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Research Article

Analysis of Bacterial Community Structure and Diversity in Different Restoration Methods in Qixing River Wetland -

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ABSTRACT

Returning farmland to wetland is a strategic measure for restoration of wetland ecosystems. Bacterial community structure and bacterial diversity in soil are important to the restoration of wetland ecosystems. However, very little research has been conducted to study bacterial communities in natural wetland restoration, particularly the bacterial community composition and diversity of returning farmland to wetland. We used high-throughput sequencing technology to compare the bacterial community structure and diversity of Natural Wetland (NW) soil with Returning Farmland to Wetland (RFW) and Returning Farmland to Forest (RFF) soils in Qixing River wetland. The following phyla were present in all samples: *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Cyanobacteria*, and *Saccharibacteria*. Of these, *Proteobacteria* (20.8–32.6%) *Acidobacteria* (14.4–33.9%) and *Actinobacteria* (11.4–19.0%) had the highest relative abundance in all the soil samples. The relative abundance values of *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were similar between RFW and NW soils. In addition, study of alpha diversity indices indicated that the ACE, Chao, and Shannon indices were significantly higher in RFW soils than NW and RFF soils. Redundancy Analysis (RDA) revealed that the total salt, organic matter, and total nitrogen significantly affected the bacterial community composition, while the total salt, organic matter, and total nitrogen were the dominant environmental factors in RFW soils. The present research indicated that RFW improved bacterial community diversity and the total salt, organic matter, and total nitrogen contents.

INTRODUCTION

Wetlands exist in the transitional zone between terrestrial and aquatic environments, and play important roles in water storage and treatment, global climate regulation, and wildlife habitat [1]. Studies have shown that plants, animals, as well as microorganisms are abundant in wetland ecosystems [2]. Microbes play the role of decomposers in these ecosystem and their primary function is energy flow and substance transformation. Meanwhile, microorganisms influence the succession and differentiation of wetland ecosystems [3]. Previous studies have demonstrated that bacterial communities constitute the majority of microorganisms in wetland habitats, [4]. Dynamic changes in the bacterial community point to changes in the wetland ecosystem environment and directly correspond to changes in soil health [5]. Studying the change of soil microorganisms under wetland ecological restoration are beneficial to realize the stage of wetland restoration [6]. A previous study in Yellow River wetland indicated that bacteria represent the largest group of microorganisms inhabiting the soil, accounting for 62.87–96.64% of the total population of soil microorganisms after returning farmland to wetland [7]. Environmental factors such as the total salt, pH, and soil water content have been reported to influence bacterial growth, which is very sensitive to changes in the soil quality [8]. It is essential to research the relationship between the bacterial community structure and environmental changes [9].

Following recent advances in sequencing technology, high-throughput sequencing has been widely used to study community structures of bacteria in soil, sediments, and freshwater [10–12]. Wang et al. reported that total organic matter, total phosphorus, salinity, and total nitrogen significantly influenced bacterial community structure under different soil environments [13]. Bolhuis and Stal studied bacterial community composition in coastal microbial mats via 16S rRNA gene tag sequencing technology and reported that bacterial diversity is influenced by seasonal variation and salinity via [14]. Furthermore, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the dominant phylum in the Winogradsky columns by testing bacterial diversity using high-throughput sequencing technology [15].

The Qixing River wetland is a freshwater marsh in the Sanjiang Plain, in Northeast China. Qixing River wetland has been listed in the Ramsar Convention in 2011 as a wetland of international importance [16]. The wetland covers about 2×10^4 ha and it is an important component of the wetland ecosystem in the cold temperature

zone [16]. The Qixing River wetland is categorized into three types, including Returning Farmland to Wetland (RFW), Natural Wetland (NW), and Returning Farmland to Forest (RFF). RFW is a strategic measure for wetland ecological restoration [17]. However, no studies have investigated the bacterial community structure in Qixing River wetland. Therefore, in the present study we aimed to study the correlation between bacterial community composition and environmental factors during the restoration of Qixing River wetland using high-throughput sequencing technology. Through this research, we hope to shed insight into the restoration process of returning farmland to wetland, which could serve as a scientific basis for returning farmland to wetland and help in the scientific management of wetland restoration in the Sanjiang Plain.

MATERIALS AND METHODS

Study area description

Qixing River wetland is located in Sanjiang Plain, Heilongjiang Province, China (46°40'–46°52' N, 132°05'–132°26' E). The wetland includes regions with ongoing Returning Farmland to Wetland (RFW), Natural Wetland (NW), and Returning Farmland to Forest (RFF) projects. It has a semi-humid continental monsoon climate with a mean annual air temperature is 2.35' [18] and average annual rainfall of 551.5 mm. The Qixing River wetland has different soil types, primarily consisting of pulp soil and swamp soil [19]. *Phragmites australis* and *Calamagrostis angustifolia* are the dominant plant species in the wetland.

Sample collection

In the RFW region of Qixing River wetland (46°43'10.8' N, 132°11'27.3' E), samples were collected in July 2014 (RFW1), October 2014 (RFW2), and May 2015 (RFW3) three times. *C. angustifolia* is the dominant plant species in RFW. According to the previous studies, the bacterial diversity was the highest in autumn [20]. Therefore, in order to compare the bacterial community composition between the NW (46°43'03.0" N, 132°11'23.7" E) and RFF (46°42'20.2" N, 132°06'03.4" E) regions, we collected soil samples from these two areas in October 2014. The dominant plant species in NW was *P. australis* and the main plant species in RFF was *Populus L.* There were three RFW, NW, and RFF plots, and three replicates were carried out for each plot. All soil samples were collected at a soil depth of 0–20 cm. The soil samples collected from each plot were thoroughly mixed, and stored in aseptic bags at 0–4' during transport to the laboratory.



In the laboratory, one part of the samples was dried for testing soil characteristics and the other part was stored at -80°C until analysis of bacterial community structure.

Analysis of soil physicochemical properties

To measure the soil Water Content (WC), soil samples were dried at 105°C for 24 h. Soil pH was determined using a compound electrode with a soil to water ratio of 1:2.5. Soil Organic Matter (OM) was determined using the potassium dichromate oxidation-outer heating method. Soil Total Nitrogen (TN) was measured by Kjeldahl digestion. Soil Total Phosphorus (TP) was measured using molybdenum antimony colorimetry. Soil Total Salt (TS) was determined using quality method.

DNA extraction and PCR amplification of soil bacteria

Bacterial DNA was extracted from the samples of Qixing River wetland by using the E.Z.N.A. [®] soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The V4-V5 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95' for 3 min, followed by 27 cycles at 95' for 30 s, 55' for 30 s, and 72' for 45 s and a final extension at 72' for 10 min) using primers 338F (5'-barcode- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of $5 \times$ FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Illumina MiSeq sequencing and accession number

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor[™] -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 \times 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP*****).

Bioinformatics and statistical analyses

Raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.9.1) using the following criteria: (i) 300-bp reads were truncated at any site with an average quality score of < 20 over a 50-bp sliding window, discarding truncated reads shorter than 50 bp; (ii) exact barcode matching, two nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed, and (iii) sequences with overlaps of more than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) [21], and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the Silva (SSU115) 16S rRNA database using a confidence threshold of 70%.

Canoco 4.5 was used to identify correlation between environmental factors and bacterial community structure, and the figure was generated by CanoDrao 4.0. Data in tables were analyzed by SPSS 22.0 software. Values in the tables indicate the means of the samples.

RESULTS

Physicochemical characterization of sample sites

In Qixing River wetland, the soil nutrient content was higher in the RFW than in the RFF and NW samples, especially in the October 2014 RFW sample (RFW2). The OM, TN, TP, and TS contents were higher in RFW2 than in NW and RFF (Table 1). The highest and lowest OM values were 17.50% and 9.38% and were found in RFW2 and RFF, respectively. The TS values ranged from 0.57 g/kg in RFF to 2.07 g/kg in RFW2. The TN increased from 0.35% in RFF to 0.73% in RFW2. The TP values increased from 0.067% in RFF to 0.154% in RFW2. The WC ranged from 22.30% in RFF to 44.30% in NW. However, the pH values of all the samples were nearly neutral (6.80–7.30).

Bacterial alpha diversity indices

Figure 1 presents a rarefaction curve of bacterial community at similarity level of 0.97. The OTU curve of the five samples tended to plateau when the sequence numbers reached 18567. The current results showed that the samples were reasonable and the sequencing depth covered all species in the five samples.

A total of 1, 85, 601 sequences were obtained from the five samples by high-throughput sequencing technology. A similarity

Table 1: Physical and chemical character of soil in Qixing River wetland.

Sample	pH	OM (%)	TN(%)	TP(%)	TS(g/kg)	WC(%)
RFW1	7.10	14.60	0.63	0.152	1.30	37.00
RFW2	7.10	17.50	0.73	0.154	2.07	29.30
RFW3	6.80	15.30	0.67	0.141	1.30	36.70
NW	7.30	12.60	0.53	0.069	1.18	44.30
RFF	7.10	9.38	0.35	0.067	0.57	22.30

Abbreviations: OM: Organic Matter; TN: Total Nitrogen; TP: Total Phosphorus; TS: Total Salt; WC: soil Water Content; RFW: Returning Farmland to Wetland. RFW1, RFW2, and RFW3 represent the time of collecting samples in July, October 2014 and May 2015. NW: Natural Wetland (October 2014), RFF: Returning Farmland to Forest (October 2014).

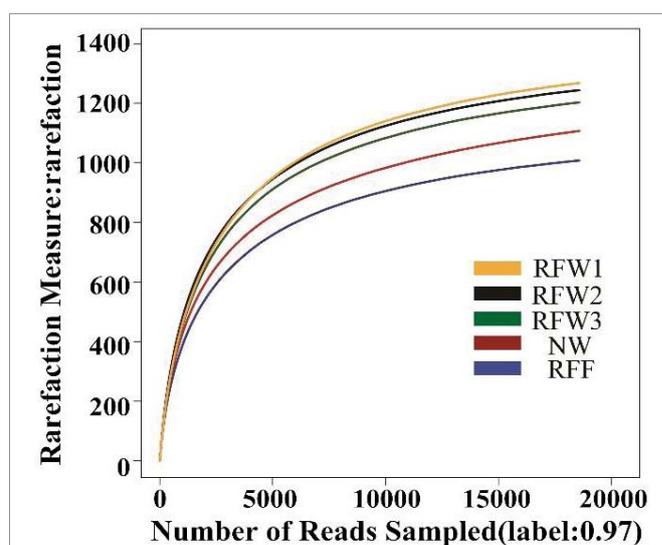


Figure 1: Rarefaction curve. The horizontal axis represents the number of number of reads sampled, and the vertical axis represents the observed number of Operational Taxonomic Units (OTU).



level of 97% was used to identify OTUs and to estimate diversity (Figure 2, Table 2). The results revealed that coverage was higher than 0.99 in all the samples. Therefore, the results of the high-throughput sequencing could adequately represent the bacterial community structure in Qixing River wetland (Table 2). As shown in figure 2, the Shannon diversity index was the highest in RFW2 and the lowest in RFF (Figure 2).

The ACE estimate of bacterial diversity ranged from 1087 to 1356 and the Chao estimate ranged from 1102 to 1380 (Table 2). The ACE and Chao values were significantly higher in RFW2 than in NW and RFF. The Shannon diversity estimate ranged from 5.89 in RFF to 6.36 in RFW2. The Simpson diversity estimate ranged from 0.0032 in RFW2 to 0.0059 in RFF. The Shannon and Simpson indices were significantly higher in RFW2 than in NW and RFF (Table 2).

Phylum-level taxonomic distribution

As shown in figure 3, based on the results of the high-throughput sequencing technology, the bacterial species in RFW1, RFW2, RFW3, NW, and RFF belonged to 12 phyla, including *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia*, *Gemmatimonadetes*, *Nitrospiras*, *Planctomycetes*, *Cyanobacteria*, and *Saccharibacteria* were included in five samples. *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*. Of these,

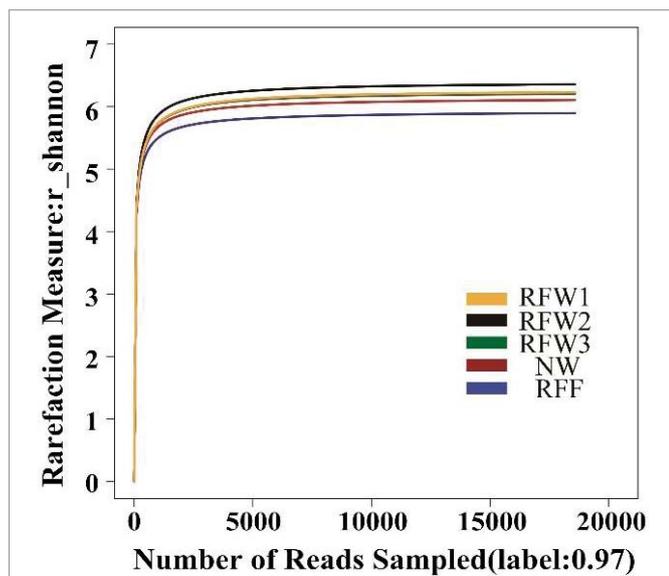


Figure 2: Shannon diversity index. The horizontal axis represents the number of reads sampled, and the vertical axis represents the Shannon-Wiener index.

Table 2: Alpha-diversity indices in different samples (0.97 level).

Sample	OTUs	Diversity Estimator		Richness Estimator		Coverage
		ACE	Chao	Shannon	Simpson	
RFW1	1268	1356d	1380b	6.23c	0.0046b	0.990629
RFW2	1244	1327c	1338b	6.36d	0.0032a	0.991436
RFW3	1203	1294c	1306b	6.2c	0.0045b	0.991113
NW	1107	1221b	1237a	6.1b	0.0045b	0.990359
RFF	1008	1087a	1102a	5.89a	0.0059c	0.992298

Abbreviations: ACE: Abundance Based-Coverage Estimator; OTUs: Operational Taxonomic Units. The same letters indicates that the values are not significantly different ($P < 0.05$).

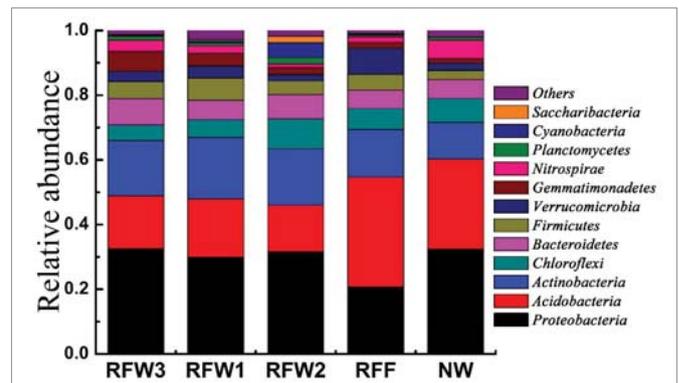


Figure 3: Analysis of the bacterial community at the phylum level

Proteobacteria had the highest relative abundance in RFW1, RFW2, RFW3, and NW, ranging from 29.9% to 32.6%. *Acidobacteria* was the highest abundance in RFF with a relative abundance of 33.9%. The relative abundance of *Actinobacteria* ranged from 11.4% to 19.0%.

Genus-level distribution

As shown in figure 4, the relative abundance of *Subgroup_6_norank* was the highest in all these zones, ranging from 8.5% to 20%. Furthermore, *Anaerolineaceae_uncultured* was relatively abundant in RFW2; the relative abundance values of *GR-WP33-30_norank*, *Gemmatimonadaceae_uncultured*, and *Gaiellales_uncultured* were higher in RFW3; those of *RB41_norank* and *DA101_soil_group_norank* were higher in RFF; and those of *GR-WP33-30_norank* and *Nitrospira* were over 5% in NW.

In addition, as shown in figure 4 (from *Actinobacteria_norank* to *Subgroup_6_norank*), the relative abundance values of *Anaerolineaceae_uncultured* and *Xanthomonadales_uncultured* were higher in RFW2 than in the other sampling groups. Additionally, from *Sh765B-TzT-29_norank* to *Bacteria_unclassified* (Figure 4, *Acidimicrobiaceae_uncultured*, *OPB35_soil_group_norank*, *Parcubacteria_norank*, *Blastococcus*, *Rhizobiales_unclassified*, *Comamonadaceae_unclassified*, and *Gitt-GS-136_norank* all had relatively higher abundance in RFW2 than in RFW1, FRW3, NW, and RFF. From *Pedomicrobium* to *Xanthomonadaceae_uncultured* (Figure 4), the relative abundance values of *Xanthomonadaceae_uncultured*, *Rivularia*, *Woodsholea*, *Leptolyngbya*, *SM1A02*, *SubsectionIII_FamilyI_unclassified*, *Porphyrobacter*, *Saccharibacteria_norank*, *Roseiflexus*, *A0839_norank*, *Flavobacterium*, *Cytophagaceae_uncultured*, *env.OPS_17_norank*, *Propionibacteriaceae_unclassified*, *JG34-KF-161_norank*, and *Pedomicrobium* were significantly higher in RFW2 than in the other zones.

Relationship between bacterial community composition and environmental factors

We used correlation analysis to investigate the relationship between bacterial diversity and environmental factors (Table 3). As shown in the table, there were significantly positive correlations between TP, TS and Shannon indices ($P < 0.01$) and between OM, TN, and Shannon ($P < 0.05$; Table 3). There were significantly positive correlations between ACE and TP ($P < 0.05$). However, negative correlations were observed between ACE, Shannon, and pH (pH value: 6.8–7.3). The results suggested that high nutrient content benefited bacterial growth in the Qixing River wetland and contributed to improve diversity in the bacterial community.

Figure 5 illustrates the relationships between the environmental factors and the sample plots. RDA was used to determine the correlation between the bacterial community structure and environmental factors. Six environmental factors were selected and included in the RDA (Figure 5). Among the environmental factors

influencing the bacterial community composition, TS, OM, and TN were the dominant environmental factors in this study. TS, OM, and TN significantly influenced the bacterial groups in RFW2 (Figure 5, Table 4). However, these factors only slightly influenced the bacterial community structure in RFF (Figure 5). At the phylum level, *SM2F11* and *Caldiserica* as well as *Candidate_division_WS6* and *Gracilibacteria* had little differences in Qixing River wetland (Figure 5).

DISCUSSIONS

A previous study reported that estimation of the alpha diversity, which reflects species diversity of the single sample, is an effective method to analyze bacterial group composition (Paul FK and Josephine Y A, 2004) Meanwhile, alpha diversity reacted bacterial community richness and diversity, including diversity estimators (ACE and Chao) as well as richness estimators (Shannon and Simpson) [22,23]. Higher values of ACE and Chao indices indicate greater diversity within the bacterial community. Higher values of the Shannon index indicates greater richness of the bacterial community, while a lower Simpson index represents greater richness. In the present research, we investigated bacterial diversity during the restoration process of the RFW, NW, and RFF zones in Qixing River wetland. The results revealed significant differences in bacterial diversity, especially in the ACE, Chao, and Shannon indices in RFW2, which were significantly higher than the corresponding values in NW and RFF. On the other hand, the Simpson index was lower in RFW2 than in NW and RFF (Table 2). Together, these results demonstrated that bacterial diversity was the highest in RFW, indicating that returning farmland to wetland was a reasonable approach for wetland restoration. The results of the present study revealed that RFW contributed to wetland reconstruction during the restoration of Qixing River wetland. A previous study found that higher bacterial diversity could be related to vegetation type and physicochemical characteristics [24].

The results of the high-throughput sequencing revealed that *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the dominant phyla in RFW1, RFW2, RFW3, NW, and RFF (Figure 3). These results are consistent with those of the previous study [27]. *Proteobacteria* (29.9–32.6%) exhibited the highest abundance in RFW1, RFW2, RFW3, and NW. Studies have already reported that *Proteobacteria* survive not only in acidic soil in the natural wetland of Virginia [25] but also in alkaline soil in the inland wetland of La Brava [26]. The soil pH of Qixing River wetland was found to be nearly neutral. Therefore, *Proteobacteria* was widely distributed in this habitat, which is similar

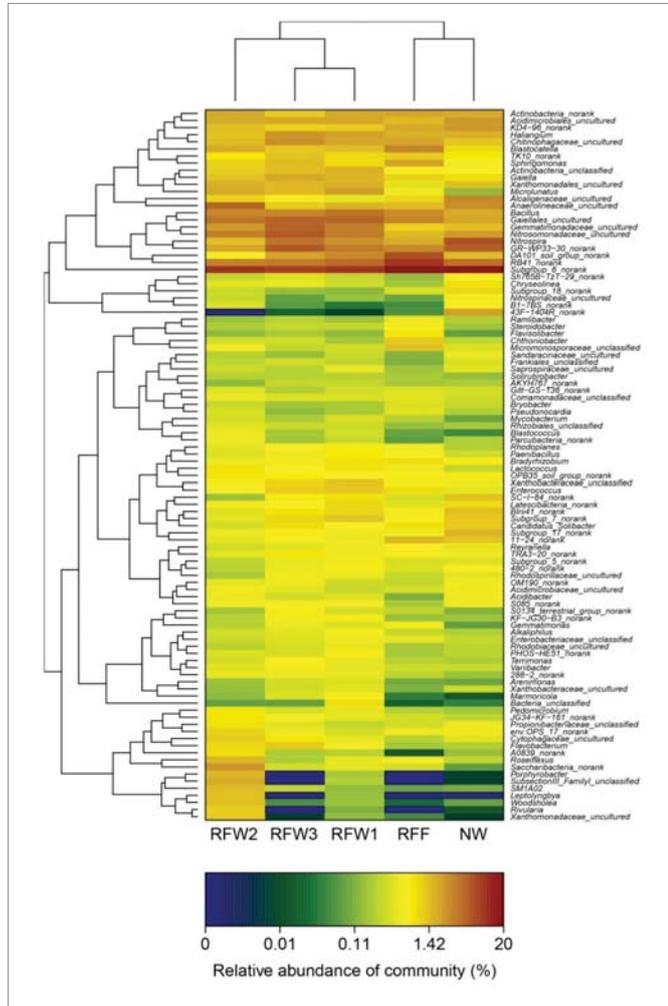


Figure 4: Analysis at the genus level. A color-scale heat map showing the top 100 representative 16s rRNA gene-based bacterial sequences classified at the genus level. Red indicates higher abundance, while green and blue represent progressively decreasing abundance.

Table 3: The correlation between bacterial diversity and matrix of soil physical and chemical characters.

	pH	OM	TN	TP	TS	WC	ACE	Chao	Shannon	Simpson
pH	1.000									
OM	-0.447	1.000								
TN	-0.447	1.000**	1.000							
TP	-0.224	0.900*	0.900*	1.000						
TS	-0.334	0.975**	0.975**	0.975**	1.000					
WC	0.447	0.000	0.000	0.100	0.051	1.000				
Ace	-0.224	0.700	0.700	0.900*	0.821	0.300	1.000			
Chao	0.224	0.500	0.500	0.800	0.667	0.500	0.900*	1.000		
Shannon	-0.224	0.900*	0.900*	1.000*	0.975**	0.100	0.900*	0.800	1.000	
Simpson	0.000	-0.821	-0.821	-0.667	-0.763	-0.205	-0.359	-0.359	-0.667	1.000

*indicates significantly differences ($P < 0.05$), **indicates significantly differences ($P < 0.01$).

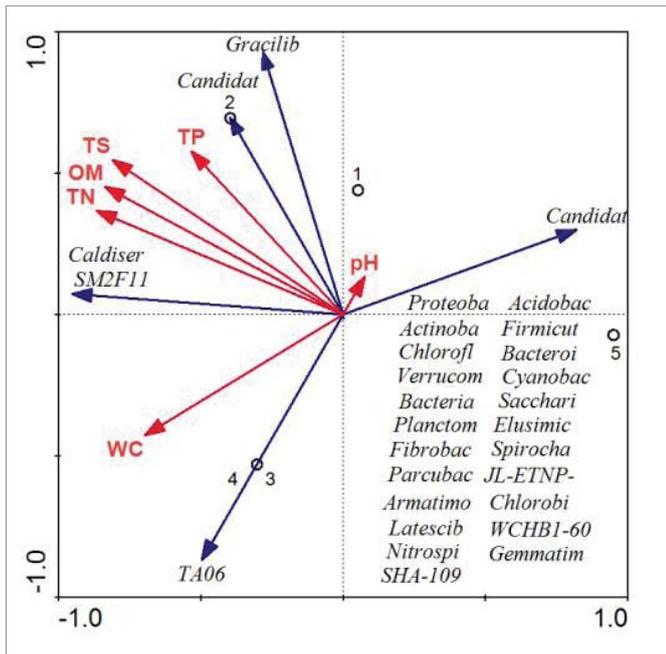


Figure 5: Results of the Redundancy Analysis (RDA) to evaluate the relationship between the bacterial community structure and environmental factors. RDA was used to evaluate the relationship between environmental factors and the bacterial community. Red arrows represent various environmental factors such as OM, TN, TP, TS, WC, and pH. Blue arrow represent the bacterial community. The length of the arrow indicates the correlation between bacterial distribution and environmental factors, i.e., a longer line indicates a greater correlation. The hollow dots labeled 1, 2, 3, 4, and 5 represent the sampling sites RFW1, RFW2, RFW3, NW, and RFF, respectively.

Table 4: Correlation coefficients of environmental factors with the first two axes of RDA.

Environmental factors	Axes 1	Axes 2
pH	0.0758	0.1330
OM	-0.8358	0.4513
TN	-0.8658	0.3666
TP	-0.5325	0.5767
TS	-0.8094	0.5460
WC	-0.6961	-0.4276

to the findings reported by Sun et al. [11]. Generally, *Acidobacteria* is a highly abundant and diverse phylum of the domain Bacteria [27,28], and it accounts for 20–46% of the total bacteria in soil [27,29]. Based on our study, *Acidobacteria* (33.9%) had the highest relative abundance among all the phyla in RFF. The distribution of *Acidobacteria* in soil was decreased with increasing soil moisture content [30]. *Actinobacteria* has been previously detected as the main phylum in wetland soils, with a relative abundance exceeding 10% [31]. The relative abundance values of *Actinobacteria* were higher in RFW than in the other wetland zones NW and RFF, indicating that the environmental conditions of RFW contributed supported the distribution of *Actinobacteria*. The relative abundance values of *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* in RFW1, RFW2, and RFW3 were similar to the corresponding values in NW (Figure 3). The results indicated that returning farmland to wetland was the most efficient restoration project in Qixing River wetland. Furthermore, as RFF did not recover at the same level as RFW, RFF may require a longer time to achieve NW status.

The bacterial community structure and bacterial diversity were both affected by environmental factors in Qixing River wetland (Figure 5, Table 3). As shown by the results of the RDA, the TS, OM, and TN were the main environmental factors influencing the structure and diversity of the bacterial community in the restoration process of Qixing River wetland. Previous studies have reported that the bacterial community composition is primarily affected by TS and TN at the global scale [31,32]. The TS, OM, and TN values were higher in RFW than in NW and RFF (Table 1), suggesting that returning farmland to wetland was the most effective method to achieve restoration in Qixing River wetland. Among the soil environmental factors, TS, OM, and TN were found to be the most important contributors to bacterial community in RFW2. However, TS, OM, and TN only slightly influenced the bacterial community structure in RFF. This may be because additional environmental factors (such as plant species and other uncertain environment variables) may contribute to the differences in bacterial community composition in RFF [33]. Therefore, returning farmland to wetland is the most effective reconstruction strategy for Qixing River wetland.

CONCLUSION

Here, we used high-throughput sequencing technology to investigate bacterial communities in areas of the Qixing River wetland undergoing RFW, NW, and RFF projects. At the phylum level, *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia*, *Gemmatimonadetes*, *Nitrospiras*, *Planctomycetes*, *Cyanobacteria*, and *Saccharibacteria* were present in the RFW1, RFW2, RFW3, NW, and RFF zones. Of these, *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the dominant phyla in all the samples. The ACE and Chao indices were higher in RFW than in NW and RFF. The Shannon index was significantly higher in RFW than in NW and RFF. Correlation analysis revealed a significantly positive correlation between TS, TP and the Shannon index ($P < 0.01$). Results of the RDA suggested that TS, OM, and TN significantly affected bacterial community composition in Qixing River wetland, especially in RFW2. Our results indicated that RFW is an effective method for restoration of Qixing River wetland. However, long-term studies are needed to verify the efficacy of restoration of RFF.

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