



Nitrogen deposition enhances the deterministic process of the prokaryotic community and increases the complexity of the microbial co-network in coastal wetlands



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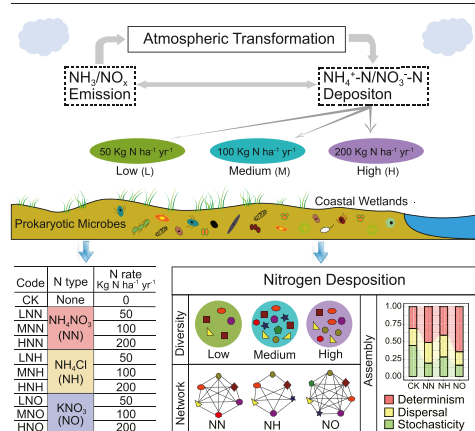
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HIGHLIGHTS

- The α diversity of the microbial communities significantly increased under nitrogen deposition ($P < 0.05$).
- Nitrogen deposition strengthened the deterministic process of community assembly and increased the complexity of the microbial co-network ($P < 0.05$).
- Long-term nitrogen deposition may affect microbial communities functions associated with soil C/N element cycling ($P < 0.05$).

GRAPHICAL ABSTRACT



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ABSTRACT

Global nitrogen deposition has increased significantly in recent years. At present, research on the effects of different amounts and types of nitrogen deposition on soil microorganisms in coastal wetlands is scarce. In this study, based on 7 years of simulated nitrogen deposition at multiple levels (low, medium, high) and of multiple types (NH_4NO_3 , NH_4Cl , KNO_3), the effects of different nitrogen deposition conditions on the diversity, community assembly processes, co-networks, and community function of soil prokaryotes in coastal wetlands were examined. The results showed that, compared with that in control, the microbial α diversity increased significantly under nitrogen deposition ($P < 0.05$). However, it decreased significantly in the high- NH_4NO_3 and high- NH_4Cl treatments ($P < 0.05$). The deterministic

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process of community assembly was strengthened under the different types of nitrogen deposition. Compared with that under NH_4^+ -N deposition, the microbial co-network under NO_3^- -N deposition was more complex. Network stability significantly decreased under different NH_4^+ -N deposition levels. In addition, the results of FAPROTAX functional prediction showed that microbial community functional groups associated with carbon and nitrogen cycling changed significantly ($P < 0.05$). In conclusion, our results emphasize that nitrogen deposition environments cause changes in soil microbial community structure and interactions, and may also affect soil carbon and nitrogen cycling, but the effects of different forms and levels of nitrogen deposition are not consistent. This study provides new insights for evaluating the changes in soil microbial communities in coastal wetlands caused by different types of long-term nitrogen deposition, and has scientific significance for assessing the ecological effects of long-term nitrogen deposition.

1. Introduction

Nitrogen in the atmosphere exists mainly in the form of nitrogen (N_2), which cannot be used directly by organisms and can only be converted into reactive nitrogen (Nr) to participate in the circulation between the atmosphere and the surface (Galloway et al., 2004). With the consumption of fossil fuels, the expansion of agricultural land, and human activities such as motor vehicles and industrial sources, the amount of Nr in the atmosphere has increased rapidly (Galloway et al., 2008; Zeng et al., 2018). Currently, the amount of Nr produced by human activities has exceeded the nitrogen fixation in nature (Vitousek et al., 2013). Under the business-as-usual scenario, anthropogenic Nr emissions will more than double in 2050 compared with 2010 (Gu et al., 2015). Two dominant types of Nr occur frequently, including the reduced state (mainly in the form of NH_4^+ -N) and the oxidized state (mainly in the form of NO_3^- -N) (Gurmesa et al., 2022; Zhang et al., 2017b). Atmospheric nitrogen deposition is considered to be the result of Nr emissions and atmospheric transport (Galloway et al., 2008). From 1980 to 2020, atmospheric nitrogen deposition in China increased (Liu et al., 2013). The nitrogen source provided by atmospheric deposition is an essential nutrients source in ecosystems and may stimulate primary production in the ecosystem if it does not exceed the critical load of the ecosystem (Huang et al., 2021; Liu et al., 2011; Liu et al., 2010; Xu et al., 2015). However, as nitrogen deposition continues to increase, serious ecological problems can arise, e.g., the eutrophication of water bodies (Zhang et al., 2017b), changes in soil physical and chemical characteristics (Nie et al., 2018; Shi et al., 2018), changes in the greenhouse gas balance (Matson et al., 2002), and reductions in biological diversity (Clark and Tilman, 2008). Moreover, the deposition of Nr to the land surface can in turn affect biogeochemical cycles (Yang et al., 2021).

Microorganisms play crucial roles in the global biogeochemical cycle (cycling of carbon, nitrogen, phosphorus, sulfur and other biogenic elements), ecological functions, and health of terrestrial organisms (Wei et al., 2016; Wei et al., 2020). Excessive nitrogen input can have multiple effects on the growth, composition and function of soil microorganisms (Zhang et al., 2018). For example, long-term nitrogen additions can inhibit microbial respiration rates and reduce microbial biomass and diversity (Zhang et al., 2017a). Then, nitrogen addition may directly or indirectly induce shifts in the life history strategies of key microorganisms in favor of more active symbiotic microbial communities, which could alter the relative abundance of microbial species (Fierer et al., 2012). In addition, nitrogen addition also reduced the metabolic capacity of the soil bacterial community (Kearns et al., 2016). Coastal wetlands are located in the land-sea interaction zone, which is a part of the coastal zone and provides important ecosystem services for marine and terrestrial organisms (Chmura et al., 2003). At present, the effects of different amounts and types of nitrogen deposition on soil microorganisms corresponding to research in coastal wetlands are seldom studied. Analyzing the response of soil microbes in coastal wetlands to different types of nitrogen deposition is essential for predicting the effects of anthropogenic nitrogen additions on wetland ecosystems.

Revealing the basic mechanisms of microbial community assembly and species coexistence is the core of ecology and is crucial for determining the link between microbial community stability and ecosystem function. Community assembly is usually explained by deterministic and stochastic

processes (Nemergut et al., 2013; Zhou and Ning, 2017). Deterministic processes are related to ecological selection, and the existence and relative abundance of species are determined by deterministic factors, including biological factors (e.g., intraspecific competition and predation) and abiotic factors (e.g., environmental conditions pH, and temperature) (Vellend, 2010; Zhang et al., 2019). Stochastic processes mainly refer to unpredictable perturbations, probabilistic dispersal, and random birth-death events that determine species change (Chase and Myers, 2011; Dini-Andreote et al., 2015). Revealing the mechanisms that regulate the balance between stochastic and deterministic processes under environmental microperturbations could contribute to understanding of community assembly processes (Jiao et al., 2022). For example, at high salinity levels, the assembly process of the microeukaryotic plankton community is mainly driven by deterministic processes (Mo et al., 2021). Warming may shape the assembly process of soil bacterial communities with enhanced homogeneous selection, which had the highest correlations with drought and precipitation (Ning et al., 2020). It is still unclear how changes in the nitrogen deposition environment mediate the community assembly process of prokaryotic microbes in coastal wetlands.

In general, molecular ecological networks (MENs) are used to reveal the patterns of microbial community coexistence and interactions among species (including cooperation, predation, and competition) (Deng et al., 2012; Feng et al., 2017). However, environmental changes can affect species interactions and the stability of the co-network. Zhou et al. (2021) by using the inter-domain ecological network (IDEN) approach, found that the fungal-prokaryotic bipartite MEN was simplified under warming. Yuan et al. (2021) revealed that warming significantly enhanced the MEN complexity of grassland soil microbial communities and improved the stability of ecosystem functions. Despite the emergence of a large number of new and insightful network analysis methods in ecology, there are still significant knowledge gaps in how the coexistence mechanisms of microbial communities respond to microperturbations in the nitrogen deposition environment. Microbial communities are characterized by high complexity and a large number of species, and it is important to identify keystone taxa that may have the greatest influence on the structure and potential function of microbial communities (Yuan et al., 2021). Previous studies have identified keystone taxa of soil microbial communities in different ecotypes by network analysis methods (Feng et al., 2017). For example, in grassland ecosystems, most keystone taxa belong to *Actinobacteria* and *Proteobacteria* (Banerjee et al., 2018; Zhou et al., 2011). Additionally, *Rhizobiales* and *Burkholderiales* have been identified as keystone species in agricultural ecosystems (Banerjee et al., 2018; Liang et al., 2016; Wang et al., 2017). However, in fragile coastal wetland ecosystems, whether and how keystone taxa change under long-term nitrogen deposition also requires further identification.

The wetland of the Yellow River Delta is the youngest estuarine wetland in the world, and is also a large area of coastal wetlands in a relatively original state (Guan et al., 2019). Atmospheric nitrogen deposition is one of the main nitrogen sources of coastal wetlands in this region (Lu et al., 2021). It is important to systematically study the effects of different amounts and types of nitrogen deposition on microbial community diversity, composition, ecological functions, community assembly processes, and interaction relationships in coastal wetlands, but corresponding research is rare. In this study, the Yellow River Delta Ecological Experiment Station was

selected, and we investigated the responses of prokaryotic microbial communities to different amounts and types of long-term nitrogen deposition. Our main questions were as follows: (1) How do the diversity, structural composition, and assembly processes of soil prokaryotic microbial communities change in coastal wetlands under different amounts and types of long-term nitrogen deposition? (2) How do the complexity and stability of the microbial interaction network change under different nitrogen deposition conditions? (3) How does the nitrogen deposition environment affect microbial community functions? This study also considered the effects of different amounts and types of nitrogen deposition on microbial communities, and the results can provide a scientific and theoretical basis for the implementation of ecological restoration and management policies in wetland of the Yellow River Estuary.

2. Methods and materials

2.1. Experimental site and sample collection

The nitrogen deposition experimental site is located at the Yellow River Delta Coastal Wetland Ecological Experimental Station in Kenli, Dongying,

Shandong, China (37°45'36" N, 118°58'39" E) (Fig. 1). The study area has a typical continental monsoon climate, with an average annual temperature of 11.7–12.8 °C and an annual precipitation amount of 530–630 mm (Guan et al., 2019; Zhou et al., 2021). And it is covered mainly by solonchak and solonetz. The natural vegetation in this region is relatively simple, and the most dominant plant is *Phragmites australis* (Yu et al., 2014). The experimental platform (built in 2012) consists of 50 randomly located 6 × 4 m plots. We selected three nitrogen sources, NH₄NO₃(NN), NH₄Cl(NH), and KNO₃(NO) to simulate mixed nitrogen with ammonium nitrate, ammonium nitrogen dominated (NH₄⁺-N), and nitrate nitrogen dominated (NO₃⁻-N). We set three nitrogen deposition levels for each nitrogen source: low nitrogen (50 kg N ha⁻¹ yr⁻¹) (LNN, LNH, and LNO), medium nitrogen (100 kg N ha⁻¹ yr⁻¹) (MNN, MNH, and MNO), high nitrogen (200 kg N ha⁻¹ yr⁻¹) (HNN, HNH, and HNO). The level of nitrogen addition was selected based on the current nitrogen deposition rate and the amount of nitrogen deposition that is likely to occur in the future (Yu et al., 2014). In addition, a control group (CK) was established without nitrogen fertilizer. Overall, there were 10 treatments in the experiment with 5 replicates for each treatment. To avoid interactions between different nitrogen addition treatments, a randomized block design was used, that is, 5 replicates of each treatment were

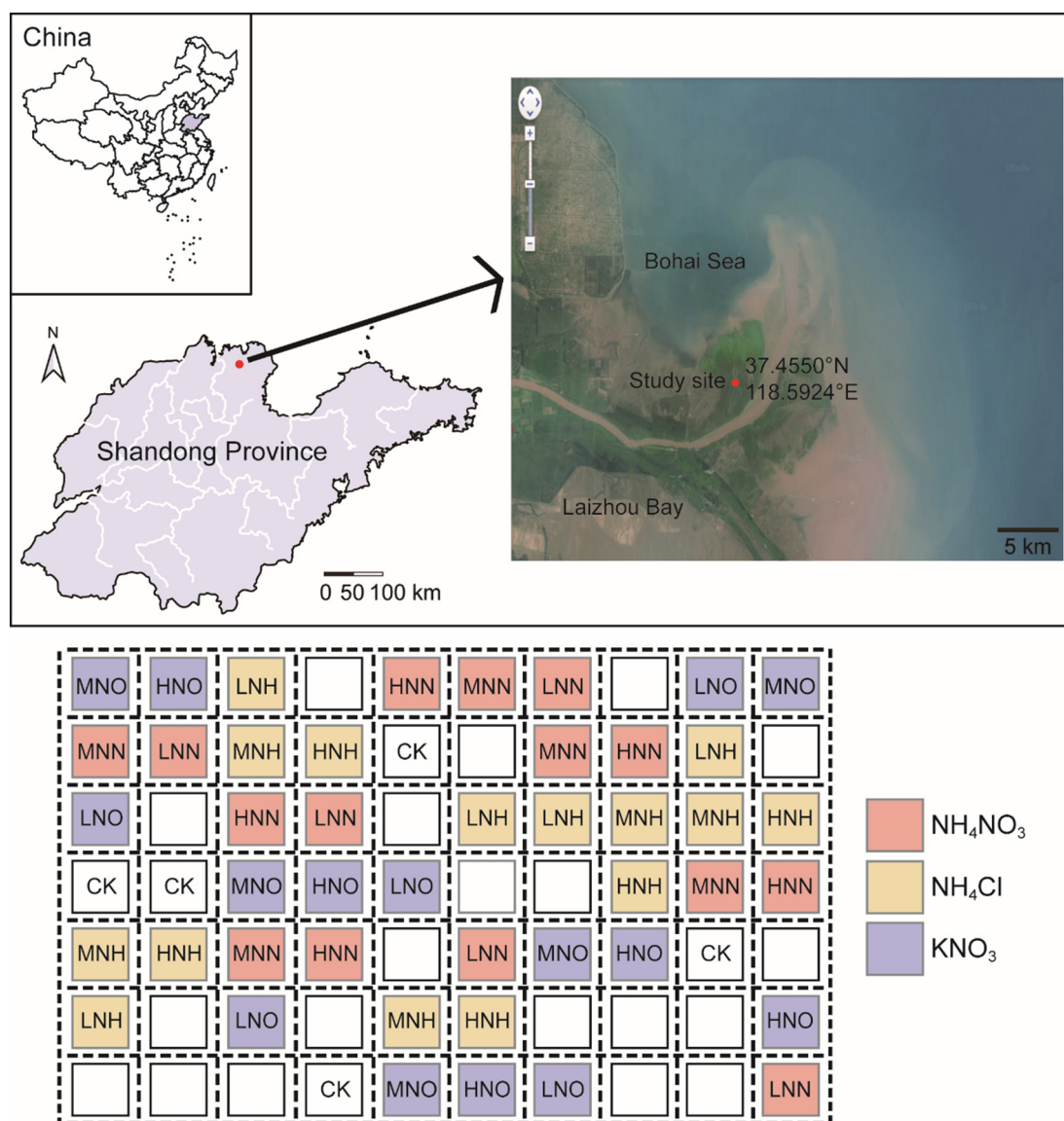


Fig. 1. Map of the Yellow River Delta site and the experimental platform. NN represents NH₄NO₃ treatment, NH represents NH₄Cl treatment, and NO represents KNO₃ treatment. L represents low nitrogen amount: 50 kg N ha⁻¹ yr⁻¹, M represents medium nitrogen amount: 100 kg N ha⁻¹ yr⁻¹, and H represents high nitrogen amount: 200 kg N ha⁻¹ yr⁻¹.

randomly distributed in 50 plots. More details about the fertilization are shown in the supplementary files.

Topsoil (0–20 cm deep) were collected from each plot using a clean soil auger with a diameter of 3.5 cm in September 2019. Two soil samples were collected from each plot, and each soil sample consisted of a mixture of 5 subsamples (at the four corners of each plot and at a central point). Ten samples were taken from each treatment, and a total of 100 samples were collected. Then, the soil samples were immediately delivered to the laboratory in an ice box. Each sample was divided into two parts, and one part was used for DNA extraction and stored at -80°C . The other part was used for the determination of soil physical-chemical factors and stored at 4°C .

2.2. Physical and chemical determination of soil samples

The wet soil was naturally wind dried before soil biochemical property analysis. The pH of the soil samples was determined using a pH electrode (Leici, Shanghai, China) with a soil: water ratio of 1:2.5 (Li et al., 2018). The moisture content was measured by oven-drying fresh soil at 105°C to a constant weight (Wei et al., 2020). The ammonium and nitrate contents of the soil samples were extracted using 2 M KCl solution at a ratio of 1:5. Nessler's Reagent Colorimetric Method was used for ammonia nitrogen ($\text{NH}_4^+\text{-N}$), and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) was measured by a spectrophotometric method (Liu et al., 2021). The soil organic matter (SOM) was measured using the potassium dichromate oxidation iron salt titration method (Zhou et al., 2021). The soil salt content (SAL) was extracted using water solution at a ratio of 1:5, and the extracted filtrate was measured using oven drying at 105°C to a constant weight (Guan et al., 2019).

2.3. DNA extraction and PCR amplification

Total DNA from the soil samples was extracted using the Qiagen DNeasy Power Soil Kit (Qiagen DNeasy Power Soil Kit). The DNA content and quality was determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). To obtain the V4 region of 16S rRNA, the corresponding fragment was amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTW-TCTAAT-3') (Yao et al., 2014). The target fragment was detected by 1 % agarose gel electrophoresis. The 25 μL polymerase chain reaction (PCR) system included 2.5 μL $10 \times$ PCR buffer (Mg^{2+} plus), 2 μL dNTP mixture (10 mM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.15 μL Taq DNA polymerase (5 U/ μL), 1 μL template DNA (20–30 ng/ μL) and ddH₂O (filling the system to 25 μL). The amplification conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 40 s, 53°C for 40 s, and 72°C for 45 s, and a final extension at 72°C for 10 min.

2.4. Illumina MiSeq sequencing of 16S rRNA gene amplicons and analysis

The purified amplicons were sequenced using the NovaSeq 6000 platform (Illumina) at Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China). A total of 17,567,635 16S rRNA raw reads were obtained from the sequenced samples. The raw data were processed using the Galaxy platform (<http://mem.rcees.ac.cn:8080>) (Feng et al., 2017), which is a publicly available pipeline that integrates various bioinformatics service tools. More details about the raw data processing are shown in the supplementary files.

2.5. Ecological and statistical analysis

In this study, the Shannon and Chao1 indexes were used to measure the α diversity of prokaryotic microbial communities under different treatments, and the indexes were calculated using the “vegan” package in R. The significant differences in α diversity were examined using one-way analysis of variance (ANOVA) with Duncan's HSD test in SPSS Statistics software. Boxplots were plotted using the “ggplot2” package in R (Wei et al., 2020). The beta diversity was calculated using the “betapart” package in R (Legendre, 2014), which visualizes differences in community composition across samples in each treatment. The pairwise weighted UniFrac

distance was calculated, which was conducted in QIIME using beta diversity through plots.py (Zhou et al., 2021). The differences in community structure under different treatments were visualized using principal coordinates analysis (PCoA) (Wei et al., 2020). Permutational multivariate analysis of variances (PERMANOVA) analyses with the “vegan” package in R was used to analyze the significant differences in community structure. At the phylum level, species abundance was counted. The abundant taxa were defined as operational taxonomic units (OTUs) with a relative abundance $\geq 1\%$ in all samples (Xue et al., 2018). Grouped stacked columns were drawn using the “ggplot2” package in R.

To quantify the impact of nitrogen deposition on the assembly process of prokaryotic microbial communities, we calculated the beta nearest taxon index (βNTI) and the Raup-Crick index (RC_{Bray}) using the null model analysis developed by Stegen et al. (2013). Null model analysis was carried out using the Galaxy platform (<http://mem.rcees.ac.cn:8080>) (Zhang et al., 2019), including the calculation of betaMNTD, betaNTI, Bray-Curtis, and RC_{Bray} values. $|\beta\text{NTI}| > 2$ indicates deterministic processes that include homogeneous selection ($\beta\text{NTI} < -2$) and heterogeneous selection ($\beta\text{NTI} > 2$). $|\beta\text{NTI}| < 2$ and $|\text{RC}_{\text{Bray}}| < 0.95$ indicate stochastic processes, mainly referring to drift (Stegen et al., 2013; Zhou and Ning, 2017). $|\beta\text{NTI}| < 2$ and $|\text{RC}| > 0.95$ represent diffusion effects that include homogeneous diffusion ($|\beta\text{NTI}| < 2$ and $\text{RC} < -0.95$) and heterogeneous diffusion ($|\beta\text{NTI}| < 2$ and $\text{RC} > 0.95$). The diffusion effect can depend on deterministic and stochastic factors, so it cannot be clearly distinguished as deterministic or stochastic (Ning et al., 2020).

To address the interaction of microbial communities under nitrogen deposition and control, we then constructed MENs using a publicly available pipeline (<http://ieg4.rccc.ou.edu/mena>) (Deng et al., 2012; Feng et al., 2022), which is a molecular ecological network analysis pipeline. First, the networks under different types and different amounts of nitrogen deposition were constructed on the basis of random matrix theory (RMT) with Spearman correlations of non-log-transformed OTU abundances. Then, only OTUs that appeared in $>80\%$ of the samples were retained. A series of thresholds from 0.01 to 0.95 with 0.01 intervals were obtained and applied to the matrix. To compare the properties of the networks, uniform threshold values of 0.81 and 0.94 were selected to construct networks of different types and different amounts of nitrogen deposition, respectively. Some network properties were calculated in the publicly available pipeline, including total nodes, total links, average connectivity, average clustering coefficient, average path distance, geodesic efficiency, density and proportion (positive/total). The Student's *t*-test was performed on the network under different treatments by the mean and standard deviation of topological properties from the random network (Deng et al., 2012). We used keystone nodes to refer to OTUs that were topologically important in the network. For each node, the intra-module connectivity (Z_i) and inter-module connectivity (P_i) were calculated, and this information was used to classify keystone roles in the network (Yuan et al., 2021). The network stability was directly measured by a robustness index. We used random node removal to measure the robustness index, which was calculated by R code provided by Yuan et al. Finally, networks were visualized in Gephi.

FAPROTAX is mainly used for predicting functional profiles of biogeochemical cycling processes (especially the cycling of carbon, hydrogen, nitrogen, phosphorus, sulfur and other elements) in environmental samples (such as oceans and lakes) (Louca et al., 2016). To evaluate the potential ecological function changes of soil prokaryotic microbial communities under nitrogen deposition, we used FAPROTAX to engage in functional predictions on the Galaxy platform (<http://mem.rcees.ac.cn:8080>). More details about the raw data processing are shown in the supplementary files.

3. Results

3.1. Nitrogen deposition significantly affected the diversity of prokaryotic communities

Our results showed that compared with that in the CK, the prokaryotic α diversity indexes of Chao1 and Shannon significantly increased under all

nitrogen deposition treatments (ANOVA, $P < 0.05$), and the strength of the effect was as follows: $\text{NO} > \text{NN} > \text{NH}$. With the increase of nitrogen deposition, the α diversity increased nonlinearly in the NN and NH treatments, it showed a trend of rapidly increasing and then decreasing, and reaching the maximum value at medium nitrogen levels ($P < 0.05$). However, in the NO treatment, the α diversity linearly increased with increasing nitrogen levels ($P < 0.05$) (Fig. 2). The species turnover (beta diversity) of the samples obviously decreased under nitrogen deposition conditions ($P < 0.05$) (Fig. 3C). PCoA indicated that in the NN treatment, the community structure was remarkably different between different amounts of nitrogen deposition ($P < 0.05$). The first two main coordinates of PCoA (PC1 = 44.65 % and PC2 = 24.29 %) together explained 68.94 % of the variation in the community structure. However, under the NH and NO treatments, there were no significant differences with different amounts of deposition (Fig. 3, Table S2). Under different types of nitrogen deposition, the weighted UniFrac distance between samples was the smallest at high nitrogen levels, suggesting that the clustering of species was the most obvious ($P < 0.05$) (Fig. 3B).

3.2. Nitrogen deposition significantly affected the microbial community composition

A total of 69 taxa with definite classification were annotated for the soil prokaryotic microbial communities under different treatments. Among them, 10 archaea were divided, including *Thaumarchaeota*, *Nanoarchaeota*, *Euryarchaeota*, and *Crenarchaeota*. Fifty-nine bacteria were divided, including *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, etc. At the phylum level, seven abundant species (relative abundance $> 1\%$) were found: *Proteobacteria* (28.99 %), *Chloroflexi* (27.50 %), *Actinobacteria* (8.89 %), *Acidobacteria* (7.19 %), *Gemmatimonadetes* (5.84 %), *Planctomycetes* (5.78 %), and *Bacteroidetes* (4.41 %). Under different types of nitrogen deposition, compared with CK, the relative abundance of *Proteobacteria* obviously increased in the NO treatment and decreased in the NH treatment ($P < 0.05$). The relative abundance of *Actinobacteria* significantly improved in the NN treatment ($P < 0.05$). The relative abundance of *Planctomycetes* obviously increased under the NH and NO treatments ($P < 0.05$) (Fig. 4). Under different amounts of nitrogen deposition, the maximum relative

abundances of *Proteobacteria*, *Actinobacteria* and *Planctomycetes* were observed in MNO (34.67 %), MNN (10.73 %), and MNH (7.46 %), respectively (Fig. S1).

3.3. Nitrogen deposition increased the determinism of community assembly, with different drivers under different treatments

The assembly of microbial communities determines the existence and richness of species (Ning et al., 2020), and it is important to clarify the contributions of deterministic and stochastic processes in driving community assembly. This study showed that under nitrogen deposition, the relative contribution of deterministic processes increased and that of stochastic processes diminished. Under different types of nitrogen deposition, the percentages of deterministic processes were 31.11 % for CK, 51.11 % for NN, 40.74 % for NH, and 64.44 % for NO. The percentages of stochastic processes were 44.44 % for CK, 19.26 % for NN, 28.15 % for NH, and 16.30 % for NO. Compared with CK (24.44 %), the diffusion effect increased in NN (29.63 %) and NH (31.11 %) and decreased in NO (19.26 %) (Fig. 5). Under different amounts of nitrogen deposition, deterministic processes accounted for the largest proportion at high nitrogen levels (HNN, HNH, HNO), mainly showing homogeneous selection (Fig. S2). Furthermore, to evaluate the key environmental factors in shaping community construction, Mantel tests were used to examine the correlation between microbial communities and environmental factors under different treatments. We found that among the physicochemical properties measured in this study (Table S1), SAL was the key factor driving community change in CK ($P < 0.05$). NO_3^- -N and SOM were significantly associated with community changes in NH ($P < 0.05$). In addition, SOM affected the microbial community in NO ($P < 0.05$). However, no significant effect of physicochemical factors on the microbial community was found in NN (Table 1).

3.4. The network was the most complex in the NO treatment

Co-network analysis can reveal potential interactions between microbial taxa (Deng et al., 2012). To analyze the changes in interactions between species under different amounts and types of nitrogen deposition, a series of microbial interaction networks were constructed based on 16S

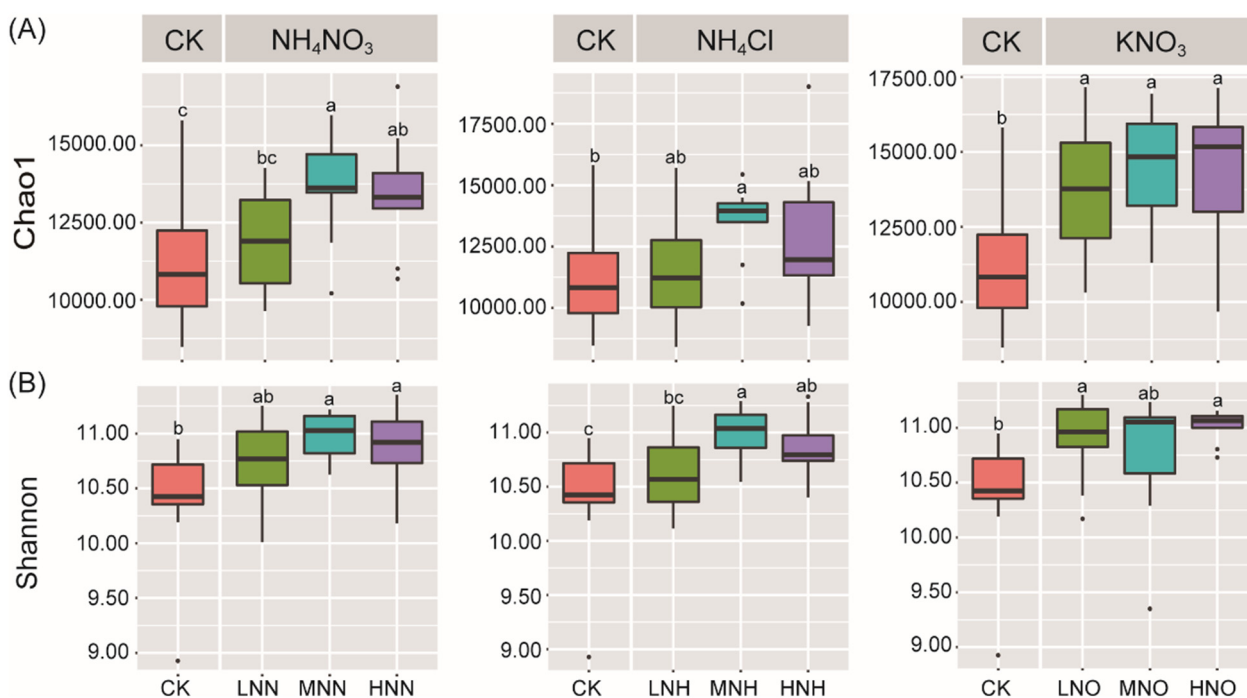


Fig. 2. Comparison of Shannon (A) and Chao1 (B) indices of prokaryotic microorganisms under control and nitrogen deposition conditions. Different labeled letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's multiple range tests ($P < 0.05$).

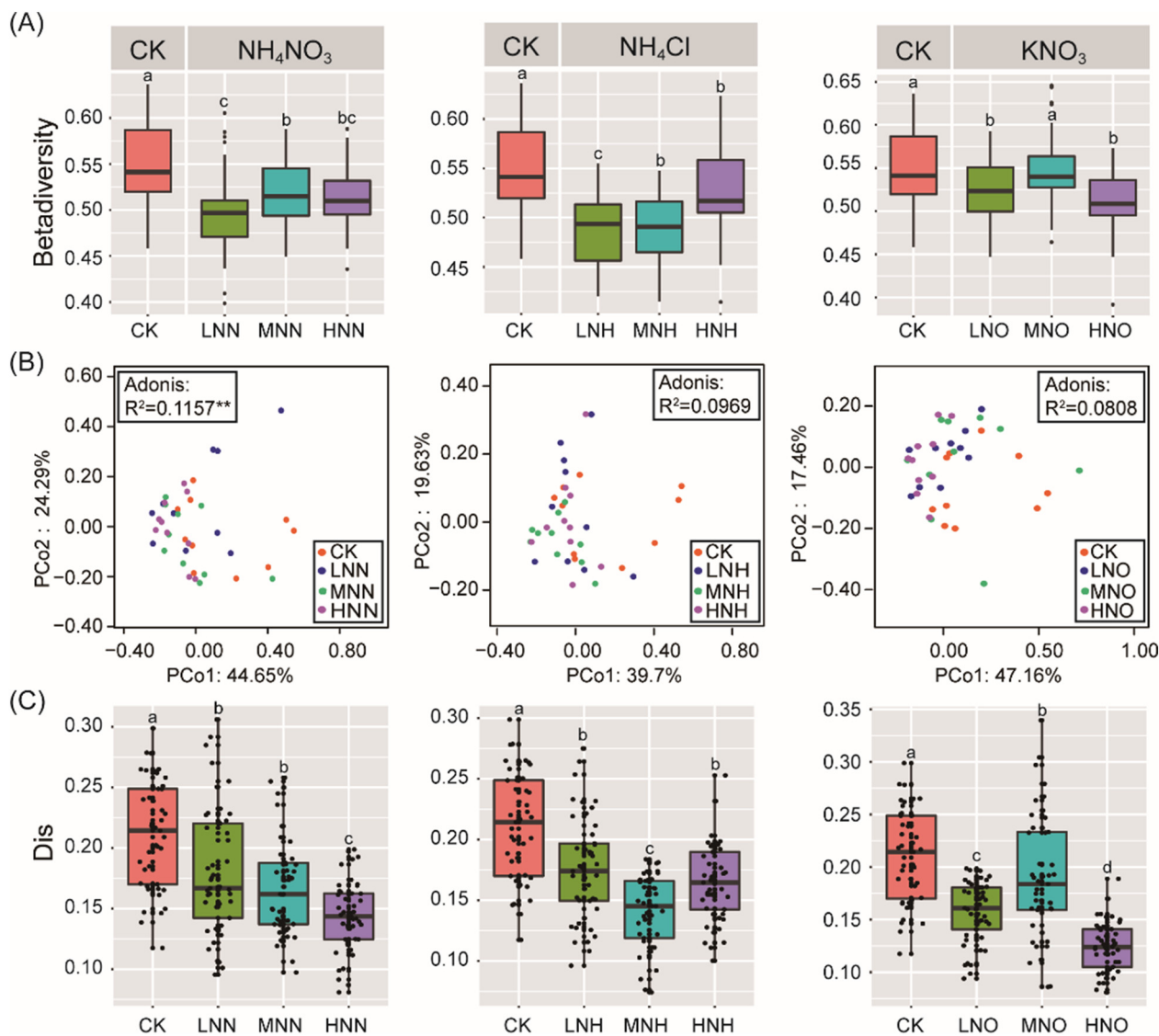


Fig. 3. Analysis of differences in prokaryotic microbial community structure under different treatments. Species turnover (beta diversity) (A). Principal coordinate analysis (PCoA) based on the weighted unifrac distance matrix (B). The weighted unifrac distance matrix between samples was obtained by comparing the analysis between two samples (C). Different colors represent different treatments. Different labeled letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's multiple range tests ($P < 0.05$).

rRNA gene (region V3-V4) data (see methods for details) (Fig. 6). Under different types of nitrogen deposition, the average connectivity and density values of the networks in the NN and NO treatments were larger than those in NH. At different amounts of nitrogen deposition, compared with CK, the node and edge of the network were increased. The average clustering coefficient and average connectivity values of networks at high levels of nitrogen deposition were significantly larger than those of the CK. The average shortest path length and geodesic efficiency were significantly reduced in the NN and NO treatments. In contrast, they increased significantly in the NH treatment ($P < 0.05$). Moreover, the negative associations of networks were enhanced, except in the MNN treatment (Table 2). In this study, we considered the topologically important OTUs in the networks as keystone nodes. At the phylum level, members of the *Proteobacteria*, *Chloroflexi*, and *Gemmatimonadetes* were the most abundant keystone nodes, accounting for 27.13 %, 25.13 %, and 13.13 % of all module hubs and connectors, respectively. The highest number of module hubs was observed at high nitrogen levels, including 12 module hubs in CK, 17 in HNN and HNH, and 18 in HNO (Fig. S3A, Table S3). We further counted the shared keystone nodes of networks under the same type and different

amounts of nitrogen deposition. In the NN treatment, two common keystone nodes were counted between the LNN and HNN treatments. One common keystone node was found between the MNH and HNH treatments in NH. No keystone node was shared in the NO treatment (Fig. S3B). When fewer keystone nodes were shared between the two networks, there were greater changes in the network. In this study, only a few shared keystone nodes were found across the different treatments. Therefore, the co-network in the nitrogen deposition environment is highly dynamic. Finally, compared with CK, the network stability was significantly reduced under different treatments except for LNN and MNO ($P < 0.05$) (Fig. S3C).

3.5. Nitrogen deposition significantly altered some microbial community functional groups associated with nitrogen and carbon cycling

The relationship between the environment and microorganisms affects the structure of the microbial community, which can further affect the function of the ecosystem (Garcia-Palacios et al., 2018). To explore whether and how changes in nitrogen deposition affect ecosystem community function, we predicted potential ecological function changes in the soil prokaryotic

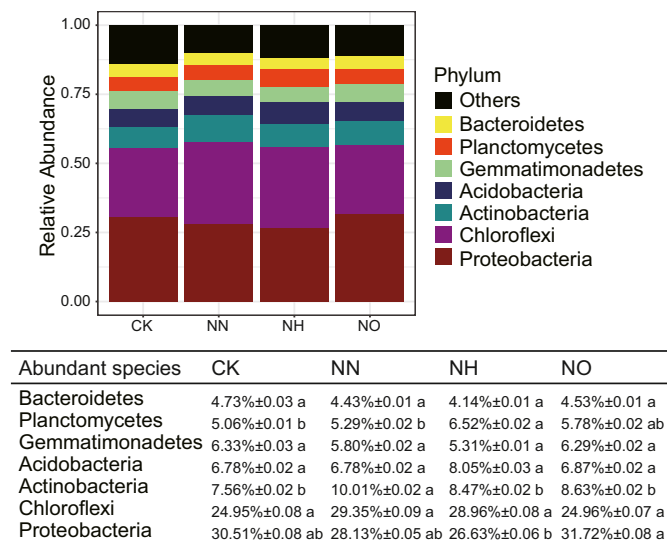


Fig. 4. At the phylum level, the relative abundance of abundant species ($\geq 1\%$) under different types of nitrogen deposition. Different colors represent different taxonomic groups. Different labeled letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's multiple range tests ($P < 0.05$).

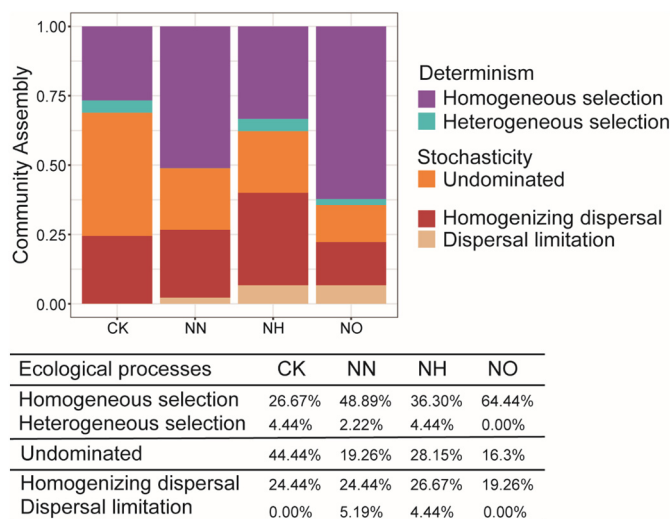


Fig. 5. The assembly process of microbial communities in control and different types of nitrogen deposition. The proportion of community assembly processes are shown below the plots.

microbial communities based on 16S rRNA gene sequences using FAPROTAX. The results showed that some nitrification- and denitrification-related functional groups changed significantly under

different types of nitrogen deposition ($P < 0.05$). However, there was no distinct rule regarding the effects of different amounts of nitrogen deposition on the functional groups (Fig. 7). Compared with CK, the nitrite metabolic function groups were significantly promoted under NN treatment ($P < 0.05$), including nitrate denitrification, nitrite denitrification, nitrous oxide denitrification, denitrification, nitrate respiration and nitrogen respiration (Fig. 7A). The aerobic nitrite oxidation, nitrite denitrification, nitrous oxide denitrification, and denitrification functional groups were significantly enhanced in NH ($P < 0.05$) (Fig. 7B). There was also significantly increased nitrite denitrification, nitrous oxide denitrification, and denitrification in NO ($P < 0.05$) (Fig. 7C). Changes in functional groups associated with ammonia oxidation were observed in the NN treatment but not in the NH and NO treatments. Notably, some functional groups related to the carbon cycle also changed under nitrogen deposition ($P < 0.05$). The methanol oxidation functional group was significantly enhanced under the NN and NO treatments ($P < 0.05$). The cellulolytic functional group was significantly increased in NH, but hydrocarbon degradation was inhibited ($P < 0.05$) (Fig. 7).

4. Discussion

With the changing global environments and human activities, the coastal wetland ecosystem of China is experiencing a continuous increase in atmospheric nitrogen deposition. Studying the response of microbial communities to simulated nitrogen deposition experiments is important for predicting the ecological consequences of future increased atmospheric nitrogen deposition and ecosystem management. In this study, a multilevel and multiform simulated nitrogen deposition platform was established to systematically explore the responses of soil prokaryotic microbial diversity and community composition to different amounts and types of long-term nitrogen deposition in coastal wetlands. The changes in community assembly and microbial interactions under nitrogen deposition were inferred. In addition, whether and how long-term nitrogen deposition would affect microbial community functions were predicted.

Different types of nitrogen, as exogenous nutrients, were deposited into the soil. If deposition reaches a critical level, it may limit the reproduction and activity of certain soil microorganisms (Zhang and Han, 2012), inhibit their growth, and cause changes in the diversity of the microbial community. Our results showed that the α diversity of the microbial community significantly increased under nitrogen deposition conditions. However, the diversity decreased significantly at high nitrogen levels in the NN and NH treatments ($P < 0.05$) (Fig. 2), suggesting that there was a threshold for the microbial community to respond to the deposited nitrogen levels. On the one hand, the nature of species interactions may shift toward more competition and away from mutualism as nutrient availability increases (Hoek et al., 2016), and resource competition can lead to a decline in species diversity (Harpole et al., 2016). On the other hand, sufficiently high nutrient inputs may disrupt interaction species (Bertness and Callaway, 1994), which can further affect the loss of species embedded in higher-order interactions. Furthermore, the loss of higher-order interaction species may cause the loss of many other taxa and contribute to the formation of a low-diversity community state (Fig. 2) (Bailey et al., 2016; Levine et al., 2017). However, the α diversity increased linearly with increasing nitrogen application in the NO treatment ($P < 0.05$). It was possible that NH_4^+ -N had more significant effects

Table 1

The Mantel tests on microbial communities vs. the physicochemical factors measured in this study based on Spearman correlation.

	CK		NH_4NO_3		NH_4Cl		KNO_3	
	P	r	P	r	P	r	P	r
NH_4^+ -N (mg/Kg)	0.622	-0.9684	0.281	0.0507	0.059	0.1541	0.979	-0.1761
NO_3^- -N(mg/Kg)	0.738	-0.1248	0.365	0.0299	0.046	0.1525	0.897	-0.1154
WC (%)	0.354	0.0614	0.477	0.0012	0.537	-0.0154	0.322	0.0408
pH	0.522	-0.0371	0.055	0.1553	0.464	0.0052	0.907	-0.1217
SOM (%)	0.436	0.0139	0.142	0.0737	<0.001	0.2119	0.029	0.1399
SAL (%)	0.014	0.5658	0.087	0.0932	0.414	0.0160	0.283	0.0451

The bold numbers are significant values.

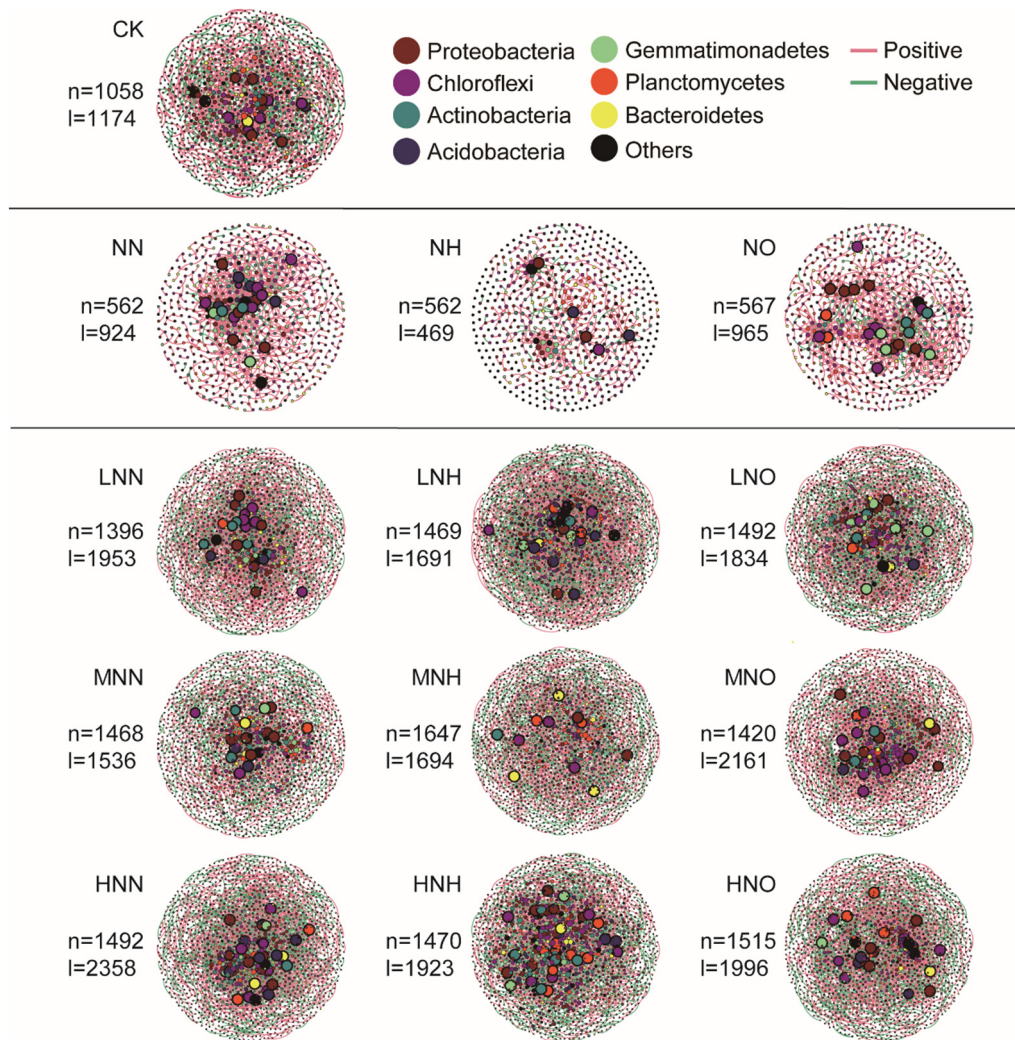


Fig. 6. Visualization of constructed MENs in different amounts and types of nitrogen deposition. n represents the number of nodes; l represents the number of edges. And the enlarged nodes represent keystone OTUs.

on soil microbial activity than did NO_3^- -N (Yan et al., 2017). Nitrogen deposition significantly altered the composition of soil microbial communities ($P < 0.05$) (Figs. 4, S1), however, the responses of different taxa to nitrogen addition were inconsistent in different studies. For example, in the present study, the relative abundance of *Planctomycetes* was significantly increased in the NH and NO treatments compared with CK, while in grassland soils, it responded negatively to nitrogen addition (Leff et al., 2015). In addition, the relative abundance of *Proteobacteria* was significantly reduced in NH and increased in NO. This seemingly contradictory result may have been because bacterial responses to nitrogen are highly environment-dependent or selectively evolutionarily conserved (Isobe et al., 2019). Nitrogen deposition promotes plant aerial portion growth, increases the litter amount and C/N ratio (Hyvönen et al., 2008; Zhu et al., 2020), and leads to changes in soil microbial habitat and carbon resources (Ramirez et al., 2010), which are transformed into changes in the soil microbial community (Liu et al., 2018), and then manifest as an increase or decrease in relative abundance. In addition, we observed significant changes in microbial community structure ($P < 0.05$) (Fig. 3), which may be because changes in microbial community diversity and composition induced by nitrogen deposition causes slight changes in community structure.

Ma et al. proposed that when responding to a common environmental factor, coexisting species would either show similar reactions to the environmental factor or interact with each other (Ma et al., 2016; Wang et al., 2021). In our study, the deterministic process of community assembly increased under nitrogen deposition and was mainly manifested by the

enhancement of homogenous selection (Figs. 5, S2), indicating that microbial communities showed a convergent response, which promoted community similarity (Gossner et al., 2016; Karp et al., 2012), thereby reducing community beta diversity (Fig. 3). However, convergence effects may also increase the competitive relationships between species. Several studies have confirmed that environmental changes affect community assembly processes (Gao et al., 2020; Mo et al., 2021; Ning et al., 2020). The highest percentage of deterministic processes was found in the NO treatment (Fig. 5), which may result from NO_3^- being more mobile and readily leached than NH_4^+ , which increased the homogeneity of the soil. Deterministic processes include biotic and abiotic factors, namely interspecific interactions (e.g., competition, predation, symbiosis, and trade-offs) and environmental filtering (e.g., salinity, pH, and temperature), which together affect the community composition (Jiao et al., 2022). We found that the soil physicochemical variables measured in this study had little effect on the microbial communities (Table 1). Therefore, biological interactions may be more important for community aggregation and ecosystem function in this study. Then, we further constructed microbial co-networks to determine the strength of interactions between microorganisms.

Generally, high topological feature values (e.g., nodes and edges) represent more complex networks (Mo et al., 2021). In this study, compared with CK, network nodes and edges were increased under different amounts of nitrogen deposition (Fig. 6), which means that nitrogen deposition increased the complexity of the microbial co-network. This phenomenon may be partly due to the increased species diversity under the nitrogen deposition

Table 2
Topological properties of the networks within each treatment.

Treatments	NN	NH	NO	CK	LNN	MNN	HNN	LNH	MNH	HNH	LNO	MNO	HNO
Empirical network indexes													
Threshold	0.810	0.810	0.810	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940	0.940
Numbers of OTUs	2427	2433	2418	2100	2754	2938	2849	2755	3122	2593	2817	2630	2966
Average connectivity (Avgk)	3.288	1.171	3.404	2.219	2.798	2.093	3.161	2.302	2.057	2.616	2.458	3.044	2.635
Average clustering coefficient (Avgcc)	0.163	0.116	0.178	0.159***	0.183***	0.122***	0.182***	0.160***	0.125***	0.175***	0.164***	0.192***	0.156***
Average path distance (GD)	5.846	15.141	6.18	10.751***	7.703***	8.171***	8.886***	12.951***	13.620***	10.703***	8.989***	8.576***	9.350***
Geodesic efficiency (E)	0.212	0.117	0.201	0.133***	0.162***	0.153***	0.144***	0.104***	0.100***	0.116***	0.140***	0.144***	0.135***
Density	0.006	0.005	0.006	0.002	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.002	0.002
Positive/total	87.99 %	89.55 %	89.95 %	69.68 %	66.31 %	59.83 %	56.02 %	60.02 %	57.50 %	58.45 %	61.56 %	70.11 %	60.02 %
Average clustering coefficient (Avgcc)	0.015±0.004	0.005±0.003	0.012±0.003	0.002±0.001	0.003±0.001	0.001±0.001	0.004±0.001	0.002±0.001	0.001±0.001	0.002±0.001	0.002±0.001	0.004±0.001	0.003±0.001
Average path distance (GD)	4.434±0.050	5.924±0.232	4.444±0.049	7.449±0.125	5.654±0.054	7.507±0.130	5.212±0.040	7.359±0.082	8.511±0.164	6.749±0.057	6.488±0.065	5.365±0.045	6.044±0.063
Geodesic efficiency (E)	0.250±0.002	0.196±0.006	0.248±0.002	0.151±0.002	0.193±0.002	0.149±0.002	0.208±0.001	0.150±0.001	0.132±0.002	0.162±0.001	0.170±0.001	0.203±0.001	0.181±0.002

Random networks indexes: Mean ± SD based on 100 rewiring networks results.
*** P < 0.001 based on Student's t-test, significant difference in network topological properties (including: Avgcc, GD, E) between control and different amounts of nitrogen deposition treatments.

treatments, which provides more opportunities for interactions between different species. Under different types of nitrogen deposition, the highest average connectivity and density were found in the NO treatment, followed by those in the NN and NH treatments, indicating that microbial networks are more complex under NO₃⁻-N deposition than under NH₄⁺-N deposition (Deng et al., 2012). Compared with the CK, except for the MNO treatment, the negative interactions of the network were enhanced under the other treatments (Fig. 6, Table 2), suggesting that the network resembled more competitive networks and away from mutualistic. Soil nutrients are altered under nitrogen deposition environment (Guan et al., 2019), which may produce environmental selection for well-adapted taxa, enhancing their competitive advantage (Madhaiyan et al., 2010; Webb et al., 2002). However, the limited ecological niche space can cause strong interactions within the taxa when they share similar resources (Gotzenberger et al., 2012), which can lead to competition between species. A similar view was reached by the enhanced selection during community assembly under nitrogen deposition (Fig. 5) because the convergence effect increases the competitive relationships between species. Our result suggested that the average clustering coefficient and average connectivity values of the network increased at all high levels of nitrogen deposition, while the average path distance and geodesic efficiency decreased (P < 0.05) (Table 2), implying that the increased coupling between the main functional nodes in the network promoted effective communication between different members, which indicated that the moderate decrease in diversity might improve the metabolic efficiency of the soil microbiota (Shi et al., 2020). Nitrogen deposition increased the efficiency of transport between species, which caused the influence of disturbance to be rapidly distributed throughout the whole network and contributed to reduced network stability (Helbing, 2013; Yuan et al., 2021). Compared with CK, we observed that the stability was reduced under different amounts of nitrogen deposition in the NN and NH treatments, however, it did not show a consistent pattern in the NO treatment (Fig. S3C). The higher the sensitivity of soil microorganisms to environmental changes, the more unstable is the co-network is (Wu et al., 2021). The network stability of NH₄⁺-N deposition was significantly reduced in this study (P < 0.05), which showed that microorganisms were more sensitive to NH₄⁺-N than to NO₃⁻-N. Notably, function-specific keystone microbes are associated with the stability of the soil microbiota (Shi et al., 2020). Therefore, changes in keystone nodes may also affect network stability. In this study, more species were identified as keystone taxa related to microbiota stability in nitrogen deposition environments, and these species mainly belonged to *Proteobacteria*, *Chloroflexi* and *Gemmatimonadetes*. *Chloroflexi* participates in the cycle of carbon and nitrogen and other elements (Lv et al., 2014). Compared with CK, the relative abundance of *Chloroflexi* was lowest at medium nitrogen levels in the NN and NH treatments (MNN: 0.54 %, MNH: 0.58 %), in the NO treatment, the lowest relative abundance was found at low nitrogen (LNO: 2.42 %) (Fig. S3A). *Gemmatimonadetes* are involved in key steps of nitrogen assimilation and allotropy processes (Liao et al., 2021), and their relative abundance increased under different treatments. *Nitrospira*, which is associated with nitrogen metabolism (Lewis et al., 2021), and *Acidobacteria* and *Verrucomicrobia*, which have high metabolism, were also identified as keystone taxa. In this study, the relative abundance of *Acidobacteria* increased under different amounts of nitrogen deposition in the NN and NO treatments. However, *Verrucomicrobia* was identified as a keystone species only under low levels of nitrogen deposition (Fig. S3A, Table S3). We hypothesize that perhaps these keystone taxa may have a broader distribution, so environmental microperturbations causing changes in the relative abundance of keystone taxa may have negatively impacted the stability of the microbial community.

The changes of soil microbial community structure and interactions in nitrogen deposition environments may affect soil element cycling, which in turn threatens the stability of ecosystems (Hu et al., 2021). The current study showed that different types of long-term nitrogen deposition environments significantly affected the functional groups of communities associated with carbon and nitrogen cycling (Fig. 7). This result may be due to the influence of nitrogen deposition on the main processes of soil nitrogen cycling (e.g., nitrification and denitrification) and the abundance of related

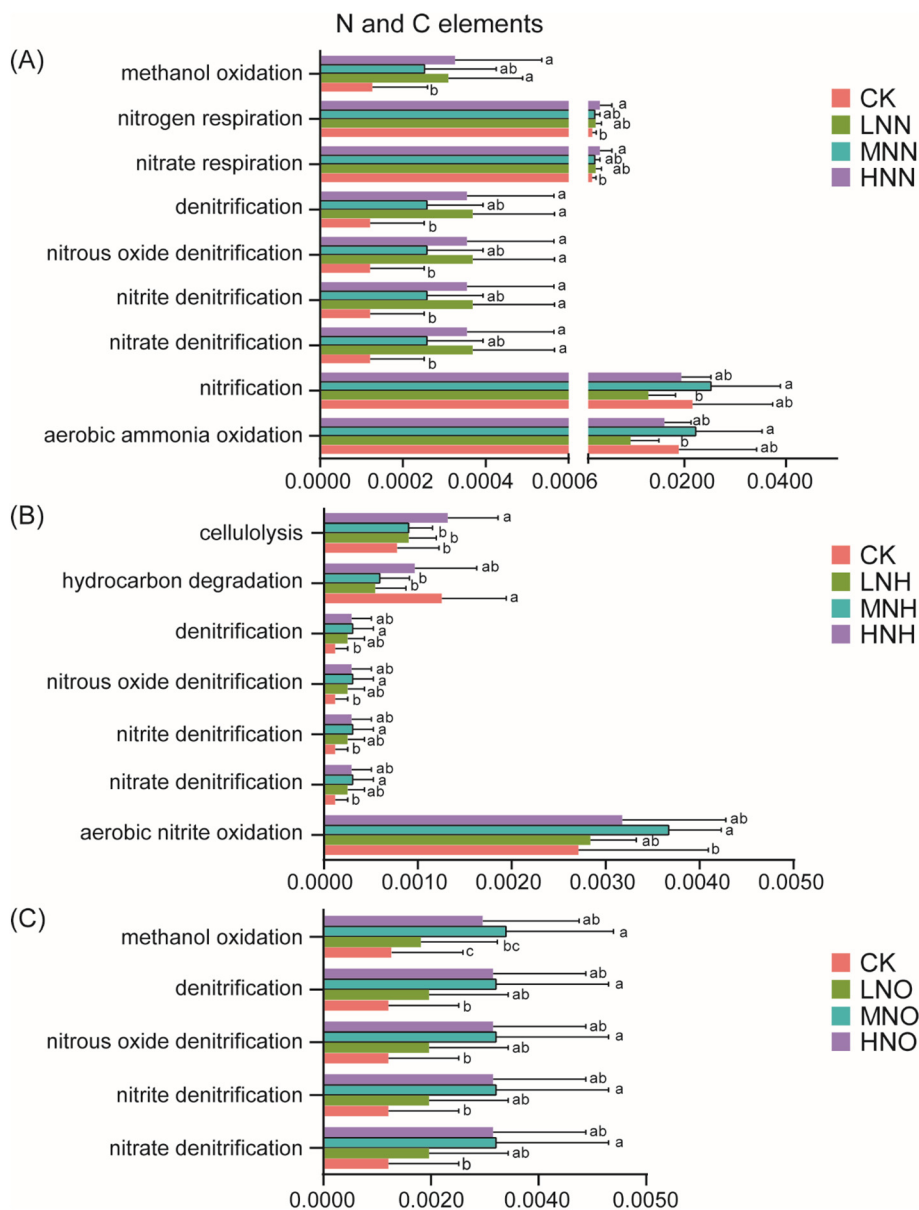


Fig. 7. The potential ecological function changes of soil prokaryotic microbial communities under different treatments as predicted by FAPROTAX.

functional microbial communities (Chen et al., 2010; Shi et al., 2018). In this study, the greatest changes in microbial community functions occurred in the NN treatment, followed by those in the NH and NO treatments, which could be due to the synergistic effects of NH_4^+ -N or NO_3^- -N input on the function of soil microbial communities. NO_3^- -N is the reaction substrate for denitrification (Li et al., 2022), and the concentration of NH_4^+ is an important factor affecting nitrification (Prosser and Nicol, 2012). Thus, in the NN and NH treatments, significant changes were observed in the functional groups associated with nitrification and denitrification ($P < 0.05$). However, only the functional groups related to denitrification changed significantly in the NO treatment ($P < 0.05$). N_2O production in soils is mainly controlled by microbial nitrification and denitrification. In the nitrification process, N_2O is released as an intermediate product when nitrifying bacteria oxidize NH_4^+ to NO_3^- . Under denitrification, the denitrifying bacteria produce N_2O in the process of reducing NO_3^- or NO_2^- (Dalal et al., 2003). We observed a significant increase in nitrification and denitrification related functional groups compared with CK (Fig. 7), indicating that the addition of NO_3^- or NH_4^+ increased the emission of N_2O , mainly due to the increased nitrogen supply to nitrifying and denitrifying bacteria (Keller et al., 2005). However, the toxicity of nitrite (NO_2^-) produced by nitrification or

denitrification processes may also inhibit CH_4 oxidation, which results in an increase in the atmospheric CH_4 concentration (Liu and Greaver, 2009; Schnell and King, 1994). In addition, nitrogen deposition has a certain effect on the carbon cycle pathway. The results of this study showed that compared with CK, the methanol oxidation functional group significantly increased under nitrogen deposition conditions ($P < 0.05$) (Fig. 7). Methanol is an intermediate product of methane oxidation, which is further oxidized to release CO_2 (Madhaiyan et al., 2010). CO_2 , CH_4 , and N_2O are the main greenhouse gases (Sanchez-Rodriguez et al., 2022), so an increase in nitrogen could change the atmospheric greenhouse gas load, which would further contribute to global warming. Under different types of nitrogen deposition, the photosynthetic autotrophic function group were significantly increased (Fig. S5), which was conducive to the increase in the soil carbon pool. However, autotrophic function was weakened under the high nitrogen level of the NN treatment, indicating that more heterotrophic bacteria would be enriched in high nitrogen environments, thus reducing the effect of nitrogen and carbon cycling in the soil. It should be noted that these methods based on the amplicon of 16S rRNA genes usually have limited predictive power. Therefore, additional methods that explain the internal mechanisms of microbial communities should be explored in the

future. Finally, the V4 region primer (515F/806R) has a high coverage of bacteria and can also detect some archaea simultaneously, but it cannot fully reflect the information for archaea.

5. Conclusion

This study demonstrated that NH_4^+ -N and NO_3^- -N deposition had significant effects on the soil prokaryotic microbial community in coastal wetlands. Nitrogen deposition significantly increased the α diversity of the microbial community ($P < 0.05$). Specifically, the α diversity reached a maximum at medium nitrogen levels under NH_4^+ -N deposition, however, it linearly increased with increasing nitrogen levels under NO_3^- -N deposition. Deterministic processes of community assembly contributed more under nitrogen deposition, which increased the competitive relationships between species and resulted in more complex ecological networks. Compared with NH_4^+ -N, the species co-network was more complex under NO_3^- -N deposition conditions. Finally, the functional groups of communities associated with carbon and nitrogen cycling changed significantly under nitrogen deposition, which will affect the stability of the ecosystem. In conclusion, these results can provide a scientific basis for predicting and evaluating the functional stability of wetland ecosystems under nitrogen deposition. However, the fate of the two types of N forms within the ecosystem differs. Therefore, we will further quantify the fate of different forms of deposited nitrogen and their contributions in the coastal wetland system.

Statement of human and animal rights

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Mengyue Sun: Study conception and design, Conduct study, Data collection and analysis, Writing - original draft. **Mingcong Li:** Study conception and design, Conduct study, Supervision, Writing - review & editing. **Yuqi Zhou:** Methodology, Writing - review & editing. **Jiailiu Liu:** Methodology, Writing - review & editing. **Wenchong Shi:** Methodology, Writing - review & editing. **Xiaoliang Wu:** Writing - review & editing. **Baohua Xie:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Ye Deng:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Zheng Gao:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

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Data accessibility

The raw reads were stored in the NCBI database with accession number PRJNA842502.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.158939>.

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