SEASONAL VARIATIONS IN DENITRIFYING BACTERIAL COMMUNITIES IN SEDIMENTS, WATER, AND EPIPHYTIC BIOFILMS OF MACROPHYTIC LAKE

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Abstract. Microbially driven plays a crucial role in nitrogen cycling within aquatic ecosystems. However, seasonal variations in denitrifying bacterial communities (DBC) in aquatic environments remain insufficiently understood. This study used high-throughput sequencing to examine seasonal shifts in DBC within the sediments, water, and epiphytic biofilms (EB) of Caohai Lake, China. Phylum-level analysis revealed that unclassified bacteria and Proteobacteria were the dominant groups in both *nirS*-type and *nirK*-type DBC. Significant structural differences were found among DBC in different habitats, with sediments and water exhibiting higher diversity compared to EB. Moreover, the co-occurrence networks of *nirS*-type and *nirK*-type DBC were more complex in EB during winter than in summer. These findings contribute to a deeper understanding of DBC ecology in macrophytic lake systems.

Keywords: *denitrifying communities, NirS gene, NirK gene, co-occurrence network*

Introduction

Human activities and natural factors have led to a significant increase in nitrogen input into water bodies, such as lakes and oceans. This excess nitrogen contributes to a variety of environmental issues, including water quality degradation and eutrophication. Eutrophication can trigger algal blooms, reduce submerged plant growth, and decrease biodiversity, ultimately disrupting the structure and function of freshwater ecosystems (Yan et al., 2018; Mu et al., 2020). Globally, the number of large eutrophic lakes and reservoirs accounts for 63.1% of the natural water resources (Wang et al., 2018), which is a matter of concern.

Previous studies have shown no significant differences in the absolute and relative abundance of *nirS* and *nirK* denitrifying bacteria across lake, river, and wetland soils, suggesting that soil type does not significantly influence the abundance of denitrifying bacterial communities (Jiang, 2021). In our study, a high relative abundance of unclassified bacteria was observed in both *nirS* and *nirK* denitrifying bacteria, with many bacterial species remaining unidentified. This aligns with findings from research on denitrifying bacteria in Tibetan wetland soils, where 35.6% and 21.1% of *nirS* denitrifying bacteria were unclassified or identified as environmental samples (Jiang, 2021). Similarly, in a large shallow eutrophic

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reservoir in northern China, Proteobacteria dominated the *nirS* denitrifying bacterial community, with an average relative abundance of 66.28%, while unclassified bacteria and environmental samples contributed 26.33% and 7.39%, respectively (Zhou et al., 2016). In contrast, most genera of *nirS* and *nirK* denitrifying bacteria were identifiable in composting systems. For instance, *Luteimonas sp.* and *Achromobacter sp.* were identified as dominant *nirK* denitrifying bacteria, while *Alcaligenes faecalis* and *Pseudomonas stutzeri* predominated the *nirS*-type denitrifiers (Zhong et al., 2020).

Eutrophication caused by nitrogen enrichment has become a hot research topic across the country and even the world. Under natural conditions, excess nitrogen is usually removed through absorption and utilization by aquatic plants and algae, sediment deposition, and microbial-mediated denitrification processes. However, nitrogen-fixation through biological absorption and deposition is only a temporary solution. Apoptosis of aquatic plants and algae eventually leads to return of nitrogen to water body in the form of nutrients. Furthermore, disturbance in sediments by wind and waves or fish feeding also causes nutrients to be reintroduced into the aquatic environment. It has been reported that the denitrification process driven by nitrifying and denitrifying microorganisms is the most crucial denitrification pathway in wetland ecosystems, accounting for 60%–90% of the total denitrification (Truu et al., 2009). This excess nitrogen can be removed by microbial denitrification, which is currently a hot research topic. Therefore, in-depth research on denitrifying bacterial communities in aquatic ecosystems is extremely crucial to address the issues related to nitrogen cycle and eutrophication.

While numerous studies have focused on denitrifying communities in sediments, recent advances in molecular biology techniques have revealed that substantial denitrifying bacterial communities are also present in water bodies and biofilms on submerged plants (Yan et al., 2018). Earlier research primarily concentrated on soil and sediment-based denitrifying communities, where factors such as nitrogen fertilizer application, soil pH, and total nitrogen content were found to significantly influence bacterial abundance and community composition, especially for *nirS* and *nirK* genes (Yang et al., 2017). In the sediments of the Yangtze Estuary, salinity was a major determinant of *nirS* denitrifying bacterial diversity, although no significant seasonal differences were observed (Zheng et al., 2015).

During microbial denitrification, microorganisms gradually reduce the NO_3^- to N_2 using denitrification enzymes. For example, nitrite reductase (*Nir*) is an enzyme that plays a key role in denitrification by catalyzing the reduction of nitrite (Kraft et al., 2011; Shrewsbury et al., 2016; Chen et al., 2017). In the lakes, denitrification mainly occurs in sediments, water, and epiphytic biofilms. Denitrification is the primary effective way to remove nitrogen from natural water bodies. The denitrifying bacteria community is widely distributed and mainly classified as bacteria and archaea. The main group of *nirS* denitrification community in river sediments, eutrophic reservoirs and wastewater treatment plants is *Proteobacteria* (Yu et al., 2021). Proteobacteria is also the dominant group of *nirK*-type denitrifying bacteria in aquatic ecosystems (Yu et al., 2020). Earlier studies have demonstrated that *Proteobacteria* play a crucial role in both the carbon and nitrogen cycles (Hou et al., 2018; Zhou et al., 2020). Denitrifying functional bacteria from the class *Betaproteobacteria* have been detected in many biological treatment systems (such as municipal sewage treatment and kitchen waste treatment systems, etc.) (Figuerola et al., 2007; Ma et al., 2015), this group can perform denitrification as frequently as Alphaproteobacteria (Gao et al., 2019; Zumft, 1997). The composition of denitrifying bacterial communities varied across different habitats. Liu et al. (2018) identified

Rhodobacter as a typical denitrifying bacterium in alkaline copper mine wastewater. In Fu et al.'s (2019) study, aerobic denitrifying bacteria such as *Pseudomonas* and *Acinetobacter* were shown to efficiently facilitate denitrification, removing over 90% of total nitrogen in wetland ecosystems. *Thauera* and *Azoarcus* are important denitrifying bacteria in quinoline denitrification removal bioreactors, accounting for 74% (Liu et al., 2006). Guo et al. (2021) conducted a mechanistic study and found that the abundance of Dechloromonas in electrolysis sludge (with a relative abundance of 5.45%) can enhance nitrate reduction via electrolytic Fe(II), effectively removing total nitrogen in the process. ASV kinetics of bacteria in denitrifying granular sludge bioreactor showed that high carbon or organic carbon concentration could promote the growth of *Acidovorax* (Zhou et al., 2021). When the carbon source is organic, it is easier to enrich *Acidovorax* genus of *Comamonadaceae* (Lu et al., 2014). The genus *Paracoccus* uses pyridine biodegradation to reduce nitrogen levels in coking wastewater (Zhou et al., 2018). *Sinorhizobium, Mesorhizobium, Rhizobium, Devosia, and Bosea* (all belonging to the Rhizobiales order) were identified as *nirK*-type denitrifiers in the roots of *T. angustifolia* and *S. triqueter* (Zhang et al., 2021). The denitrifying bacterial community in the biofilm is primarily dominated by *Proteobacteria*. Despite the high diversity of denitrifying bacteria in the biofilm, a large portion remains unculturable in the medium (Zhang et al., 2016), possibly due to the unique microenvironment within the biofilm. However, many studies have primarily focused on denitrifying bacteria in sediment environments. Comparative studies on denitrifying bacterial communities in water and epiphytic biofilms are scarce. Therefore, the denitrifying bacterial communities of the lake ecosystems need to be further explored by comparatively analyzing various types of samples collected from these ecosystems.

To address this gap, this study collected sediment, water, and biofilm samples from Lake Caohai during both summer and winter seasons to examine the *nirS*- and *nirK*-type denitrifying bacterial communities. The main objectives were to: (1) characterize the denitrifying bacterial communities in the sediments, water, and epiphytic biofilms of a macrophytic lake; (2) assess the structural and diversity differences among these communities across the different sample types; and (3) explore seasonal variations in the co-occurrence networks for denitrifying bacterial communities.

Materials and methods

Study area

The study area is located in Caohai, Weining County, Guizhou Province, China. As the largest natural plateau lake in the province, Caohai holds significant ecological importance. Caohai is also an important habitat and hibernating site for the rare and endemic black-necked cranes (*Grus nigricollis*). The region features a subtropical plateau monsoon climate, receiving about 950.9 mm of rainfall annually. It is situated at an altitude of 2171 m, covering a water area of 29.91 km^2. Caohai is home to many rare birds and massive aquatic plants that exist in diverse life forms. It is not only an important research site but also a protected national nature reserve.

Sample collection and processing

The sampling sites of the study area have been shown in *Figure 1*. Sediment, water, and submerged plant (*Potamogeton lucens* and *Najas marina*) samples were collected in summer (July) and autumn (November) seasons in 2020. A hand-held columnar mud collector was employed to collect approximately 10 g of surface sediments. Sediment samples were collected, kept at low temperature during transport, and stored at -20°C upon arrival at the laboratory. A 1.5 L water sample was collected 0.5 m underwater using a water collector. The collected water sample was transported to the laboratory, filtered using a 0.22 μm membrane, and then stored at -20°C. For each submerged plant species, samples of 3 different plants (about 10 g fresh weight) were collected, placed in a phosphate buffer, and transported to laboratory under low temperature conditions. In the laboratory, plant samples were ultrasonicated for 3 min, followed by 30 min of shaking, and then ultrasonicated again for 3 min. Finally, the submerged plants were removed, and the eluate was suction-filtered through a 0.22 μm membrane to collect the biofilm on the filter membrane. The filter membrane was subsequently stored at -20°C. We took equal number of samples from each site and we got 36 plant samples, 12 sediment samples,12 water samples in total.

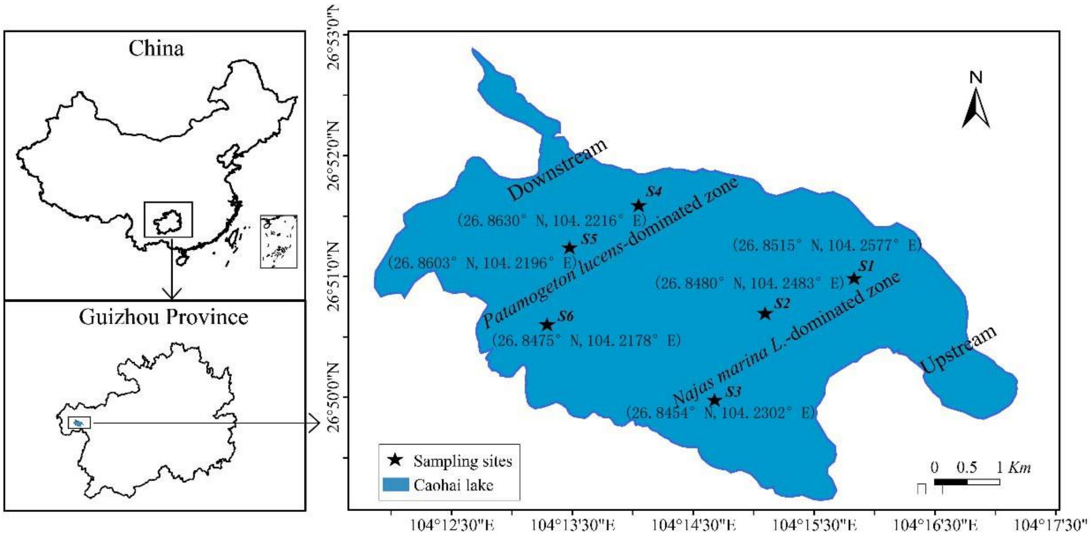

Figure 1. *Sampling site layout of Caohai Lake, Weining, Guizhou*

High throughput DNA sequencing

Bacterial DNA was extracted from the biofilm on the filter membrane using the FastDNA® Spin Kit for Soil samples (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer’s instructions. The samples were mixed and centrifuged, after which DNA concentration and purity were assessed using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA integrity was checked by running a 1% agarose gel electrophoresis at 5 V/cm for 20 min. The qualified DNA samples were then stored at -80°C for subsequent analyses.

Primer pairs cd3aF/R3cd and F1aCu/R3Cu were used for amplifying and constructing clone libraries of the *nirS* and *nirK* genes via polymerase chain reaction (PCR) (*Table 1*). The PCR reactions were prepared using TransGen AP221-02: TransStart Fastpfu DNA Polymerase in a 20 μL system, which included 4 μL of 5×FastPfu Buffer, 2 μL of dNTPs (2.5 mM), 0.8 μL each of forward and reverse primers (5 μM), 0.4 μL of polymerase, 0.2 μL of BSA, 10 ng of template DNA, and ddH2O. Amplification was performed on an ABI GeneAmp® 9700 thermal cycler with initial denaturation at 95°C for 3 min, followed by cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 5 min.

Table 1. *Primers used for PCR amplification*

Genes	Primers	Primer sequences (5’ →3’)	References
nirS	cd3aF	GTSAACGTSAAGGARACSGG	(Throbäck et al., 2004)
	R3cdR	GASTTCGGRTGSGTCTTGA	
nirK	F1aCu	ATCATGGTSCTGCCGCG	(Hallin and Lindgren,1999)
	R3Cu	GCCTCGATCAG RTTGTGGTT	

High-throughput sequencing and library preparation were conducted using the Illumina MiSeq platform (Illumina, San Diego, USA) at Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). To optimize the sequence data, duplicate sequences were dereplicated (http://drive5.com/usearch/manual/dereplication.html), and singletons were removed (http://drive5.com/usearch/manual/singletons.html). Operational taxonomic units (OTUs) were clustered at 97% similarity after filtering out chimeric sequences, and representative sequences were selected. Finally, sequences with at least 99% similarity to the representative OTUs were mapped to generate OTU tables for downstream analysis.

Statistical analysis

The sampling site map was created using ArcGIS (version 10.6), while R (version 4.0.1, https://www.r-project.org/) was used for subsequent data visualization and analysis. Histograms illustrating the relative abundance of denitrifying bacteria at the phylum level were generated, and Venn analysis was performed to compare community similarities across sample groups. Alpha diversity, measured using the Shannon and ACE indices, was calculated to assess community diversity and richness. Statistical differences in alpha diversity among groups were analyzed using one-way ANOVA with the “aov” function (choosing Tukey HSD to conduct the post-hoc test), and results were visualized through the “ggplot2” (Wickham, 2016) package in R.

Beta diversity was examined through principal coordinate analysis (PCoA) based on Bray-Curtis distance matrices, revealing structural variations among bacterial communities. Statistical significance of these variations was confirmed using permutational multivariate analysis of variance (PERMANOVA), with visual outputs also created using “ggplot2.” To explore interactions among denitrifying bacteria, co-occurrence networks were constructed from relative abundance data at the OTU level using the WGCNA (Langfelder and Horvath, 2008, 2012) package in R and visualized with Gephi (version 0.9.2).

Results

Denitrifying bacterial communities: composition and dynamics

In Caohai Lake, 2014 and 2181 OTUs were identified as belonging to *nirS*-type and *nirK*-type denitrifying bacterial communities, respectively, across sediments, water, and epiphytic biofilms of submerged plants. At the phylum level, unclassified bacteria and *Proteobacteria* were the dominant taxa in both communities (*Fig. 2*). While the composition of *nirS*-type bacteria showed no significant seasonal changes, the abundance of Proteobacteria in *nirK*-type communities was notably higher during winter (*Fig. 2a*, *b*). Venn diagrams further revealed substantial species differences among habitats and seasons, with low overlap of OTUs and a high percentage of unique taxa (*Fig. 2c*, *d*). Sediments and water samples generally exhibited more unique OTUs compared to epiphytic biofilms.

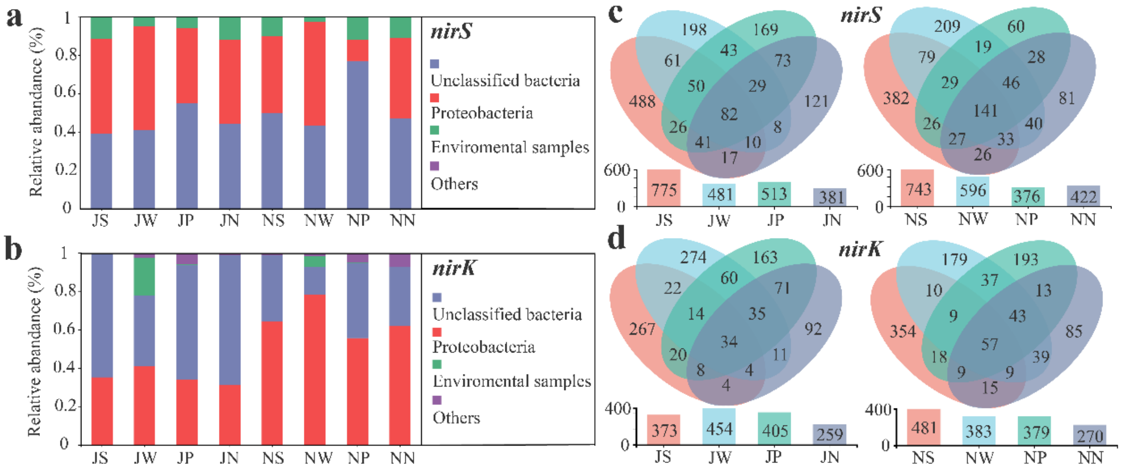

Figure 2. *(a) Distribution of bacterial phyla in nirS-type denitrifying bacterial community; (b) Distribution of bacterial phyla in nirK-type denitrifying bacterial community; (c) Venn diagram of the nirS-type denitrifying bacterial community at the OTU level; (d) Venn diagram of the nirK-type denitrifying bacterial community at the OTU level. Here, JS, JW, JP, and JN represent the sediment, water, epiphytic biofilms of Potamogeton lucens and Najas marina collected in July, respectively. NS, NW, NP, and NN refer to the sediment, water, epiphytic biofilms of P. lucens and N. marina collected in November, respectively*

Variations in denitrifying bacterial community diversity

The Shannon index was used to assess the diversity of denitrifying bacterial communities across different habitats in Caohai during summer and winter. For *nirS*-type communities, no significant seasonal changes in diversity were observed within the same habitat. However, in winter, sediments and water samples showed significantly higher diversity compared to epiphytic biofilms of *Potamogeton lucens* (*Fig. 3a*). In contrast, the diversity of *nirK*-type communities in epiphytic biofilms of *Najas marina* varied significantly between seasons ($P < 0.05$, *Fig. 3c*).

The ACE index was used to evaluate the richness of denitrifying bacterial communities. In summer, *nirS*-type communities in sediments exhibited significantly higher richness compared to those in water and epiphytic biofilms ($P < 0.05$, *Fig. 3b*). However, no notable differences were found between sediment and water samples. Similarly, for *nirK*-type communities, the ACE index showed no significant variation across sediments, water, and epiphytic biofilms (*Fig. 3d*).

Denitrifying bacterial communities: structural insights

PCoA analysis demonstrated clear structural differences among *nirS*-type denitrifying bacterial communities in sediments, water, and epiphytic biofilms, with these distinctions further validated by PERMANOVA ($P < 0.05$, *Fig. 4a*). A similar trend was observed for *nirK*-type communities, which also exhibited significant variation across different habitats ($P < 0.05$, *Fig. 4b*). Seasonal analysis showed no significant changes in *nirS*-type community structures within sediments or biofilms of *Najas marina*, whereas notable differences were detected between water samples and *Potamogeton lucens* biofilms (*Fig. A1*).

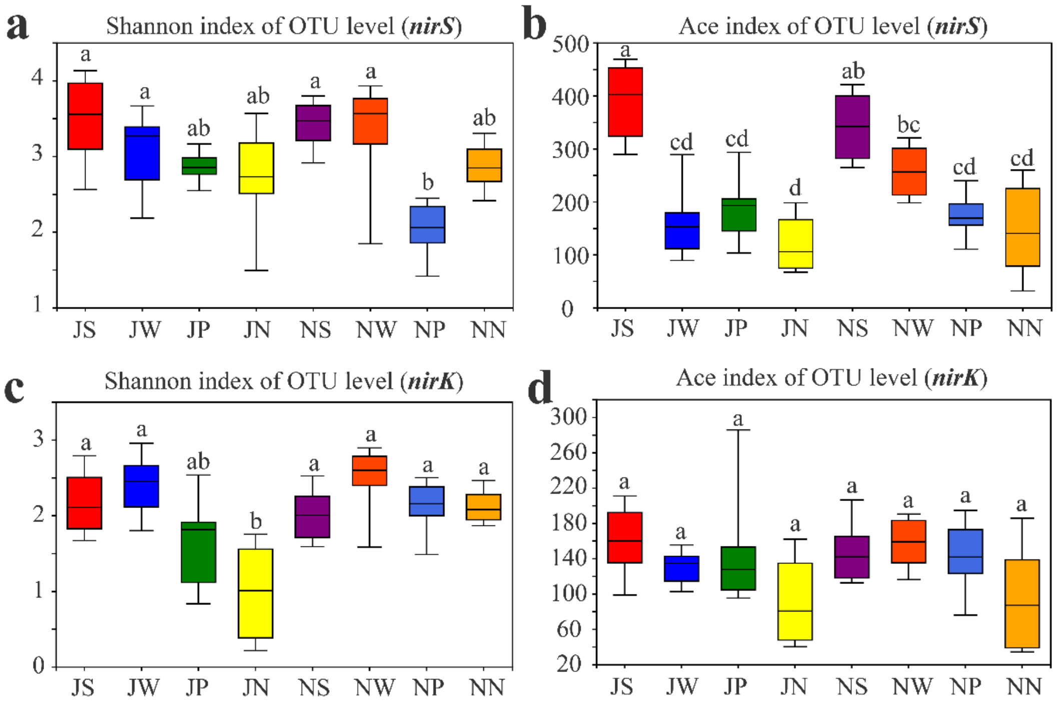

Figure 3. *Shannon (a) and ACE (b) indices of nirS-type denitrifying bacterial communities; Shannon (c) and ACE (d) indices of nirK-type denitrifying bacterial communities in different samples. Here, JS, JW, JP, and JN represent the sediment, water, epiphytic biofilms of Potamogeton lucens and Najas marina collected in July, respectively. NS, NW, NP, and NN refer to the sediment, water, epiphytic biofilms of P. lucens and N. marina collected in November, respectively*

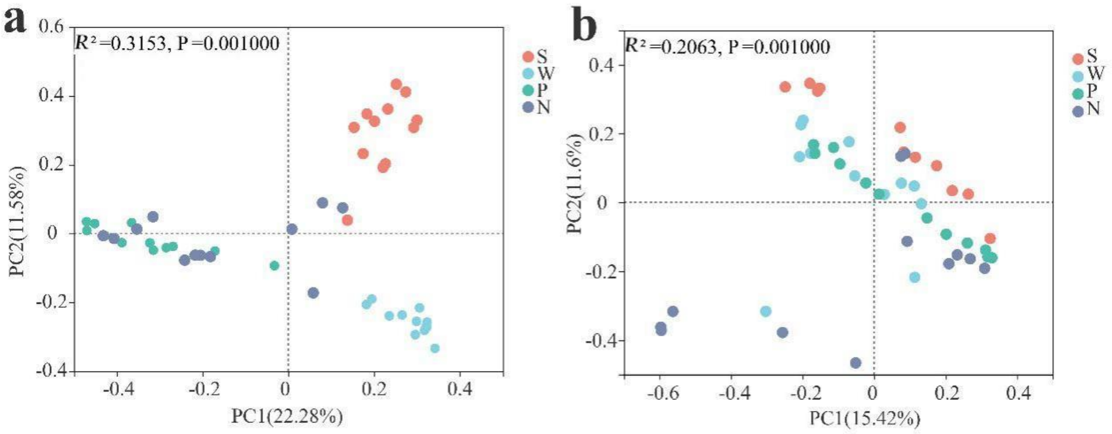

Figure 4. *PCoA analysis of (a) nirS-type and (b) nirK-type denitrifying bacterial communities in different samples*

For *nirK*-type communities, structural differences were consistent across habitats, with no seasonal variation in sediments or *Potamogeton lucens* biofilms. However, water samples and Najas marina biofilms showed significant seasonal shifts ($P < 0.05$, *Fig. A2*). These results highlight the influence of habitat and season on the structural dynamics of denitrifying bacterial communities.

Interconnectedness of denitrifying bacterial communities

The co-occurrence network analysis revealed seasonal differences in the interaction patterns of denitrifying bacteria. In sediments and water samples, the weighted average degree and proportion of positive correlation edges for *nirS*-type bacteria were higher in summer compared to winter (*Table 2*). For *nirK*-type bacteria, the weighted average degree in sediments was also higher during summer, but an inverse trend was observed in water samples.

Table 2. *Co-occurrence network attributes of denitrifying bacterial communities*

Genes	Samples	Nodes	Edges	Average degree	Weighted average degree	Modularity	Average clustering	Proportion of positive correlation edges
nirS	JS	419	1264	6.033	12.076	0.866	0.127	94.62%
	NS	364	1065	5.852	11.703	0.823	0.154	87.51%
	JW	168	657	7.821	15.643	0.582	0.222	91.48%
	NW	235	526	4.477	8.953	0.835	0.328	76.62%
	JP	194	545	5.619	11.237	0.779	0.271	84.77%
	NP	105	530	10.095	20.19	0.568	0.463	75.66%
	JN	128	407	6.359	12.719	0.714	0.207	91.89%
	NN	180	2673	29.7	59.4	0.254	0.345	96.71%
nirK	JS	158	433	5.481	10.962	0.76	0.397	87.53%
	NS	155	785	10.169	20.258	0.53	0.497	69.68%
	JW	436	4786	21.594	43.908	0.68	0.072	94.94%
	NW	125	179	2.864	5.728	0.872	0.283	82.12%
	JP	117	351	6	12	0.695	0.369	82.34%
	NP	105	530	10.095	20.19	0.566	0.463	75.66%
	JN	234	1522	13.009	26.017	0.574	0.083	95.80%
	NN	242	3458	28.597	57.157	0.283	0.138	95.52%

In epiphytic biofilms, the weighted average degrees of *nirS*-type and *nirK*-type networks were generally lower in summer. Notably, *Potamogeton lucens* biofilms had a higher proportion of positive correlation edges for *nirS*-type bacteria in summer, whereas *Najas marina* biofilms showed the opposite trend. Within the same season, the weighted average degree of denitrifying bacteria networks was greater in *Najas marina* biofilms than in *Potamogeton lucens* biofilms (*Figs. 5* and *6*).

Discussion

Due to the combined impact of human activities and natural factors, eutrophication has become a global issue affecting most lakes worldwide (Wang et al., 2018). In China, the surface water quality has been deteriorating significantly, necessitating a deeper understanding of the composition and ecological functions of denitrifying microbial communities in natural water bodies (Liu et al., 2022). These communities, which play a central role in the nitrogen cycle, are composed of diverse and widely distributed microorganisms, primarily bacteria and archaea (Lu et al., 2014).

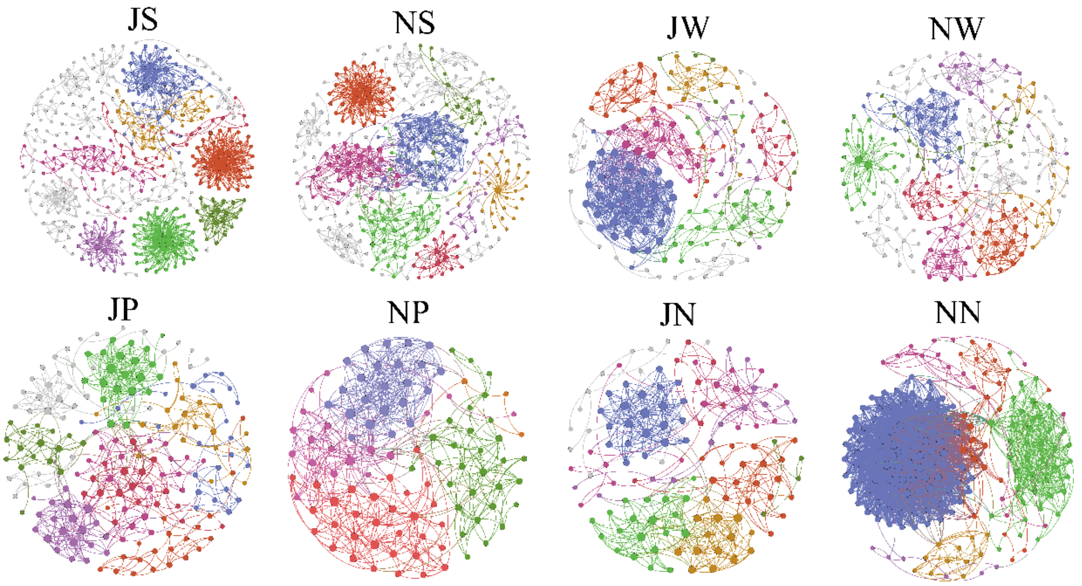

Figure 5. *Co-occurrence network of nirS-type denitrifying bacterial communities. JS, JW, JP, and JN represent the sediment, water, epiphytic biofilms of Potamogeton lucens and Najas marina collected in July, respectively. NS, NW, NP, and NN refer to the sediment, water, epiphytic biofilms of P. lucens and N. marina collected in November, respectively*

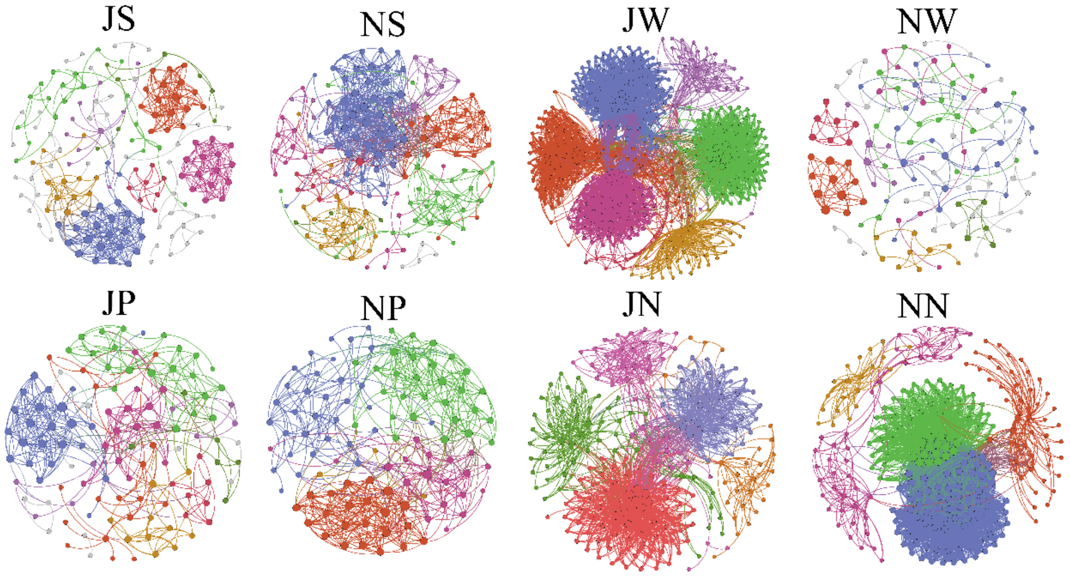

Figure 6. *Co-occurrence network of nirK-type denitrifying bacterial communities. JS, JW, JP, and JN represent the sediment, water, epiphytic biofilms of Potamogeton lucens and Najas marina collected in July, respectively. NS, NW, NP, and NN refer to the sediment, water, epiphytic biofilms of P. lucens and N. marina collected in November, respectively*

In various environments, such as river sediments, eutrophic reservoirs, and wastewater treatment systems, *nirS*-type denitrifying bacteria are mainly affiliated with the *Proteobacteria phylum* (Yu et al., 2021). Similarly, in aquatic ecosystems, *Proteobacteria* dominate the *nirK*-type denitrifying bacterial communities (Yu et al., 2020). *Proteobacteria* contribute significantly to both carbon and nitrogen cycling, with *Betaproteobacteria* being particularly efficient in denitrification. These bacteria are frequently identified in biological systems, including urban sewage and food waste treatment processes (Figuerola and Erijman, 2007; Ma et al., 2015). The denitrification efficiency of *Betaproteobacteria* is comparable to that of *Alphaproteobacteria*, highlighting their critical ecological role (Gao et al., 2019; Zumft, 1997).

Denitrification activity occurs on the surfaces of sediments, water, and submerged plants in aquatic environments. High-throughput sequencing of samples collected from Caohai Lake identified *Proteobacteria* and unclassified bacteria as the dominant phyla in both *nirS*- and *nirK*-type communities. Unclassified bacteria exhibited a relatively high abundance, indicating the presence of many species that remain unidentified. In a study conducted in Northern China, a large, shallow eutrophic reservoirs, the *nirS*-type denitrifying bacterial community showed an average relative abundance of 66.28% for *Proteobacteria*, with unclassified bacteria and environmental samples contributing 26.33% and 7.39%, respectively (Zhou et al., 2016).

In compost systems, where microbial diversity is often well-characterized, sequencing with similar primers revealed that *nirK*-type denitrifiers were dominated by *Luteimonas sp.* and *Achromobacter sp.*, while *nirS*-type communities primarily consisted of the same genera (Zhong et al., 2020). These results underscore the significant knowledge gaps regarding denitrifying bacterial communities in natural ecosystems. Many of these microorganisms remain unidentified, indicating a pressing need for further research to characterize their diversity, ecological roles, and potential applications.

Microbial diversity plays a pivotal role in driving ecosystem functions by influencing fundamental ecological processes, including organic matter decomposition, nutrient cycling, and gas exchange (Bastida et al., 2021). Microbial diversity is affected by various factors, such as climate change, seasonal variations, and habitat (Yan et al., 2019; Bastida et al., 2021). Consequently, it is believed that soil type does not affect the abundance of denitrifying bacterial communities. Still, Venn analysis was used in this study to investigate the similarities or dissimilarities in the communities of different habitats, such as sediments, water, and epiphytic biofilms.

Studies have shown that the shared OTUs of *nirS*-type and *nirK*-type denitrifying bacteria across sediments, water bodies, and epiphytic biofilms during both summer and winter were relatively low, highlighting substantial compositional differences among these habitats. This suggests that while sediments are traditionally recognized for their denitrification roles, epiphytic biofilms and water samples also contribute significantly to denitrification in aquatic ecosystems due to their active microbial communities. Pang et al. (2016) reported notable structural differences between bacterial communities in wetland epiphytic biofilms and sediments dominated by aquatic plants, as evidenced by cluster and principal component analyses. Similarly, Yan et al. (2019) observed distinct bacterial compositions between water and biofilm habitats, with specific taxa characterizing each.

In the present study, significant differences were confirmed in the structure of denitrifying bacterial communities across sediments, water, and epiphytic biofilms. These differences may arise from varying environmental factors such as water flow, light availability, and nutrient concentrations, as suggested by He et al. (2012). Faulwetter et al. (2013) further noted that microbial communities in aquatic plant roots differed from those in surrounding sediments, likely due to organic matter accumulation and the secretion of oxygen and other chemical compounds by the roots. Consistent with Liu et al. (2020), our findings showed that OTU richness and Shannon diversity of microbial communities in sediments were significantly higher than in epiphytic biofilms, possibly due to the structural and chemical complexity of sediment niches, which provide diverse microenvironments for bacterial colonization (Song et al., 2019).

The composition of epiphytic bacterial communities also differs from that of bacterioplankton communities, as noted by He et al. (2014), due to the presence of specialized species. Garulera et al. (2016) and Yan et al. (2019) highlighted that bacterioplankton communities act as seed banks for epiphytic bacteria, which subsequently colonize biofilm surfaces. Yan et al. (2019) further demonstrated that although there are similarities, distinct differences exist between the structures of bacterial and bacterioplankton communities in epiphytic biofilms of submerged plants.

In this study, marked differences were observed in denitrifying bacterial communities across various habitats, likely reflecting their adaptability to distinct environmental conditions. Additionally, notable variations were found between *nirS*- and *nirK*-type bacterial communities, which may result from their differing ecological strategies and environmental requirements (Shi et al., 2019). These findings emphasize the complex interactions and adaptations of denitrifying bacteria in diverse aquatic ecosystems.

Conclusion

In natural environments, complex interactions among species are mediated through the exchange of matter, energy, and information. These interconnections within bacterial communities can be effectively investigated using co-occurrence network analysis (Zhao et al., 2016). Correlation-based microbial networks have become a valuable tool for microbial ecologists to uncover co-occurrence and co-exclusion patterns within microbial communities (Ju and Zhang, 2015; Zhao et al., 2016). In this study, the co-occurrence network of epiphytic biofilms consisted of 64 nodes and 182 strongly correlated edges, which was approximately three times more complex than the sediment network, comprising 40 nodes and 57 edges. This highlights the intricate interactions within biofilms compared to sediments (Liu et al., 2020). Such findings align with prior research, which consistently shows that microbial networks in biofilms exhibit higher complexity due to their unique characteristics.

Biofilms are specialized microbial communities that foster dynamic interspecies interactions. When ecological niches overlap, species may compete or exhibit mutual exclusion under resource-limited conditions, whereas they tend to engage in positive interactions when resources are plentiful (Zhao et al., 2016). Additionally, metabolites secreted by submerged plants significantly influence the composition and diversity of bacterial communities within biofilms (Fan et al., 2016). Algal-derived carbon sources can further support the proliferation of denitrifying bacterial communities, enhancing the denitrification process in aquatic ecosystems (Lu et al., 2014). For example, in constructed wetlands with limited carbon sources, adding algae has been shown to effectively improve denitrification efficiency in a simple and practical manner (Cheng et al., 2022).

Moreover, microorganisms in biofilms, such as bacteria and metazoans, are interconnected through various ecological relationships, including symbiosis, parasitism, and predation (Zhang et al., 2020). These interactions create highly active microbial networks within biofilms, leading to their greater complexity compared to sediment networks. The intricate interplay of microbial communities in biofilms reflects their role as hotspots of biological activity in aquatic environments.

In the present study, unclassified bacteria and Proteobacteria emerged as the predominant members of both *nirS*-type and *nirK*-type denitrifying bacterial communities across sediments, water, and epiphytic biofilms. A considerable number of unidentified bacterial species were detected, accompanied by notable differences in community composition at the OTU level. The structural variations among denitrifying bacterial communities in these habitats can be attributed to differences in bacterial survival strategies and environmental adaptability. Denitrifying bacterial communities in sediments and water exhibited greater diversity compared to those in the epiphytic biofilms of submerged plants. However, the co-occurrence network within epiphytic biofilms was significantly more intricate than that in sediments, reflecting stronger interspecies interactions in biofilm habitats.

Seasonal analysis revealed that the co-occurrence networks of *nirS*-type and *nirK*-type denitrifying bacteria in epiphytic biofilms were more complex during winter. This increased complexity suggests enhanced interspecific interactions as the biofilm matures and develops. The study highlights the abundant presence of denitrifying bacteria in sediments, water, and epiphytic biofilms of Caohai Lake, underscoring their critical role in the nitrogen cycle. Despite this abundance, a significant proportion of bacterial species remains unidentified, emphasizing the need for further research to characterize these unknown members of denitrifying bacterial communities.

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REFERENCES

[1] Bastida, F., Eldridge, D. J., García, C., Kenny Png, G., Bardgett, R. D., Delgado-Baquerizo, M. (2021): Soil microbial diversity-biomass relationships are driven by soil carbon content across global biomes. – ISME J. 15: 2081-2091. https://doi.org/10.1038/s41396-021-00906-0.

[2] Chen, J., Ying, G. G., Liu, Y. S., Wei, X. D., Liu, S. S., He, L. Y., Yang, Y. Q., Chen, F. R. (2017): Nitrogen removal and its relationship with the nitrogen-cycle genes and microorganisms in the horizontal subsurface flow constructed wetlands with different design parameters. – J Environ Sci Heal A 52(8): 804-818.https://doi.org/10.1080/10934529.2017.1305181.

[3] Cheng, S., Huai, J., Zhong, F., Wu, J., Yu, S. (2022): Enhancing denitrification in constructed wetland with algae addition. – Environ Sci Pollut Res 29: 1949-1960. https://doi.org/10.1007/s11356-021-15755-9.

[4] Fan, Z., Han, R. M., Ma, J., Wang, G. X. (2016): Submerged macrophytes shape the abundance and diversity of bacterial denitrifiers in bacterioplankton and epiphyton in the Shallow Fresh Lake Taihu, China. – Environ Sci Pollut Res 23(14): 14102-14114. https://doi.org/10.1007/s11356-016-6390-1.

[5] Faulwetter, J. L., Burr, M. D., Parker, A. E., Stein, O. R., Camper, A. K. (2013): Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and

ammonia oxidizing bacteria in constructed wetland microcosms. – Microb Ecol (2013) 65: 111-127. DOI: 10.1007/s00248-012-0114-y.

[6] Figuerola, E. L. M., Erijman, L. (2007): Bacterial taxa abundance pattern in an industrial wastewater treatment system determined by the full rRNA cycle approach. – Microb Ecol 9(7): 1780-1789. https://doi.org/10.1111/j.1462-2920.2007.01298.x.

[7] Fu, G. P., Han, J. Y., Yu, T. Y., Huangshen, L. K., Zhao, L. (2019): The structure of denitrifying microbial communities in constructed mangrove wetlands in response to fluctuating salinities. – – J. Environ. Manage. 238: 1-9. https://doi.org/10.1016/j.jenvman.2019.02.029.

[8] Gao, J., Duan, Y., Liu, Y., Zhuang, X. L., Liu, Y. C., Bai, Z. H., Ma, W. L., Zhuang, G. Q. (2019): Long- and short-chain AHLs affect AOA and AOB microbial community composition and ammonia oxidation rate in activated sludge. – JE Sciences 78: 53-62. https://doi.org/10.1016/j.jes.2018.06.022.

[9] Garulera, J. B., Vila, M., Borrull, E., Riobó, P., Franco, J. M., Sala, M. M. (2016): Variability of planktonic and epiphytic vibrios in a coastal environment affected by Ostreopsis blooms. – Sci. Mar. 80S1: 97-106. DOI: http://dx.doi.org/10.3989/scimar.04405.01A.

[10] Guo, Y., Zhang, Z. Q., Shi, W. X., Zhang, B., Li, W. G., Cui, F. Y., Lens, P. N. L. (2021): Evolution of the sludge mineral composition enhances operation performance of the aerobic granular sludge reactor coupled with iron electrolysis. – J. Cleaner Prod. 295: 126394. https://doi.org/10.1016/j.jclepro.2021.126394.

[11] Hallin, S., Lindgren, P. E. (1999): PCR detection of genes encoding nitrite reductase in denitrifying bacteria. – Appl Environ Microbiol. 65(4): 1652-1657. https://doi.org/10.1128/aem.65.4.1652-1657.1999.

[12] He, D., Ren, L., Wu, Q. (2012): Epiphytic bacterial communities on two common submerged macrophytes in Taihu Lake: diversity and host-specificity. – Chin. J. Oceanol. Limnol. 30(2): 237-247. https://doi.org/10.1007/s00343-012-1084-0.

[13] He, D., Ren, L., Wu, Q. L. (2014): Contrasting diversity of epibiotic bacteria and surrounding bacterioplankton of a common submerged macrophyte, Potamogeton crispus, in freshwater lakes. – FEMS Microbiol. Ecol. 90(3): 551-562. DOI: 10.1111/1574-6941.12414.

[14] Hou, L. F., Zhou, Q., Wu, Q. P., Gu, Q. H., Sun, M., Zhang, J. M. (2018): Spatiotemporal changes in bacterial community and microbial activity in a full-scale drinking water treatment plant. – Sci. Total Environ. 625: 449-459. https://doi.org/10.1016/j.scitotenv.2017.12.301.

[15] Jiang, X. L. (2021): Community characteristics and construction mechanism of nitrifying and denitrifying microorganisms in typical wetlands. – Ph.D., Wuhan Botanical Garden, Chinese Academy of Sciences (in Chinese).

[16] Ju, F., Zhang, T. (2015): Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. – ISME J. 9(3): 683-695. 10.1038/ismej.2014.162.

[17] Kraft, B., Strous, M., Tegetmeyer, H. E. (2011): Microbial nitrate respiration—genes, enzymes and environmental distribution. – J. Biotechnol. 155(1): 104-117. https://doi.org/10.1016/j.jbiotec.2010.12.025.

[18] Langfelder, P., Horvath, S. (2008): WGCNA: an R package for weighted correlation network analysis. – BMC Bioinformatics 9: 559. DOI: 10.1186/1471-2105-9-559.

[19] Langfelder, P., Horvath, S. (2012): Fast R functions for robust correlations and hierarchical clustering. – Journal of Statistical Software 46(11): 1-17. http://www.jstatsoft.org/v46/i11/.

[20] Liu, B. B., Zhang, F., Feng, X. X., Liu, Y. D., Yan, X., Zhang, X. J., Wang, L. H., Zhao, L. P. (2006): Thauera and Azoarcus as functionally important genera in a denitrifying quinoline-removal bioreactor as revealed by microbial community structure comparison. –

FEMS Microbiol. Ecol. 55(2): 274-286. https://doi.org/10.1111/j.1574-6941.2005.00033.x.

[21] Liu, J. X., Li, C., Jing, J. H., Zhao, P. Y., Luo, Z. M., Cao, M. W., Ma, Z. Z., Jia, T., Chai, B. F. (2018): Ecological patterns and adaptability of bacterial communities in alkaline copper mine drainage. – Water Res. 133: 99-109. https://doi.org/10.1016/j.watres.2018.01.014.

[22] Liu, S., Hou, J., Suo, C., Chen, J., Liu, X., Fu, R., Wu, F. (2022): Molecular-level composition of dissolved organic matter in distinct trophic states in Chinese lakes: implications for eutrophic lake management and the global carbon cycle. – Water Res. 217: 118438. https://doi.org/10.1016/j.watres.2022.118438.

[23] Liu, Y., Gong, L., Mu, X., Zhang, Z., Zhou, T., Zhang, S. (2020): Characterization and co-occurrence of microbial community in epiphytic biofilms and surface sediments of wetlands with submersed macrophytes. – Sci. Total Environ. 715: 136950. https://doi.org/10.1016/j.scitotenv.2020.136950.

[24] Lu, H. J., Chandran, K., Stensel, D. (2014): Microbial ecology of denitrification in biological wastewater treatment. – Water Res. 64: 237-254. https://doi.org/10.1016/j.watres.2014.06.042.

[25] Ma, Q., Qu, Y., Shen, W., Zhang, Z., Wang, J., Liu, Z., Li, D., Li, H., Zhou, J. (2015): Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-through put sequencing. – Bioresour. Technol. 179: 436-443. https://doi.org/10.1016/j.biortech.2014.12.041.

[26] Mu, X. Y., Lv, X. Y., Liu, W., Qiu, C. H., Ma, Y., Zhang, S. H., Jeppesen, E. (2020): Biofilms attached to Myriophyllum spicatum play a dominant role in nitrogen removal in constructed wetland mesocosms with submersed macrophytes: evidence from N-15 tracking, nitrogen budgets and metagenomics analyses. – Environ. Pollut. 266: 115203. https://doi.org/10.1016/j.envpol.2020.115203.

[27] Pang, S., Zhang, S. H., Lv, X. Y., Han, B., Liu, K. H., Qiu, C. H., Wang, C., Wang, P. F., Toland, H., He, Z. L. (2016): Characterization of bacterial community in biofilm and sediments of wetlands dominated by aquatic macrophytes. – Ecol. Eng. 97: 242-250. https://doi.org/10.1016/j.ecoleng.2016.10.011.

[28] Shi, R. J., Xu, S. M., Qi, Z. H., Huang, H. H., Liang, Q. Y. (2019): Seasonal patterns and environmental drivers of nirS- and nirK-encoding denitrifiers in sediments of Daya Bay, China. – Oceanologia. 61(3): 308-320. https://doi.org/10.1016/j.oceano.2019.01.002.

[29] Shrewsbury, L. H., Smith, J. L., Huggins, D. R., Carpenter-Boggs, L., Reardon, C. L. (2016): Denitrifier abundance has a greater influence on denitrification rates at larger landscape scales but is a lesser driver than environmental variables. – Soil Biol. Biochem.103: 221-231. https://doi.org/10.1016/j.soilbio.2016.08.016.

[30] Song, W., Qi, R., Zhao, L., Xue, N., Wang, L., Yang, Y. (2019): Bacterial community rather than metals shaping metal resistance genes in water, sediment and biofilm in lakes from arid northwestern China. – Environ. Pollut. 254: 113041. https://doi.org/10.1016/j.envpol.2019.113041.

[31] Throbäck, I. N., Enwall, K., Jarvis, Å., Hallin, S. (2004): Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. – FEMS Microbiol. Ecol. 49(3): 401-417. https://doi.org/10.1016/j.femsec.2004.04.011.

[32] Truu, M., Juhanson, J., Truu, J. (2009): Microbial biomass, activity and community composition in constructed wetlands. – Sci. Total Environ. 407(13): 3958-3971. https://doi.org/10.1016/j.scitotenv.2008.11.036.

[33] Wang, S. L., Li, J. S., Zhang, B., Spyrakos, E., Tyler, A. N., Shen, Q., Zhang, F. F., Kuster, T., Lehmann, M. K., Wu, Y. H., Peng, D. L. (2018): Trophic state assessment of global inland waters using a MODIS-derived Forel-Ule index. – Remote Sens Environ 217: 444-460. https://doi.org/10.1016/j.rse.2018.08.026.

[34] Wickham, H. (2016): ggplot2: Elegant Graphics for Data Analysis. – Springer-Verlag, New York.

[35] Yan, D. B., Xia, P. H., Song, X., Lin, T., Cao, H. P. (2019): Community structure and functional diversity of epiphytic bacteria and planktonic bacteria on submerged macrophytes in Caohai Lake, southwest of China. – Ann Microbiol 69(9): 933-944. https://doi.org/10.1007/s13213-019-01485-4.

[36] Yan, L., Zhang, S., Lin, D., Guo, C., Yan, L., Wang, S., He, Z. (2018): Nitrogen loading affects microbes, nitrifiers and denitrifiers attached to submerged macrophyte in constructed wetlands. – Sci. Total Environ. 622-623: 121-126. https://doi.org/10.1016/j.scitotenv.2017.11.234.

[37] Yang, Y. D., Zhao, J., Jiang, Y., Hu, Y. G., Zhang, M. C., Zeng, Z. H. (2017): Response of bacteria harboring nirS and nirK genes to different N fertilization rates in an alkaline northern Chinese soil. – Eur. J. Soil Biol. 82: 1-9. https://doi.org/10.1016/j.ejsobi.2017.05.006.

[38] Yu, Q. L., Zhou, R., Wang, Y. J., Feng, T. S., Li, H. (2020): Corpse decomposition increases nitrogen pollution and alters the succession of nirK-type denitrifying communities in different water types. – Sci. Total Environ. 747: 141472. https://doi.org/10.1016/j.scitotenv.2020.141472.

[39] Yu, Q., Zhou, R., Wang, Y., Su, W., Yang, J., Feng, T., Dou, Y., Li, H. (2021): Carcass decay deteriorates water quality and modifies the nirS denitrifying communities in different degradation stages. – Sci. Total Environ. 785: 147185. https://doi.org/10.1016/j.scitotenv.2021.147185.

[40] Zhang, S., Pang, S., Wang, P., Wang, C., Guo, C., Addo, F. G., Li, Y. (2016): Responses of bacterial community structure and denitrifying bacteria in biofilm to submerged macrophytes and nitrate. – Sci. Rep. 6(1): 36178. https://doi.org/10.1038/srep36178.

[41] Zhang, S. H., Cui, J., Zhang, M., Liu, J. M., Wang, L. X., Zhao, J., Bao, Z. H. (2021): Diversity of active anaerobic ammonium oxidation (ANAMMOX) and nirK-type denitrifying bacteria in macrophyte roots in a eutrophic wetland. – J. Soils Sediments 21: 2465-2473. https://doi.org/10.1007/s11368-021-02926-x.

[42] Zhang, Z., Chen, H., Mu, X., Zhang, S., Pang, S., Ohore, O. E. (2020): Nitrate application decreased microbial biodiversity but stimulated denitrifiers in epiphytic biofilms on Ceratophyllum demersum. – J. Environ. Manage. 269: 110814. https://doi.org/10.1016/j.jenvman.2020.110814.

[43] Zhao, D., Shen, F., Zeng, J., Huang, R., Yu, Z., Wu, Q. L. (2016): Network analysis reveals seasonal variation of co-occurrence correlations between Cyanobacteria and other bacterioplankton. – Sci. Total Environ. 573: 817-825. https://doi.org/10.1016/j.scitotenv.2016.08.150.

[44] Zheng, Y. L., Hou, L. J., Liu, M., Gao, J., Yin, G. Y., Li, X. F., Deng, F. Y., Lin, X. B., Jiang, X. F., Chen, F., Zong, H. B., Zhou, J. L. (2015): Diversity, abundance, and distribution of nirS-harboring denitrifiers in intertidal sediments of the Yangtze estuary. – Microb. Ecol. 70(1): 30-40. https://doi.org/10.1007/s00248-015-0567-x.

[45] Zhong, X. Z., Zeng, Y., Wang, S. P., Sun, Z. Y., Tang, Y. Q., Kida, K. (2020): Insight into the microbiology of nitrogen cycle in the dairy manure composting process revealed by combining high-throughput sequencing and quantitative PCR. – Bioresour. Technol. 301: 122760. https://doi.org/10.1016/j.biortech.2020.122760.

[46] Zhou, J. R., Sun, H. D., Ali, A., Rott, P. C., Javed, T., Fu, H. Y., Gao, S. J. (2021): Quantitative proteomic analysis of the sugarcane defense responses incited by Acidovorax avenae subsp. avenae causing red stripe. – Ind. Crops Prod. 162: 113275. https://doi.org/10.1016/j.indcrop.2021.113275.

[47] Zhou, S. L., Huang, T. L., Zhang, C. H., Fang, K. K., Xia, C., Bai, S. Y., Zeng, M. Z., Qiu, X. P. (2016): Illumina MiSeq sequencing reveals the community composition of NirS-type

and NirK-type denitrifiers in Zhoucun reservoir—a large shallow eutrophic reservoir in northern China. – RSC Advances 6(94): 91517-91528. https://doi.org/10.1039/c6ra18017e.

[48] Zhou, S. L., Sun, Y., Zhang, Y. R., Huang, T. L., Zhou, Z. Z., Li, Y., Li, Z. X. (2020): Pollutant removal performance and microbial enhancement mechanism by water-lifting and aeration technology in a drinking water reservoir ecosystem. – Sci. Total Environ. 709: 135848. https://doi.org/10.1016/j.scitotenv.2019.135848.

[49] Zhou, X., Jin, W. B., Sun, C. F., Gao, S. H., Chen, C., Wang, Q., Han, S. F., Tu, R. J., Latif M. A., Wang, Q. L. (2018): Microbial degradation of, N,N-dimethylformamide by Paracoccus sp strain DMF-3 from activated sludge. – Chem. Eng. J. 343: 324-330. https://doi.org/10.1016/j.cej.2018.03.023.

[50] Zumft, W. G. (1997): Cell biology and molecular basis of denitrification. – Microbiol. Mol. Biol. Rev. 61: 553-616. https://doi.org/10.1016/j.ccr.2004.08.030.

APPENDIX

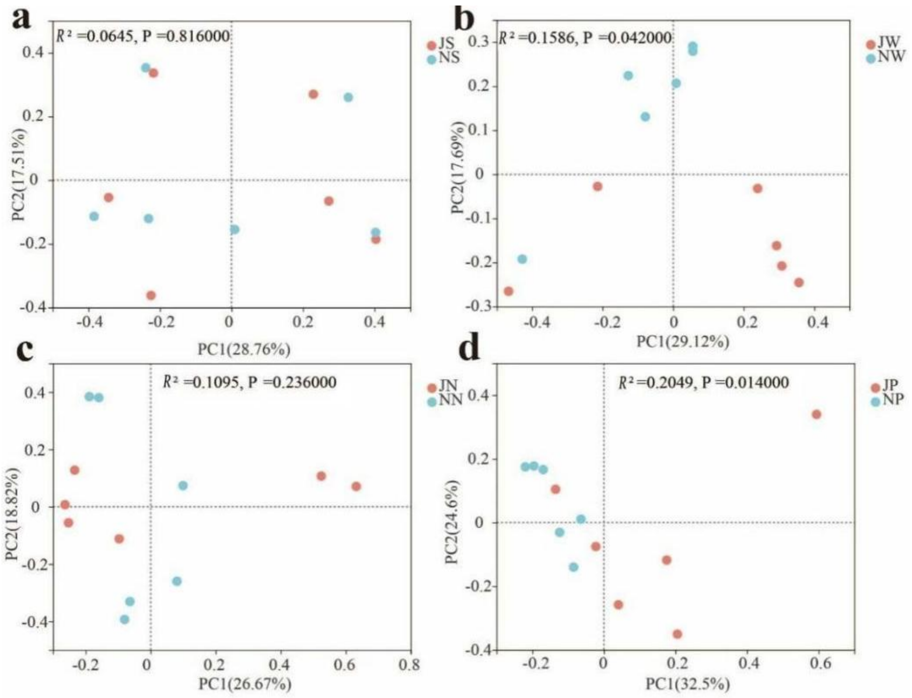

Figure A1. *PCoA analysis of nirS-type denitrifying bacteria*

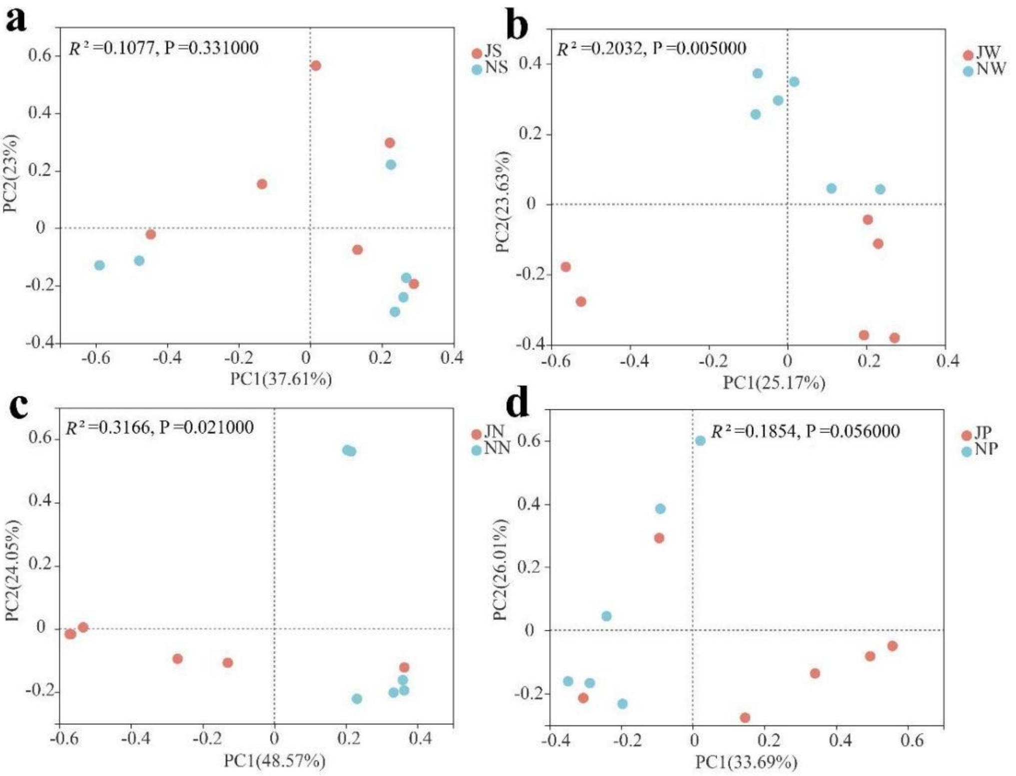

Figure A2. *PCoA analysis of nirK-type denitrifying bacteria*