum specimen box, and oven-dried at 105°C for 24 h. To estimate vegetation variables, we randomly selected a 1 m × 1 m square area in each plot and recorded vegetation type, abundance, and average height according to the method of Wang et al. (2012). Subsequently, a 25 cm × 25 cm area of vegetation was trimmed, dried, and weighed to obtain above-ground biomass data (AGB). Vegetation diversity was measured by the Shannon-Wiener diversity index (SW).

## DNA Extraction and PCR and DNA Sequencing

We performed DNA extraction and polymerase chain reaction (PCR), and DNA sequencing as described previously (Li et al., 2016). The DNA was extracted from 0.5 g of soil using a soil-specific FastDNA spin kit according to the manufacturer's instructions. The DNA quality and quantity were assessed using a Nano-Drop ND-1000 spectrophotometer. Subsequently, the DNA samples were diluted to 10 ng/μL and stored at –20°C. Before high-throughput sequencing, the DNA samples were subjected to PCR amplification with bacteria-specific primers targeting the V4-V5 regions of the 16S rRNA: forward 515F (5'-GTGCCAGCMGCCGCGG-3') and reverse 907R (5'-CCGTC AATTCMTTTRAGTTT-3).

The sequences in the V4 of 16S rRNA region provide comprehensive coverage (Sul et al., 2011) with the highest for taxonomical accuracy (Wang et al., 2007). Briefly, 50 μL of the amplification reaction system contained 1 × buffer, 0.2 μM of each primer, 1.5 mM MgCl<sub>2</sub>, 10 ng of template, and 1 unit of Pfu polymerase (BioVision, Mountain View, CA, United States). The amplification procedure was as follows: 2 min at 94°C, followed by 25 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension step for 10 min. After three repetitions of the reaction procedure, the resulting products were combined and separated by gel electrophoresis. The separated PCR products were extracted from the gel using the QIAquick gel extraction kit and purified using the Qiagen PCR purification kit. High-throughput sequencing was performed with the Miseq Sequencer (Illumina).

## Sequence Analysis

Sequence analysis was conducted as described previously (Li et al., 2016). Sequence processing and diversity estimation were performed using the microbial data processing platform QIIME (Quantitative Insights into Microbial Ecology). As suggested by Huse et al. (2007), sequences containing ambiguous base calls, less than 380 nt or more than 10 homologs were eliminated. A chimera check was performed with QIIME via ChimeraSlayer. There were four replicate datasets for each treatment (i.e., CK, N, W, and W&N). The pairing distance between the sequences was calculated with the distant neighbor algorithm, and the OTU was clustered by 97% sequence similarity. Singleton OTUs (with only one read) were removed, and the remaining sequences were sorted into each sample based on OTUs (Zhou et al., 2012). Subsequently, the most abundant sequence was selected from each OTU as the representative sequence. The Basic Local Alignment Search Tool (BLAST) was used for the taxonomic assignment of each representative sequence with a subset of the Silva database, and the bacterial OTU was classified. Based on a previous study, 2,000 denoised sequences per sample could interpret more than 80% of the trends in α-diversity and 95% in β-diversity in samples where 15,000–20,000 bacterial sequences were observed (Lundin et al., 2012). When sampling up to 2,100 denoised sequences, the rarified data set should be acceptable. Rarefaction was repeated 30 times, and each subsequent analysis was based on the average of 30 random trials.

## Data Analysis

Statistical analysis was performed using the software packages of vegan, picante, car and cardata in R v3.6.4. The sample was rarified to 21,000 sequences because the number of sequences between soil cores was not equal. Samples with less than 21000 sequences were not included. Samples of plots 1 (CK), 2 (N), 3 (W), and 4 (W&N) were up to this standard and were excluded. Bacterial  $\alpha$ -diversity was characterized using the Shannon-Wiener Index (SW). Species richness (SR, calculated as OTU numbers), and Pielou's evenness, and bacterial  $\beta$ -diversity was determined by the average pairwise community differences in each treatment using the Bray-Curtis distance matrix (Rodrigues et al., 2013). The distribution of OTUs in each soil core was calculated based on the number of soil cores that the OTUs evenly distributed in Rodrigues et al. (2013), whereas the distribution of each OTU was calculated as the number of soil cores in which it was detected. Tukey's HSD test was used to perform multiple comparisons of the bacterial phyla,  $\alpha$ -diversity,  $\beta$ -diversity, and relative abundance across the four treatments. Two-way analysis of variance (ANOVA) was performed to examine the effects of N application, warming, and their interactions on bacterial diversity.

Non-metric multidimensional scaling (NMDS) was used to estimate the differences in overall community composition across the treatments. A total of 16 soil and plant variables were tested to evaluate possible links between bacteria and soil and vegetation variables. Stepwise regression analysis was performed to determine the variables influencing bacterial  $\alpha$ - and  $\beta$ -diversity. For bacterial composition, the most important factors were selected based on variance inflation factors (VIF < 10) with 999 Monte Carlo permutations, as well as Mantel test and biology (Zhou et al., 2011). Finally, we fitted nine variables as vectors onto the NMDS ordination graphic to clarify the relationships among soil, vegetation and the bacterial community.

## RESULTS

### Effects of N Application and Warming on Soil Physicochemical and Plant Properties

There were no significant changes in soil pH, moisture,  $\text{NH}_4^{4+}$ -N, AK, AP, and TC caused by N addition, warming and their interaction. Nitrogen addition alone caused a significant increase in  $\text{NO}_3^{-}$ -N and AGB by 44.17 and 45.07%, respectively. Warming caused a significant increase in AGB by 81.37%, but it induced a significant decrease in plant diversity by 28.55%. Significant positive interaction effects between N addition and warming were found on AGB (123.50%). A significant decrease in plant diversity by 26.49% was found caused by interactive plots (Supplementary Table 1).

### Effects of N Application and Warming on Bacterial Diversity

The interaction between nitrogen application and warming had no significant effects on bacterial  $\alpha$ -diversity (i.e., SW,

SR, and Pielou's evenness) (Table 1). Sole N application or sole warming did not induce significant changes in bacterial  $\alpha$ -diversity (Figure 1). The responses of bacterial  $\alpha$ -diversity to N application and warming were significantly correlated with some soil (e.g., TC) and plant (plant SR and AGB) factors, which explained more than 30% of the variation in bacterial  $\alpha$ -diversity (Table 2).

Nitrogen application alone or warming alone caused no significant changes in bacterial  $\beta$ -diversity. The interaction between nitrogen application and warming also did not cause significant changes in bacterial  $\beta$ -diversity, which significantly increased in the interaction treatment compared to the nitrogen addition and warming treatments by 48.58 and 48.47%, respectively (Figure 1 and Supplementary Table 2). Interaction effects played a crucial role in altering the bacterial  $\beta$ -diversity ( $P = 0.018$ ) (Table 1). The parameters AGB,  $\text{NO}_3$ -N, and AK were critical factors influencing bacterial  $\beta$ -diversity, explaining 65% of the variation in bacterial  $\beta$ -diversity (Table 2).

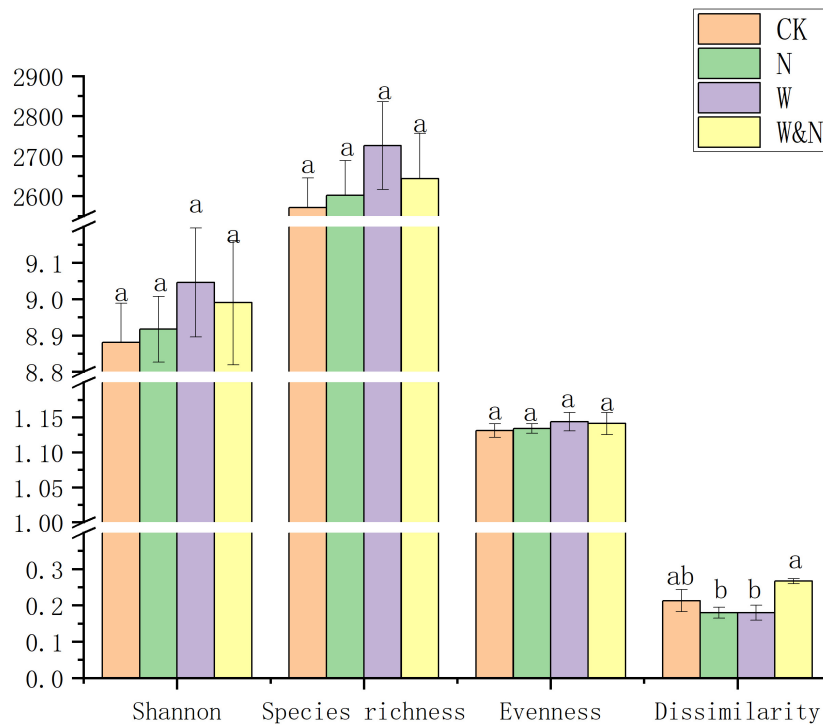
### Effects of N Application and Warming on Bacterial Community Composition

In our study site, the soil bacterial community mainly consisted of Actinobacteria followed in decreasing order of relative abundance by Proteobacteria, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes (Figure 2). Warming significantly affected the overall and rare bacterial species composition (Table 1). The NMDS analysis showed that both

**TABLE 1** | Significance tests of the treatments on bacterial community diversity, overall community, and rare species (i.e., relative abundance <0.1%) composition and phylum groups using N application (N) and warming (W) as main factors. Bacterial  $\beta$ -diversity was estimated as the average pairwise community dissimilarity within each treatment.

	N	W	N&W
Shannon-Wiener index	0.921	0.244	0.641
Species richness	0.714	0.192	0.435
Pielou's evenness	0.983	0.283	0.753
$\beta$ -diversity	0.223	0.221	<b>0.018</b>
Overall bacterial composition	0.865	<b>0.046</b>	0.690
Rare species composition	0.749	<b>0.006</b>	0.745
Actinobacteria	0.839	0.591	0.864
Proteobacteria	0.891	0.776	0.732
Alphaproteobacteria	0.841	0.361	0.739
Betaproteobacteria	0.838	0.992	0.940
Deltaproteobacteria	0.474	<b>0.001</b>	0.119
Gammaproteobacteria	0.838	0.075	0.514
Acidobacteria	0.492	0.613	0.745
Gemmatimonadetes	0.845	0.249	0.143
Nitrospirae	0.832	0.626	0.518
Bacteroidetes	0.473	0.438	0.624
Chloroflexi	0.828	0.225	0.742
Planctomycetes	0.883	<b>0.038</b>	0.958

Only phylum groups with relative abundance greater than 0.01 are shown. *P* values indicate the statistical significance. Bold values represent significant effects ( $P < 0.05$ ) of treatments.



**FIGURE 1 |** Bacterial Shannon-Wiener index, species richness, Pielou's evenness and bacterial community dissimilarity for no-N application with no-warming (CK), N application with no-warming (N), no-N application with warming (W), and N application with warming (W&N) treatment. Different letters indicate significant differences at  $p < 0.05$  level. Error bars represent standard error ( $n = 3$ ).

nitrogen application and warming deviated the sample from the control in the same direction, and that the warming effect was greater. The interaction between warming and N

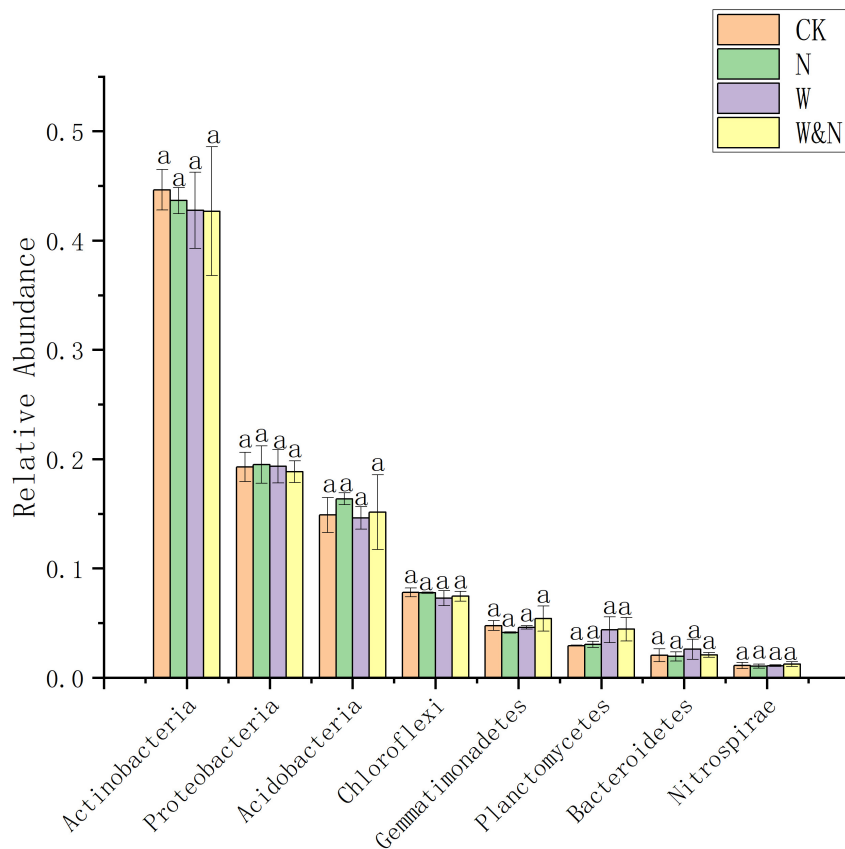
**TABLE 2 |** Stepwise regression analysis of soil and vegetation factors influencing bacterial Shannon-Wiener index (SW), species richness (SR), Pielou's evenness, and  $\beta$ -diversity.

	Variables	Beta	P	Adjusted R <sup>2</sup>
SW	Plant SW	-1.320	<b>0.01</b>	0.49
	AGB	-1.393	<b>0.04</b>	
	TC	0.913	0.06	
SR	Plant SW	-1.293	<b>0.02</b>	0.36
	AGB	-1.399	0.06	
Pielou's evenness	Plant SW	-1.246	<b>0.03</b>	0.26
	AGB	-1.367	0.08	
Beta diversity	AGB	1.443	<b>&lt; 0.01</b>	0.65
	NO <sub>3</sub> -N	0.701	<b>0.03</b>	
	AK	-0.924	<b>0.02</b>	
	AP	-0.797	0.05	

The beta value is a standardized regression coefficient, which indicates the rate of change in the dependent variable brought about by each independent variable. Bold values represent a significant correlation ( $P < 0.05$ ).

application resulted in an increasing difference among the samples (Figure 3). At the phylum level, N application alone, warming alone, and interaction between N application and warming did not significantly change the relative abundance of phylum groups with relative abundance greater than 0.01 (Figure 2). There were significant effects of warming on the phylum/subphylum composition of Planctomycetes ( $p = 0.038$ ) and Deltaproteobacteria ( $p = 0.001$ ), although the differences of relative abundances among the four treatments were not significant (Table 1).

At the 97% identity level, the resampled 34633 sequences were distributed into 4508 OTUs. A total of 85.69 and 88.58% of the OTUs (99.45 and 99.64% reads) were found in no-warming (CK and N)-warming (W and W&N), no-N application (CK and W)-N application (N and W&N) treatments (Figures 4A,D), respectively. The OTUs unique for no-warming and warming treatments accounted for 6.21% (0.20% of reads) and 8.10% (0.34% of reads) of the overall OTUs, respectively (Figure 4A). No-N application and N application treatments occupied 5.50% (0.17% reads) and 5.92% (0.19% reads) unique OTUs. Neither nitrogen application nor warming significantly affected the distribution range of shared and unique OTUs (Figures 4B,C,E,F). The two most abundant OTUs (OTU9 and OTU1) detected in the treatments belonged to the order Solirubrobacterales (class Thermoleophilia, phylum Actinobacteria). There were no significant effects of N application, warming, and their interaction on the



**FIGURE 2** | Relative abundance of bacterial phyla in no-N application with no-warming (CK), N application with no-warming (N), no-N application with warming (W), and N application with warming (W&N) treatments. Error bars represent standard error ( $n = 3$ ).

relative abundances of the top 10 most abundant OTUs (Supplementary Table 3).

## Relationship Between the Shift in Bacterial Community Composition and Environmental Variables

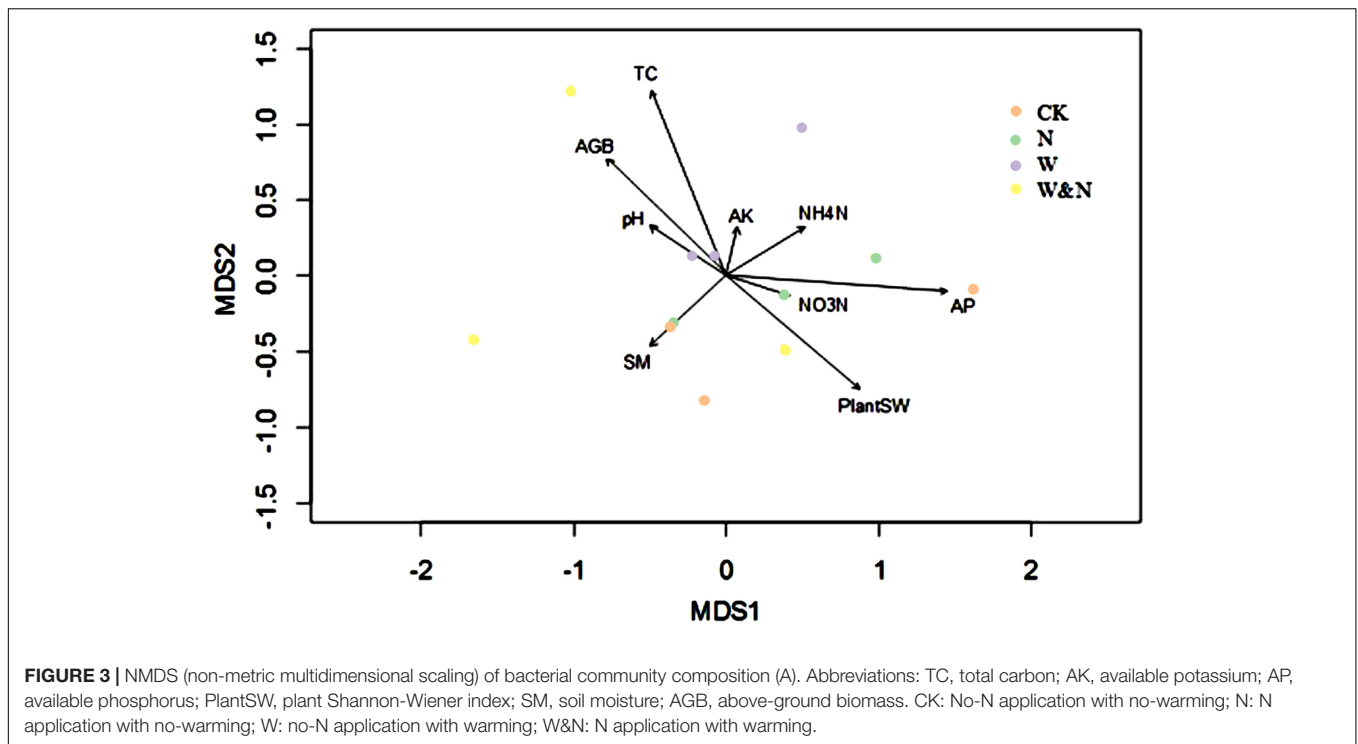
Based on the forward selection, five soil nutrient variables (pH, SM, TC,  $\text{NO}_3\text{-N}$ , and  $\text{NH}_4\text{-N}$ ), two vegetation variables (plant SR and above-ground biomass), and two available soil nutrient variables (AK and AP) were selected to interpret the interrelationships between environmental variables and the bacterial community. The NMDS analysis showed that plant variables were significantly related to bacterial community composition (Figure 3), which was also supported by the results of the Mantel test (Table 3).

## DISCUSSION

### Effect of N Application on Soil Bacterial Communities

In this study, 3 years of N application did not cause significant changes in soil microbial diversity and community structure

at the experimental site. This is slightly different from the results of some previous studies. Frey et al. (2004) found that nitrogen application reduced soil microbial species richness and community diversity indices. Meanwhile, Xue et al. (2007) found that short-term nitrogen addition significantly increased soil bacterial abundance and richness. In this study, the amount of nitrogen applied at the site did not reach a threshold sufficient to cause changes in the soil bacterial community, and this could have contributed to the contradictory results. Boxman et al. (1998) found that lower nitrogen input levels had little effect on bacterial biomass and diversity, but De Vries et al. (2006) observed a significant increase in bacterial activity with the application of higher nitrogen amounts. In this study,  $8 \text{ kg N year}^{-1} \text{ ha}^{-1}$  is similar to the global atmospheric nitrogen deposition level at this stage and may not be sufficient to produce shifts in the bacterial community. Meanwhile, this amount of N did not cause a significant decrease in soil pH. Some authors postulate that soil nitrogen fertilization may reduce microbial biomass due to the indirect effect of nitrogen fertilization on soil acidification (Janssens et al., 2010; Waring et al., 2013; Zhou et al., 2017). Other studies have shown that the species richness and diversity of soil bacteria were significantly positively correlated with pH (Tripathi et al., 2012; Shen et al., 2013). However, we did not observe similar effects in this study probably because the



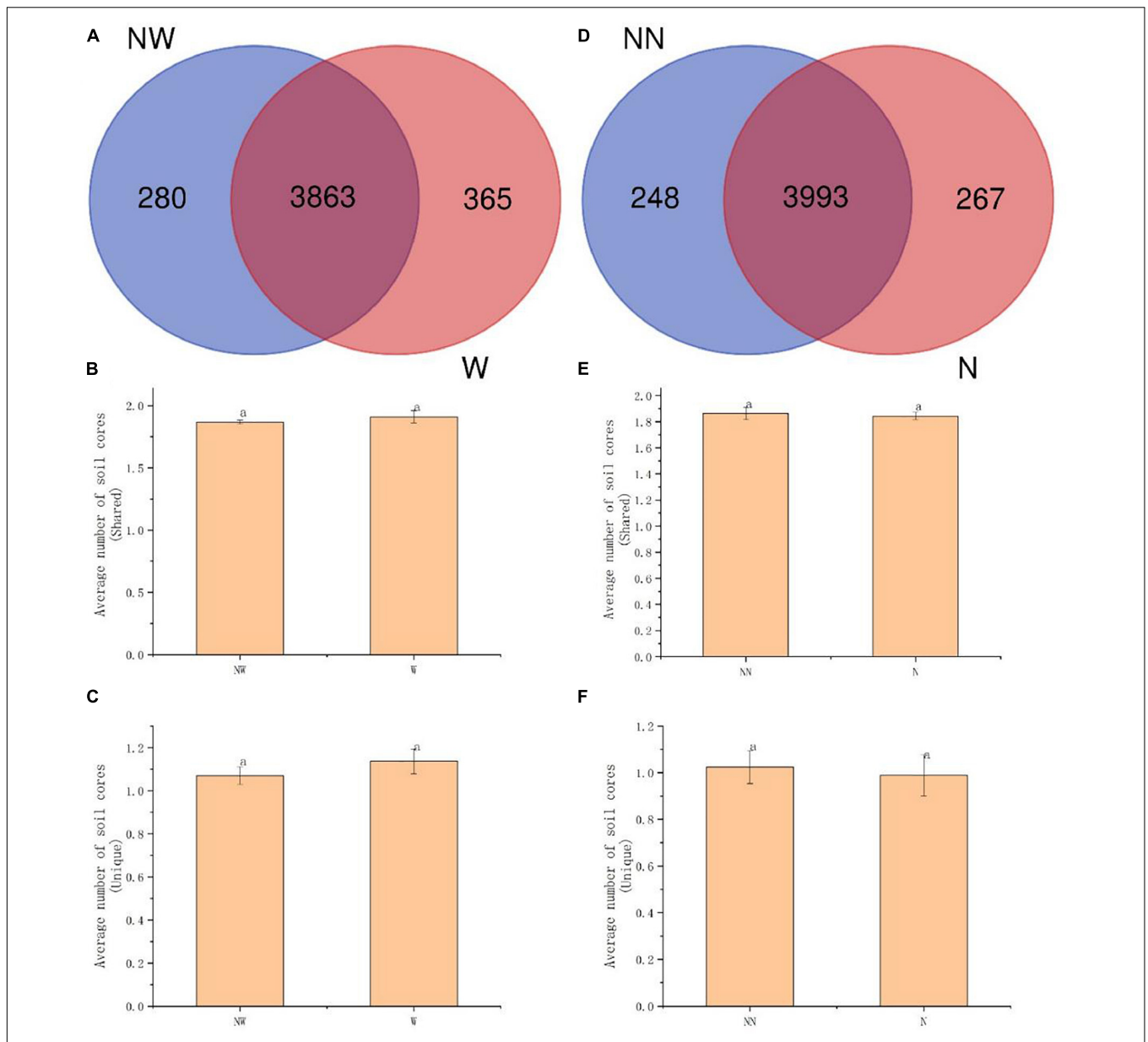
soil pH was high (about 6.76) and well buffered. We also found no significant change in soil pH due to nitrogen application, which may also contribute to the stability of the local bacterial community to some extent. Collectively, our results imply that the lower nitrogen inputs at our study site had no significant effect on soil bacteria in the short term.

### Effect of Warming on Soil Bacterial Communities

The bacterial community composition in the alpine steppe was either resistant to warming or took longer to show a response, which is consistent with the results of Zheng et al. (2012) regarding QTP grassland, which exhibited an unchanged methanotrophic community composition under warming conditions. Many previous studies in different sites have shown that long-term warming can cause significant changes in the diversity and composition of soil bacterial communities (Zhang et al., 2005; Rinnan et al., 2007, 2009; DeAngelis et al., 2015). Thus, we speculated that the short-term warming in this study might have contributed to the insignificant changes in microbial diversity. Li et al. (2016) found that warming significantly improved the evenness and similarity of the bacterial communities and broadened the distribution range of the bacteria on the QTP after 3 years of experimental warming. However, we did not observe similar changes in the 3-year warming period. Zhang Y. et al. (2016) observed that neither the warming nor the precipitation reduction treatment alone had any significant effect on soil bacterial community structure on the QTP, in contrast to the combination of the two treatments. Sheik et al. (2011) observed that warming reduced soil species richness,

evenness, and diversity during the non-drought period, whereas it increased soil species richness, evenness, and diversity during drought periods. These studies demonstrate that the effects of warming on microorganisms are dependent on water availability and that these two factors can potentially interact and affect the size and diversity of bacterial communities. Our experimental site was relatively dry, with an average annual precipitation of only 377 mm and average annual evaporation of almost four times the precipitation (1,484 mm): insignificant changes in soil water content were observed with warming. This may be attributed to the resistance of soil microbial diversity to warming. Further studies are required to discern how long this resistance will persist and which changes in bacterial community diversity and structure will eventually occur.

In this study, significant differences between the warming treatment and the CK were detected for the overall bacteria community composition and rare species composition of bacteria. However, insignificant changes were observed in the nitrogen application treatment alone or the interaction between nitrogen application and warming. This is consistent with the finding that warming significantly affected the communities of Planctomycetes and Deltaproteobacteria (Table 1), which could be attributed to the significant changes in biomass, relative abundance, activity, or other indicators of some of the more temperature-tolerant taxa in these two phyla. Planctomycetes are widespread in marine, hyper-saline, and even terrestrial soil habitats (Fuerst et al., 1997). *Isosphaera pallida*, a member of Planctomycetes, has moderately thermophilic physiology and can survive even at 55°C (Giovannoni et al., 1987). Deltaproteobacteria is an order of Proteobacteria, which is the most abundant taxon in the



**FIGURE 4 |** Distribution of bacterial OTUs across soil cores. **(A)** Venn diagrams showing the number of OTUs unique to no-warming (CK and N, NW) and warming (W and W&N, W) plots and those shared between them. **(B)** Number of soil cores that the shared OTUs were averagely distributed in for NW and W plots. Error bars indicate standard error ( $n = 3,863$ ). **(C)** Number of soil cores that the unique OTUs were averagely distributed in for NW and W plots. **(D)** Venn diagrams showing the number of OTUs unique to no-N application (CK and W, NN) and N application (N and W&N, N) plots and those shared between them. **(E)** Number of soil cores that the shared OTUs were averagely distributed in for NN and N plots. Error bars indicate standard error of the mean ( $n = 3,993$ ). **(F)** Number of soil cores that the unique OTUs were averagely distributed in for NN and N plots.

bacterial community and is divided into Alpha-, Beta-, Delta-, and Gammaproteobacteria. Previous studies have shown that taxa of this phylum and its sub-phyla are more susceptible to elevated temperatures (Sheik et al., 2011; Tuyet et al., 2015; Aydogan et al., 2018; Tao et al., 2020). These findings infer that increased temperatures induce the emergence and expansion of microbial communities that are more adapted to such conditions. Notably, increased temperature can enhance the competitive advantage of these communities, significantly

altering the overall community composition and structure. The temperature-sensitive bacterial taxa are of particular interest for future study.

### Interactive Effects of N Application and Warming on Soil Bacterial Communities

Against the background of climate change, previous studies have focused on the effects of individual climate change, such



**TABLE 3** | Significance tests of the correlation between soil and vegetation variables and bacterial community composition as shown by Mantel tests.

Groups	Variables	P
Total Soil Nutrition	NO <sub>3</sub> N	0.790
	NH <sub>4</sub> N	0.415
	pH	0.504
	SM	0.375
	TC	0.139
Available Soil Nutrition	AP	0.065
	AK	0.754
Plant	PlantSW	0.375
	AGB	<b>0.037</b>

Values are the *P*-values of the Mantel test. Significant correlations ( $P < 0.05$ ) are indicated in bold.

as nitrogen deposition, elevated temperature, and precipitation, among others, on soil microbes. However, it is still unclear how subsurface microbial communities respond to the interactions of these factors. NMDS analysis showed that the interaction of N-application and warming resulted in a significant difference among the samples, which corroborated the conclusion of Xiong et al. (2016) that the combined treatment of warming and nitrogen application can significantly promote soil bacterial diversity and relative abundance in a high-elevation alpine meadow. Two-way ANOVA showed that nitrogen application and warming had significant effects on the dissimilarity, i.e.,  $\beta$ -diversity, of the bacterial community (Table 1). The  $\beta$ -diversity of the bacterial community was significantly changed by the interaction of nitrogen application and warming in comparison with nitrogen application and warming alone (Figure 1). Admittedly, the different responses of many taxa to nitrogen application and warming treatments may be attributed to the complex interactions among soil, plants, and microorganisms because of their different sensitivities to various environmental factors (Singh et al., 2010). The combination of nitrogen application and warming may induce indirect effects of soil and vegetation properties on soil bacterial communities, which are likely to be greater than the direct effect (Xiong et al., 2016). In this study, stepwise regression analysis (Table 2) and the Mantel test (Figure 3) verified that vegetation diversity and above-ground biomass potentially influence soil bacterial diversity and community composition, suggesting that above-ground vegetation significantly affects below-ground bacteria. Previous studies have reported a strong link between soil bacterial communities and above-ground biomass and diversity (Stephan et al., 2000; Chung et al., 2007; Li et al., 2013). These effects reflect changes in vegetation and suggest that plant and microbial responses to nitrogen addition and warming are closely coupled. Meanwhile, previous studies involving warming treatments on the alpine grasslands showed positive effects on bacterial biomass in spring and winter, but opposite effects in summer and autumn, indicating that the impact of warming on soil microbial communities are seasonal (Belay-Tedla et al., 2009; Liu et al., 2009; Schadt, 2010). In summer and early autumn (growing season), the

vigorous plant growth at our experimental site could have affected the turnover and cycling of soil nutrient reservoirs, soil water availability, and other changes in the soil micro-environment, leading to intensified interactions between plants and microorganisms.

There is substantial evidence that climate change stressors significantly influence the composition and function of soil bacterial and even microbial communities in the grasslands of the QTP (Yang et al., 2014; Zhang et al., 2015). In this study, soil bacterial community in the alpine steppe showed some resistance to 3 years of nitrogen application and warming treatments, indicating that the soil bacterial community in the alpine steppe is stable under short-term nitrogen and warming treatments. Although we did not observe any significant changes in soil bacteria under short-term nitrogen addition and warming conditions, we cannot conclude that the soil microbial community will remain in this steady-state over the long term. Indeed, it may take longer to observe further changes in community composition and distribution in our experimental site. We did, however, observe a significant effect of warming on certain microbial taxa, suggesting that climate change potentially affects the soil microbial community structure by shifting species that can adapt to rapid environmental changes, increasing their dispersal opportunities, and, thus, changing ecosystem functions (Fierer et al., 2003; Gray et al., 2011). Therefore, it was crucial for us to further identify and study the roles and functions of rare microorganisms in the soil bacterial community. However, more in-depth and long-term studies are needed to examine the effects of nitrogen deposition and warming on soil microorganisms.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject: PRJDB11767/DDBJ DRA012263.

## AUTHOR CONTRIBUTIONS

ZM, SD, and YL contributed to conception and design of the study. SL and ZZ organized the database. ZM performed the statistical analysis and wrote the first draft of the manuscript. HS, JZ, YH, and YX wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.709518/full#supplementary-material>

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