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Effects of simulated nitrogen deposition on soil microbial community diversity in coastal wetland of the Yellow River Delta

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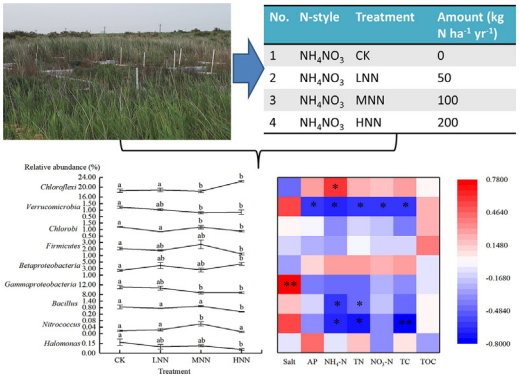
HIGHLIGHTS

- Nitrogen deposition affected the composition of the soil microbial community.
- The salinity was significantly correlated with the relative abundance of *Gammaproteobacteria*.
- Nitrogen deposition reduced the relative abundance of halophilic *Halomonas*.
- NH_4^+-N significantly decreased soil microbial diversity.

GRAPHICAL ABSTRACT



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ABSTRACT

Due to the enhancement of human activities on the global scale, the total amount of atmospheric nitrogen (N) deposition and the rate keep increasing, which seriously affect the structure and function of terrestrial ecosystems. In order to study the effects of N deposition on the soil structure and function of coastal saline wetlands, we established a long-term nitrogen deposition simulation platform in 2012 in the Yellow River delta (YRD). Herein, we analyzed the composition and diversity of the soil microbial community under different N deposition treatments (LNN, MNN and HNN, which stand for 50 kg N ha^{-1} yr^{-1}, 100 kg N ha^{-1} yr^{-1}, and 200 kg N ha^{-1} yr^{-1}) and in a water-only control (CK). The results showed that with the increasing level of N deposition, α-diversity (Shannon and Simpson indices) decreased significantly, and the composition of the microbial community changed. At the phylum level, compared with CK, the relative abundance of *Chloroflexi* increased significantly under the treatment of HNN ($P = 0.002$), but the relative abundance of *Chlorobi* ($P = 0.013$) and *Verrucomicrobia* ($P = 0.035$) decreased significantly. At the genus level, compared with CK, the relative abundance of *Bacillus* ($P = 0.01$) and *Halomonas* ($P = 0.042$) increased significantly with HNN treatment. *Bacillus* and *Nitrococcus* showed a significant correlation with soil NH_4^+-N. The results suggest that the response of microorganisms to N deposition treatments varied by the concentration, and the deposition of a high concentration would increase the nutrients in the soil, but reduce the diversity of soil microorganisms, causing a negative impact on the coastal wetland ecosystem of the YRD.

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1. Introduction

Nitrogen is a key factor affecting the structure, dynamics, ecological diversity and function of many ecosystems. It is also one of the most important nutrients in the ecosystem (Huang et al., 2020a, 2020b). The mobility of available nitrogen species means that their impact spreads regionally and globally (Vitousek et al., 1997). The average soil nitrogen in natural ecosystems in China increased by 8 kg N ha^{-1} yr^{-1} between the 1980s and 2000s, from 13.2 to 21.1 kg N ha^{-1} yr^{-1}, the third largest nitrogen deposition rate country next to the United States and Europe (Liu et al., 2013). According to the current trend, the global total annual nitrogen deposition will further increase to twice the current level by 2050 (Peñuelas et al., 2012). Long-term nitrogen input will lead to an excess of nitrogen in the soil and ultimately lead to increased nitrogen loss through solution leaching and trace gas emissions (Matson et al., 2002). This has been shown to affect the structure and function of ecosystems through eutrophication and acidification, leading in some cases to changes in species composition and ecosystem decline (Goulding et al., 1998; Janssens et al., 2010).

Previous studies have shown that nitrogen deposition affects the growth, reproduction and activity of soil microorganisms, changes the structure and function of soil microbial community, and affects biogeochemical cycling and nutrient availability in the soil (Liu et al., 2016). High concentrations of nitrogen can inhibit the growth of diazotrophic *Azotobacter* spp. (Zhang and Han, 2012) and significantly increase the abundance of ammonia-oxidizing bacteria (Ai et al., 2013). Guan et al. (2019) found that nitrogen deposition may improve the function of wetland ecosystems by increasing soil nutrient content, promoting soil enzyme activity and increasing plant yield based on a five-year nitrogen deposition experiment. However, studies of the effect of nitrogen deposition on microbial community diversity, especially in saline wetlands, are limited.

The Yellow River Delta is one of the most active deltas in the world in terms of land-ocean interactions and its geographic position makes it an important habitat for migrating birds. During the last several decades, the delta has experienced substantial changes attributed to human activities and climate change. The total nitrogen deposition rate of the YRD during the growing season (May to November) is 22.64 kg ha^{-1}, a relatively high level in China (Yu et al., 2014). Therefore, the YRD has become a unique area to study the effects of environmental change, such as nitrogen deposition, on the structure and function of wetland ecosystems. Previous research in the coastal wetland of the YRD has considered differences in soil microorganisms between differing vegetation communities (Lv et al., 2016; Zhang et al., 2017), the response of specific microbial functions, such as methane cycling to nitrogen deposition (Xiao et al., 2017), the microbial diversity of aging oil sludge (Kuang et al., 2018) and the responses of bacterial community to biological invasion (Zhang et al., 2020). However, the impact of nitrogen deposition on the regional soil microbial community diversity remains unknown. The study of the effects of nitrogen deposition on soil microorganisms in coastal wetland of the Yellow River Delta is necessary in order to comprehend the response of coastal wetland ecosystem function to human activities.

In May 2012, we built a long-term nitrogen deposition simulation experimental platform on a salinized reed wetland in the Yellow River Delta. After five consecutive years of nitrogen amendments, we applied high-throughput DNA sequencing techniques and ecophysiological analysis to determine the change in the microbial community diversity under different levels of nitrogen deposition. The objectives of the present study were (1) to characterize the molecular diversity and community composition of soil microorganisms among different levels of nitrogen deposition, and (2) to determine the relationships between specific microbial taxa and soil salinity and nutrients levels under different nitrogen deposition treatments.

2. Materials and methods

2.1. Sites and environmental conditions

The study site (37°45′50″N, 118°59′24″E) (Fig. 1) and experimental design of the research station, the Yellow River Delta Ecological Research Station of Coastal Wetlands, has been described previously (Guan et al., 2019). Briefly, the annual average temperature is 11.7–12.8 °C. The annual evaporation is 1900–2400 mm, and the annual precipitation 530–630 mm, of which 70% is rainfall that occurs between July and September. It is typified by distinct wet (in general, July to September) and dry (in general, October to June) seasons and remains inundated throughout the wet season.

2.2. Simulated nitrogen deposition

The experiment began in 2012 and established 16 plots, each plot (6 m × 4 m) separated by a one-meter buffer to prevent increased horizontal movement and lateral loss of N. Four levels of N fertilization were set, including: control 0 kg N ha^{-1} yr^{-1} (CK); LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}, and each treatment consisted of three repetitions used for microbial analysis. The selection of fertilization level was based on the current nitrogen deposition rate and the potential amount that may occur in the future (Yu et al., 2014). For each fertilization event, NH_4NO_3 was first dissolved in pure water, and then sprayed evenly on the target area. The same amount of pure water without nitrogen was sprayed in the control area. The treatments were applied once a month from May 2012 to May 2017.

2.3. Soil sampling and physicochemical properties analysis

Sample collection was conducted in May 2017. Subplots (0.5 m × 0.5 m) were randomly selected from each treatment site. Soil samples (0–15 cm depth) were drilled with soil sampler with a diameter of 3.5 cm. Each soil sample was cut vertically in the middle, put into a sterile self-sealing bag, and transported on ice. One half was stored in a refrigerator at −4 °C for microbial sequencing and the other half was naturally air-dried for soil physicochemical properties analysis. The soil properties and methods of analysis were previously reported in (Guan et al., 2019) and are analyzed here in conjunction with new microbial data. The soil properties included soil salinity, soil total nitrogen (TN), total carbon (TC) and total organic carbon (TOC), soil nitrate (NO_3^--N) and ammonium (NH_4^+-N), and soil available phosphorus extractable by sodium bicarbonate (AP).

2.4. Microbial diversity analysis

Total genomic DNA from sediment samples was extracted using CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng μL^{-1} using sterile water. The16S rRNA V4-V5 region was amplified using barcoded 515F/907R primers (Biddle et al., 2008; Jing et al., 2015). Briefly, these primers cover one or several high-variation regions that are sequenced and identified. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Paired-end sequencing of PCR amplicons was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). Briefly, the PCR reaction mixture of 37.5 μL contained the following components: 25 μL PCR Master Mix with GC Buffer (New England Biolabs, United States), 1.0 μL of 10 μM forward and reverse primers (each), 2.5 μL of total genomic DNA (5–10 ng) and 8.0 μL of H_2O. The amplification program consisted of 5 min at 95 °C for initial denaturation followed by 34 cycles of 94 °C for 1 min, 57 °C for 45 s, 72 °C for 1 min and a final 72 °C for 10 min for extension and 16 °C for 5 min for cooling. The PCR products were separated in 2% agarose gel and stained with

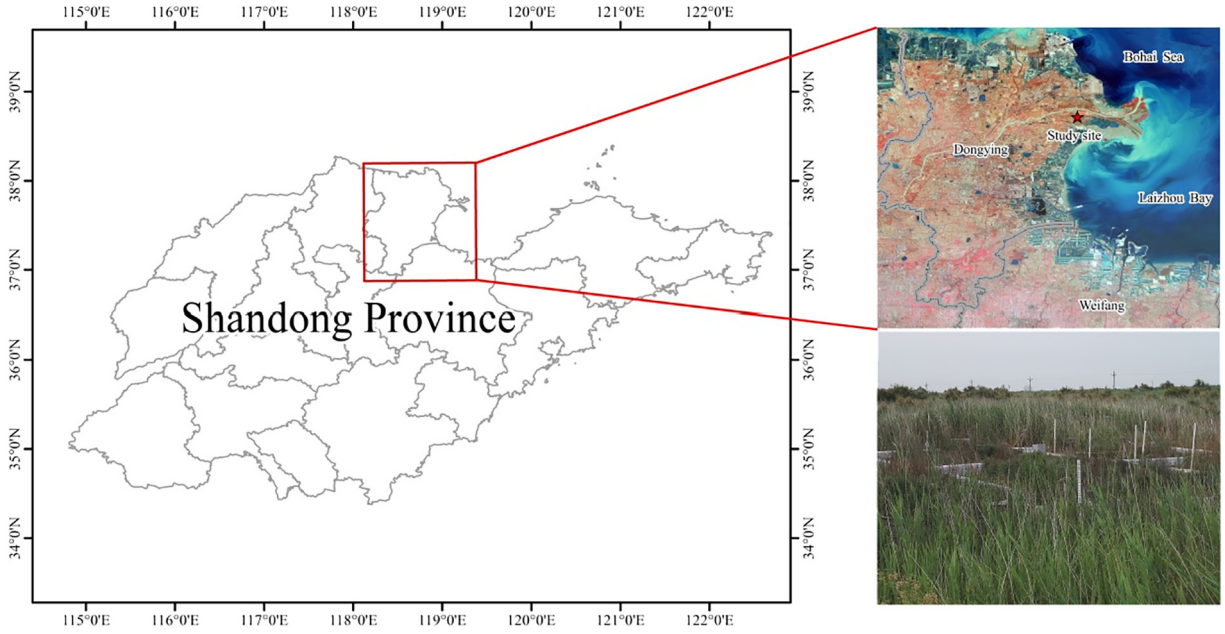

Fig. 1. Location of the Yellow River Delta and the experimental platform.

ethidium bromide. The PCR products were then purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The bar-coded PCR products from all samples were combined in equimolar amounts, then prepared for sequencing using the TruSeq™ DNA Sample Preparation Kit and sequenced using the HiSeq PE250 (Illumina) following the manufacturer's protocols.

The sequencing reads were demultiplexed, forward and reverse reads were combined, then barcode and primer sequences were removed from all the sample data and using FLASH (Magoč and Salzberg, 2011). The sequences were processed through strict quality filtering using the QIIME (version 1.7.0; Caporaso et al., 2010) script *split_libraries_fastq.py* with options to truncate from the first low-quality base site (where the quality threshold is ≤19 for the set length of 3) and filter out sequences with a continuous high-quality base length of <75%. The chimeric sequences were detected and removed using Vsearch (Rognes et al., 2016), giving the final effective sequences.

Then the Uparse (Edgar, 2013) software (version 7.0.1001, http://www.drive5.com/uparse/) was used to cluster the effective sequences into operational taxonomic units (OTUs) at 97% similarity and select the highest frequency sequence as the representative sequence. The taxonomic annotation of the OTUs was carried out with Mothur (Schloss et al., 2009) and the SSU rRNA reference sequences from the SILVA (Pruesse et al., 2007) database (version 132; http://www.arb-silva.de/) with the threshold value set at 0.8–1. The community composition of the samples was analyzed at multiple taxonomic levels: kingdom, phylum, class, order, family, genus, species. The number of sequences was normalized across all samples by rarefaction using the number corresponding to the sample with the least sequences (43896). All sequences from this study have been deposited at GenBank with accession number SRP278006.

2.5. Statistical analysis

The complexity of species diversity, or alpha diversity, was analyzed by the Shannon and Simpson metrics. The indices were calculated with QIIME (version 1.9.1; Caporaso et al., 2010) and displayed with R software by heatmap and boxplot (version 2.15.3; http://www.r-project.org).

Differences among treatments were tested using Fisher's LSD method and statistically significant correlations between taxa and environmental variables were identified using Pearson correlation. Redundancy analyses (RDA) were conducted on environment variables with microbial diversity and specific bacteria separately. All acquired data were represented by the average of three replicate measurements and standard error. Significance was tested at the 5% level. LSD and correlation analyses were done with SPSS 24.0. RDA analyses were conducted with CANOCO 4.5 software.

3. Results

3.1. Effects of N addition on microbial diversity and community composition

At the phylum level, the microbial community was dominated by *Proteobacteria* (36.2%) and *Chloroflexi* (21.6%), followed by *Bacteroidetes* (12.0%), *Acidobacteria* (8.6%), *Gemmatimonadetes* (6.7%), *Planctomycetes* (5.9%), *Actinobacteria* (4.6%), *Firmicutes* (2.2%), *Chlorobi* (1.1%) and *Verrucomicrobia* (1.1%) (Fig. 2). The relative abundance of *Chloroflexi* increased significantly under the HNN treatment ($P = 0.002$). The relative abundance of *Firmicutes* increased under the MNN treatment and decreased under HNN, and the difference between the MNN and HNN treatment was significant ($P = 0.021$). Compared with the control group, the relative abundance of *Chlorobi* significantly decreased under the treatment of LNN ($P = 0.008$) and HNN ($P = 0.013$). The relative abundance of *Verrucomicrobia* decreased significantly under the treatment of MNN ($P = 0.024$) and HNN ($P = 0.035$).

For the most abundant phylum *Proteobacteria*, we considered the relative abundance distributions at the subphyla level (Fig. 3). The primary subphyla were the *Deltaproteobacteria* (34.3%), *Gammaproteobacteria* (30.5%), *Alphaproteobacteria* (24.4%), and *Betaproteobacteria* (10.4%). The relative abundance of *Betaproteobacteria* and *Gammaproteobacteria* significantly increased ($P = 0.049$, HNN) and decreased ($P = 0.027$, HNN) under different nitrogen deposition gradients, respectively.

The relative abundance of the most abundant 35 groups at the genus level is illustrated in Fig. 4 with a clustered heat map. Compared with the control group, *Halomonas* ($P = 0.042$), *Urania-1B-19_marine_sediment_group* ($P = 0.036$), *Bacillus* ($P = 0.001$) and *Spirochaeta_2* ($P = 0.001$) showed significant differences under HNN treatment. *Bacillus* ($P = 0.041$), *Alterococcus* ($P = 0.016$) were significantly different between LNN and HNN treatment; *Deferrisoma* ($P =$

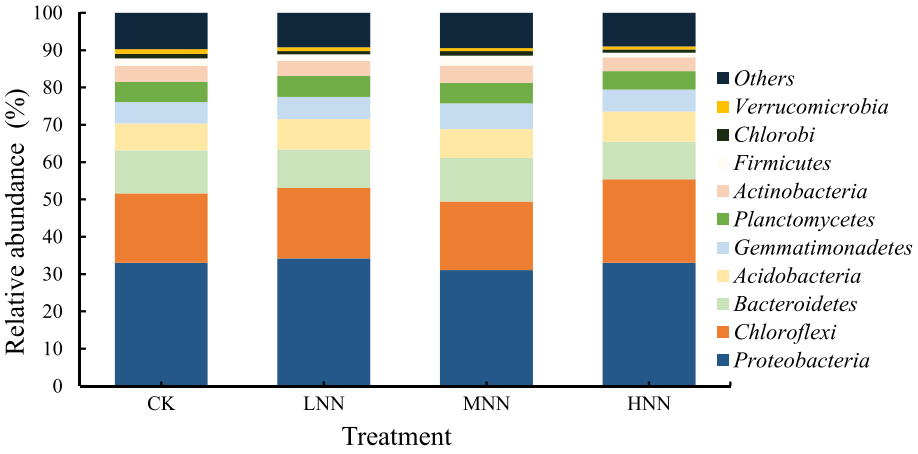

Fig. 2. The relative abundances of bacterial phyla under N deposition treatment. The four treatments respectively represent different amounts of N deposition: CK, 0 kg N ha^{-1} yr^{-1}; LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}.

0.034) and *Spirochaeta_2* ($P = 0.007$) showed significant differences between the control and the MNN treatment.

The Shannon and Simpson indices were used to estimate and compare the alpha diversity of bacterial communities among the N deposition treatments. The Shannon and Simpson indices significantly decreased with the HNN treatment (Shannon, $P = 0.026$; Simpson, $P = 0.000$) (Fig. 5; Supporting Materials Table 1).

3.2. The relationship between soil microorganisms and soil physicochemical properties under N addition

Correlation analysis was conducted between phyla (*Chloroflexi, Verrucomicrobia, Chlorobi, Firmicutes*), *Proteobacteria* subphyla (*Betaproteobacteria*, *Gammaproteobacteria*) and three genera (*Bacillus, Nitrococcus, Halomonas*) with significant changes in relative abundance and soil physicochemical properties. The relative abundance of *Bacillus* and *Halomonas* showed a decreasing trend with greater nitrogen deposition treatments. With HNN treatment *Bacillus* was significantly less abundant ($P = 0.01$) than in the three treatments and *Halomonas* was significantly less abundant ($P = 0.042$) than in the control group. The relative abundance of *Nitrococcus* significantly increased ($P = 0.04$) under the MNN treatment (Fig. 6).

Correlation analysis showed that the relative abundance of *Chloroflexi* was positively correlated with NH_4^+-N ($r = 0.584$, $P < 0.05$). The *Verrucomicrobia* was negatively correlated with AP ($r = -0.602$, $P < 0.05$), NH_4^+-N ($r = -0.645$, $P < 0.05$), NO_3^--N ($r = -0.591$, $P < 0.05$), TN ($r = -0.631$, $P < 0.05$) and TC ($r = -0.614$, $P < 0.05$) concentrations in soil. The relative abundance of *Gammaproteobacteria* was positively correlated with salt content ($r = 0.780$, $P < 0.01$). The relative abundance of *Bacillus* was positively correlated with NH_4^+-N ($r = 0.635$, $P < 0.05$) concentrations in soil. The relative abundance of *Nitrococcus* was positively correlated with NH_4^+-N ($r = -0.666$, $P < 0.01$), TN ($r = -0.745$, $P < 0.01$) and TC ($r = -0.796$, $P < 0.01$) concentrations in soil.

A RDA of soil physicochemical properties and the Shannon and Simpson indices of microbial diversity explained 95.2% of the variation in axes 1 (Fig. 7). Pearson correlation analysis showed that NH_4^+-N and NO_3^--N concentration significantly affected Shannon index ($P < 0.01$), and NH_4^+-N was also negatively correlated with Simpson index ($P < 0.05$; Supporting Materials Table 2).

4. Discussion

Microorganisms have many roles in the ecological processes of the YRD (Yu et al., 2012). They represent an important component of the soil ecosystem and can be a valuable mechanism for wetland restoration (Ma et al., 2017; Wang et al., 2010). Previous studies have found that microorganisms can work cooperatively with plants (Bentivenga and Hetrick, 1992), reduce the toxicity of heavy metals to plants (Bissonnette et al., 2010), affect plant competition and plant coexistence

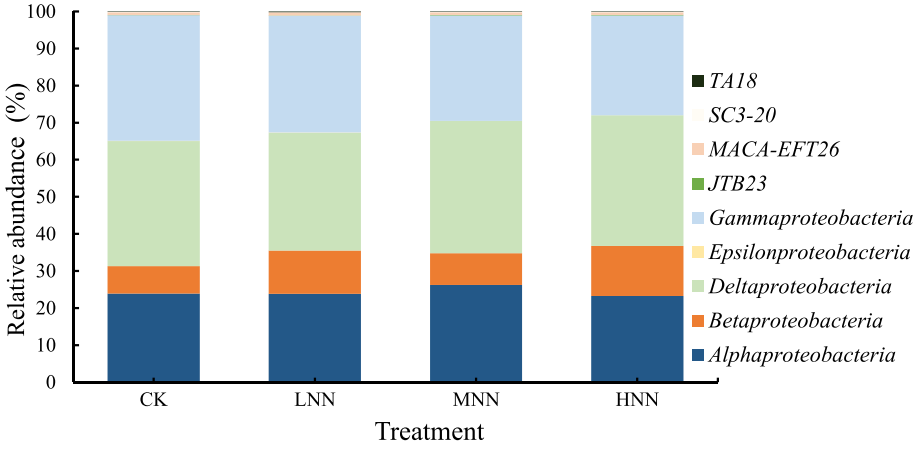

Fig. 3. The relative abundances of Proteobacterial subphyla under N deposition treatment. The four treatments respectively represent different amounts of N deposition: CK, 0 kg N ha^{-1} yr^{-1}; LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}.

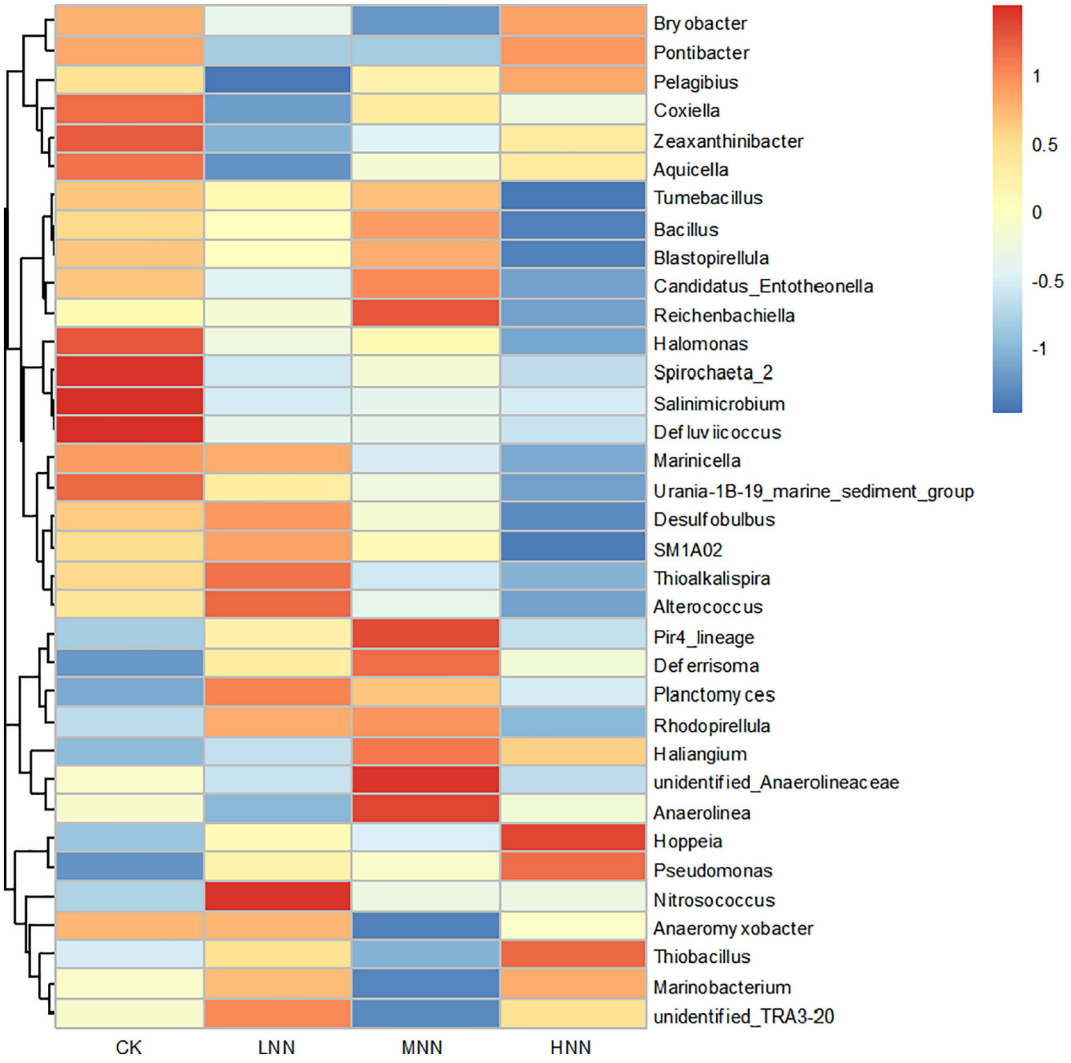

Fig. 4. The relative abundance of the first 35 most abundant genera was made into a clustering heat map. The four treatments respectively represent different amounts of N deposition: CK, 0 kg N ha^{-1} yr^{-1}; LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}.

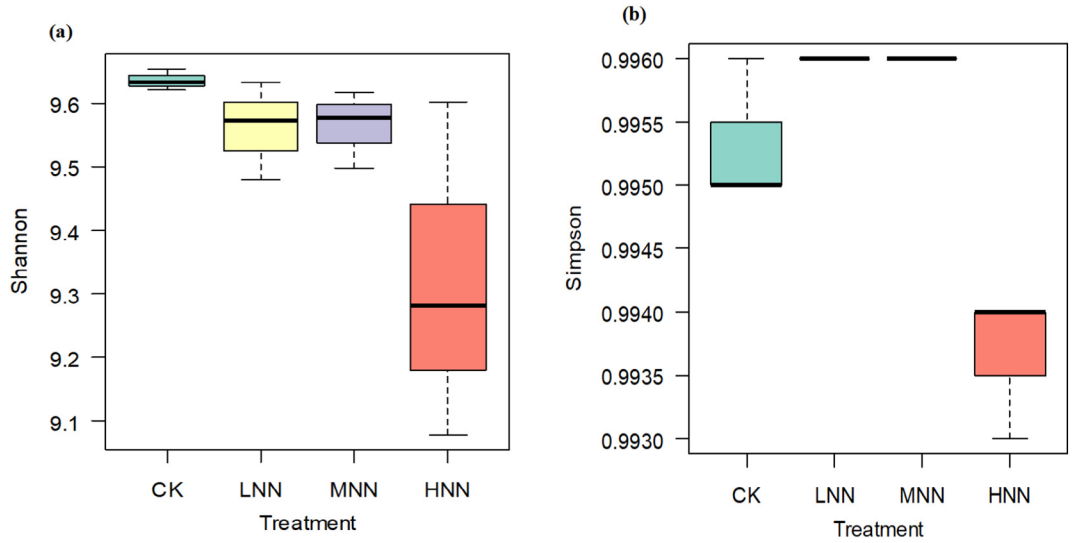

Fig. 5. (a) Shannon index analysis of soil bacteria. (b). Simpson index analysis of soil bacteria. The ends of the whiskers represent the minimum and maximum, the bottom and top of the box are the first and third quartiles, and the line inside the box is the median. The four treatments respectively represent different amounts of N deposition: CK, 0 kg N ha^{-1} yr^{-1}; LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}.

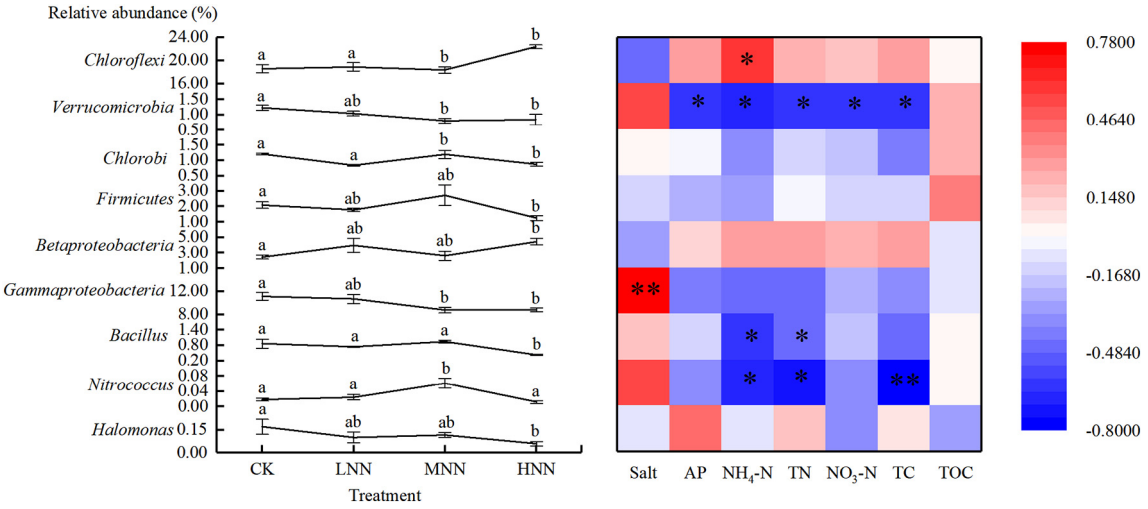

Fig. 6. Correlation analysis of microbial relative abundance and soil physio-chemical properties under N deposition treatments (mean ± s.e., $n = 3$). The four treatments respectively represent different amounts of N deposition: CK, 0 kg N ha^{-1} yr^{-1}; LNN, 50 kg N ha^{-1} yr^{-1}; MNN, 100 kg N ha^{-1} yr^{-1}; HNN, 200 kg N ha^{-1} yr^{-1}. Different letters in the line diagram on the left indicate significant differences between different treatments for each microbe (ANOVA, LSD test, $P<0.05$). Heat map on the right shows the Pearson correlation between the relative abundance of the microorganisms and environmental variables, *, $P < 0.05$; **, $P < 0.01$.

(Hetrick et al., 1989), and thus influence the structure and species diversity of plant communities.

Under different N deposition treatments, combined with soil physical and chemical indexes and microbial diversity analysis, the quantities of NO_3^--N, NH_4^+-N, TN and TC were found the most important factors affecting soil bacterial diversity. High levels of N in the HNN treatment were associated with a significant decrease in the microbial diversity compared with the other treatments. This decrease in diversity may be due to soil acidification resulting from the long-term N deposition in the supersaturated state (Chung et al., 2010). On the other hand, long-term N deposition may also decrease available organic matter of soil microorganisms, changing the use of microbial substrate model (Wallander, 1995), reducing the microbial utilization of carbon sources (Bowden et al., 2004), reducing the microbial activity and diversity (Liu et al., 2016). A decrease in microbial diversity with high N deposition was also found in previous studies. Based on a meta-analysis of data from 198 sampling points, the researchers found that nitrogen deposition reduced the soil microbial diversity of all ecosystem types to varying degrees (Wang et al., 2018). As N deposition rates increase, the alteration of ecosystem function resulting from the loss of rare species and microbial diversity might have profound feedback on global climate change (Cui et al., 2017; Liu et al., 2016). Other researchers have shown that long-term N deposition can reduce soil respiration rate, enzyme activity, and change the biomass ratio of fungi and bacteria (Frey et al., 2004; DeForest et al., 2004). Under a high N environment, bacteria may be at a disadvantage in competition with fungi. Liu et al. (2016) found that a high level of N deposition would affect the reproduction and activity of soil microorganisms, inhibit their growth, change the structure and function of soil microbial community, and affect decomposition and nutrient availability in soil.

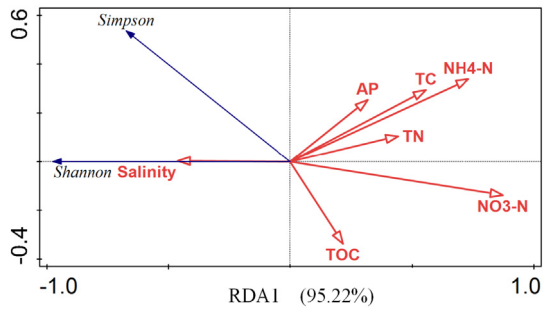

Fig. 7. Redundancy analysis (RDA) of soil physicochemical properties and the Shannon and Simpson indices of microbial diversity under N deposition treatments.

The three bacterial genera identified in this study with significant correlations to N have different life history strategies. The relative abundance of *Halomonas*, a halophile, was negatively correlated to N. The reason for the decrease in relative abundance may be that the addition of N can indirectly reduce the salt content of the soil surface (Guan et al., 2019). Both *Bacillus* and *Nitrococcus* participate in the denitrification process. *Nitrococcus* is an aerobic bacterium, while Bacillus can survive under aerobic and anaerobic conditions. Their relative abundance increases or decreases with the availability of nitrogen. It may be that N deposition directly or indirectly leads to the transformation of life cycle strategies of major microorganisms, so their response to nitrogen deposition is inconsistent (Fierer et al., 2012).

Previous study demonstrated that the change of soil microbial community composition was closely related to soil nutrients (Zhang et al., 2019). In our study, the dominant bacteria groups were *Proteobacteria,* and *Bacteroides*, at the phylum level, even at different nitrogen levels. This indicated that some phyla of soil microorganisms had certain adaptability (Zhao et al., 2020; Huang et al., 2020a, 2020b). However, the abundance of *Firmicutes*, *Chlorobacteria* and *Verrucomicrobia* were significantly decreased by nitrogen addition. Three phyla are known to be important participants in the carbon and nitrogen cycles (Buchanan and Arnon, 1990; Wahlund and Madigan, 1993; Wahlund and Tabita, 1997), and high concentration of nitrogen deposition inhibits their activity. Nitrogen deposition increases the content of NO_3^--N and number of free hydrogen ions in soil (Chen et al., 2013), thus increasing the relative abundance of *Chloroflexi*, a heterotrophic phylum growing under acidic conditions (Hug et al., 2013). In addition, Pearson correlation analysis showed that TN, TC and NO_3^--N were negatively correlated with *Firmicutes*, *Chlorobi* and *Verrucomicrobia*, and positively correlated with *Acidobacteria*. The former may be at a disadvantage in the competition under high nutrient conditions (Fierer et al., 2012). The decrease in the relative abundance of *Gammaproteobacteria* with nitrogen addition may be due to the presence of halophilic bacteria under its subphylum, such as *Thialkalivibrio Halophilus*, *Pseudoalteromonas*, etc. (Banciu et al., 2004; Novikova et al., 2013). Correlation analysis also showed that the relative abundance of *Gammaproteobacteria* was significantly correlated with salinity content, consistent with the results of a previous study (Korlević et al., 2016). However, nitrogen deposition increased the relative abundance of *Betaproteobacteria*, perhaps because nitrogen addition affected the nitrification rate of some bacteria under

its subphyla (Ma et al., 2018), and it showed a positive correlation with salinity content.

5. Conclusions

In general, nitrogen deposition significantly reduced microbial diversity in the coastal wetland of the YRD. Statistical analysis showed that TN, TC and NO_3^--N had a large influence on the microbial community, while NH_4^+-N had a great impact on some specific microbial groups such as *Nitrococcus* and *Bacillus*. Our research provides a basis for understanding the soil microbial responses to nitrogen deposition and the relationship between the soil microbial community and soil physiochemical characteristics in a salinized wetland of the YRD. These results provide theoretical support for understanding the impact of environmental changes on microbial communities in the coastal wetland ecosystem.

Declaration of competing interest

The authors have declared that no competing interests exist.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.143825.

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