

DIFFERENCES IN DENITRIFYING COMMUNITY STRUCTURE OF THE NITROUS OXIDE REDUCTASE (*nosZ I*) TYPE GENE IN A MULTI-STAGE SURFACE CONSTRUCTED WETLAND

ZHOU, J.^{1*#} – SHA, W. L.^{1#} – YU, G. H.² – ZHANG, N. X.¹ – SHU, F. Y.¹ – GONG, Z. J.¹ – KONG, Y.¹

¹*School of Life Sciences, Qufu Normal University, Jining 273165, China*

²*Institute of Dry Farming, Institute of Dry land Farming, Hebei Academy of Agriculture and Forestry Sciences, Hengshui 053000, China*

[#]*Co-first authors*

^{*}*Corresponding author*
e-mail: jingzhou-2004@163.com

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Abstract. Nitrous oxide (N₂O)-reducing microorganisms with the capacity to reduce the greenhouse gas N₂O to harmless dinitrogen gas are affected by environmental conditions. We applied pyrosequencing of the *nosZ* clade I gene to investigate denitrifying communities in a restoring constructed wetland ecosystem in China. We collected sediments from an ecological retention pond (0) and a five-stage (I, II, III, IV, and V) surface-flow constructed wetland. Content of ammonium-nitrogen (N), nitrate-N, available phosphorus, total N, organic matter, and available potassium as well as pH value in the sediment decreased in the last stage (i.e., V). Richness and diversity of *nosZ* clade I communities in stage V were slightly higher than those of other stages. The *nosZ* clade I communities in stage IV and V samples were clearly distinguished from the other four samples. The proportion of Actinobacteria and Chloroflexi was lower in wetlands than in the ecological retention pond. *Bradyrhizobium* was significantly lower in all five stages (17.9–37.8%) than in the ecological retention pond (44.7%). Of 32 abundant operational taxonomic units, 22 were significantly correlated with more than at least one of the measured physicochemical properties. Our results are essential for understanding the interrelationship between N-related microbial distribution and the pollution status of wetlands for the evaluation of constructed wetlands.

Keywords: *N₂O-reducing microorganisms, nosZ I abundance, Artificial Wetlands, ecological retention, water quality*

Introduction

As the decomposers of ecosystems, including the biosphere, microbial composition and function are closely interrelated with the surrounding environment (Cao et al., 2017), including in wetland sediments (Overbeek et al., 2019). The reducing conditions in wetland sediments favor microbial respiratory processes that use alternate electron acceptors, such as denitrification, which contribute to nitrogen (N) removal in a variety of environments (Lansdown et al., 2016; Cheng et al., 2020). In the absence of oxygen in wetlands, denitrifiers can sequentially respire nitrate (NO₃⁻) to nitrite (NO₂⁻), nitric oxide (NO), nitrous oxide (N₂O), and ultimately to dinitrogen gas (N₂) (Zhang et al., 2019). Novel N₂O-reducing microorganisms with the capacity to reduce the third most important anthropogenic greenhouse gas N₂O (Gao et al., 2021) to harmless N₂ gas are receiving increased attention due to rising N₂O emissions (Hallin et al., 2018; Gao et al., 2021). The *nosZ* gene, encodes N₂O reductase, which is related to the reduction of N₂O to N₂, and is a suitable marker for investigating the diversity and composition of the N₂O-reducer community (Li et al., 2020). A recent important discovery showed that *nosZ* phylogeny has two major clades, I and II (Conthe et al., 2018). Denitrifiers are present in both clades, but organisms in clade I, which is dominated by Proteobacteria

under all conditions (Miguel et al., 2020), were more likely to be complete denitrifiers, while a large fraction of organisms with clade II *nosZ* lacked the genetic capacity to produce N₂O (Juhanson et al., 2017). Community composition of denitrifiers within clade I could influence in situ net N₂O fluxes (Hallin et al., 2018), because these denitrifiers reduce N₂O at different rates (Wang et al., 2020).

Nansi Lake, with a total surface area of 1.2×10^3 km², is one of the largest shallow freshwater lakes (Zhang et al., 2011), and an important storage reservoir and regulation works for the South-to-North Water Transfer Project of China (Tian et al., 2013). It is becoming the main freshwater fishery production base in Shandong due to its fertile water and large lake surface (Wang et al., 2014; Zhu et al., 2015). However, this lake has been polluted by inefficiently treated effluent from industrial and municipal activities, runoff from mining sites and agriculture land and deposition of air pollutants since the 1970s (Wang et al., 2012a, 2014). Accordingly, its water quality is poor. In 2002, water quality was inferior to Class V, which refers to the categories of “polluted” and “dirty” (Kondrateva et al., 2009), according to the “China surface water quality standard (GB3838–2002)” and U.S. Environmental Protection Agency, and the chemical oxygen demand reached thousands of milligrams per liter (Wang et al., 2016).

When the eastern route of the South-To-North Water Diversion Project was completed, the government started to take measures to improve water quality, one of which was constructed wetlands (CWs). The CWs are widely used, cost-effective, and energy-efficient solutions for purifying water, and enhance a watershed’s environmental capacity to self-defend from pollution (Wang et al., 2016; Rajan et al., 2018). The “Artificial Wetlands Demonstration Project” has been widely applied in the whole basin and in an area of 120 km² in the estuaries of the Xinxue River from 2005, and has been shown to be effective in improving the water quality of rivers flowing into the lake and increasing the environmental capacity of the entire lake (Cao et al., 2015). However, the effects of pH on denitrifier community richness and diversity in sediment remain unclear. Our previous research confirmed that there was abundant bacterial population diversity in the sediments of Nansi Lake, and the bacterial population diversity is positively correlated with the total phosphorus (P) content in the environment. Among these, relative abundance of the genus *Devosia* in the class α -Proteobacteria is reduced. Most microorganisms in this group are N-fixing, and are functional in the N cycle (Lu, 2013). However, the shift in the N₂O-reducer community after ecological restoration remains unclear.

The wetland effluent is collected by the tail water collection channel and flows into Nansi Lake. This CW is a flow-through system, and the water flowing into the wetland originates from the adjacent Xinxue River. According to the landform characteristics of Xinxue River Estuary, the process scheme of the demonstration project is determined as follows: ecological detention pond (0) + multistage (I, II, III, IV, and V) surface flow CW system. The water plants in the wetland are mainly *Arundo donax* and *Phragmites australis*, the floating leaf plants are *Nymphoides peltatum*, and the submerged plants are *Potamogeton crispus*. The CW has achieved good results, and its pollutant removal effect is obvious. The standard rate of wetland effluent is 80%, and water quality is class III, which refers to the category of “moderately polluted” (Zhang et al., 2014). In this study, we collected sediments from the Xinxue Estuary CW water purification project, including 0 + five-stages CW, to investigate the changes in denitrification communities. The plan layout of Xinxue River CW is shown in *Figure A1* in the *Appendix*. We hypothesized that composition of denitrifiers would be affected by CW; such a shift may directly or indirectly derive from a shift in pH.

Materials and methods

Site description

The Xinxue River wetland (34°45'N, 117° 09'E) was built in 2005 (Zhang et al., 2008) and is located in the flood discharge area on the west side of the embankment at the entrance of Xinxue River into Nansi Lake, Shandong Province, China (Fig. A1). The Xinxue River is intercepted and stored in a rubber-lined dam, enters the diversion channel through the metering system, and successively enters the ecological detention pond and surface flow CW through the water distribution channel. The dam is 170 m long and the dam crest elevation is 33.55 m. The water level of the river is raised by the rubber dam to form a river storage pond. The river water is pretreated here to remove some suspended substances through physical sedimentation and then passes through to the river. The photosynthesis of aquatic plants in the channel pre-oxygenates the river water to reduce some pollutants. The river water raised by the rubber dam enters the ecological detention pond (0) through the diversion channel. The diversion channel has the form of a trapezoidal artificial open earth channel, with a length of about 250 m and a slope of 0.02%. At 300 m downstream of the rubber dam, the ecological detention pond was constructed using the low-lying beach and pond inside the embankment, with an area of about 333,350 m², a length of 1400 m, a width of 120–360 m, and a water level elevation of 32.75 m.

Totally, six surface sediment layers of the ecological retention pond (0) and five stages (I–V) of sampling stations were selected in September in 2018. Each station had three sampling sites at intervals of 20 m. Thus, a total of 18 surface sediment samples were collected in an icebox (4 °C) and transported back to the laboratory. One part of each sample was immediately put in a refrigerator at –80 °C for later microbial investigation, and the other part was for nutrient determination.

Physicochemical properties

Sediment samples were air-dried for 1 week at room temperature and sieved through a 1-mm screen. The sediment monitoring items were pH, NO₃⁻, ammonium (NH₄⁺), total N (TN), available P (AP), available potassium (AK), and organic matter (OM), measured by the Analysis and Testing Center in the Chinese Academy of Agricultural Sciences (IEDA, CAAS, Beijing, China) according to Chinese national standard assay methods (pH: GB 7859-1987; N: GB 7173-1987, AP: GB 7853-1987, AK: GB 7856-1987; OM: GB 9834-1988).

Pyrosequencing of nosZ clade I gene

The process of DNA extraction from 1 g of sediment (Enwall et al., 2010) was performed using a Fast DNA SPIN Kit (MP Biomedicals, Solon, OH, USA) and primers *nosZ1840-F/nosZ2090-R* (Henry et al., 2006) were used to amplify the *nosZ* clade I gene by touchdown PCR using TransGene high-fidelity enzyme. The PCR process follows: 95 °C predenaturation for 5 min, 94 °C for 30 s, annealing at 55 °C for 35 s, 72 °C for 30 s, with a total of five cycles; 94 °C for 30 s, annealing at 55–51 °C for 35 s, extending at 72 °C for 30 s, with a total of 25 cycles. The amplified PCR products were sequenced on an Illumina MiSeq PE 2 x 250 platform at BeiJing Fixgene Co., Ltd (Beijing, China). The obtained raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under accession PRJNA734135.

Bioinformatic analysis

Adaptor, barcode, and primer sequences were filtered from the raw data. The pair-end reads were then joined according to their overlapping sequences using FLASH (v1.2.7) with the properties set to a shortest length of 10 bp (Peng et al., 2019) and a maximum mismatch ratio of 0.3. Then, the remaining sequences were converted to amino acid sequences using the FunGene Pipeline of the Ribosomal Database Project according to Penton et al. (2015). The sequences encoding proteins that contained termination codons or that did not match the *nosZ* clade I protein sequence were removed (Zhou et al., 2021). High-quality sequences obtained through the above processing were rarefied and subsampled based on the lowest number of reads in a sample for subsequent alpha and beta analysis (Zhou et al., 2021). Operational taxonomic units (OTUs) were classified with a similarity of 97%. The classification system from the FunGene pipeline database was used (Penton et al., 2015), and the blastn method was used for annotation (Hou et al., 2012).

Statistical analysis

The least significant difference test was used to identify the differences in sediment physicochemical properties, absolute *nosZ* I gene abundance, and alpha diversity among the six sediments at $P < 0.05$ (Liang et al., 2020), using IBM Statistic SPSS 21. A non-metric multidimensional scaling (NMDS) and hierarchical clustering based on pairwise Bray–Curtis dissimilarities was performed to determine the relationship between individual sediment samples using PAST (version 3.01, folk.uio.no/ohammer/past/). Permutational MANOVA (PERMANOVA) using Bray–Curtis dissimilarity was used to compare differences in *nosZ* clade I community composition among samples.

We analyzed *nosZ* clade I taxa at the genus level to identify, which were different among the six sediment samples. We identified genera whose proportions significantly differed ($P < 0.05$). We use the term ‘X-enriched taxon’ to refer to a genus found in significantly higher proportion in that group compared to the other group (Zhou and Fong, 2021). A phylogenetic tree was inferred for these enriched genera using the neighbor-joining method in MEGA and displayed using the iTOL (Interactive Tree of Life, <https://itol.embl.de/>) (Wei et al., 2020; Zhou and Fong, 2021), together with data on genera average relative abundance. We identified all observed genera whose proportions significantly differed (FDR-corrected, $P < 0.05$, Wilcoxon rank-sum test) for 0 and V. Additionally, Pearson’s correlation between ‘X-enriched taxon’ and physicochemical properties was determined using IBM Statistic SPSS 21 and visualized with Origin Pro, 2019b. Redundancy analysis (RDA) was used to explain the relative effects of soil properties on the microbial community (at OTU level) using CANOCO 5.0 (Zhou, 2016).

Results

Sequencing results

In analysis of the 13 sediment samples, we obtained a total of 2,571,816 raw *nosZ* clade I sequences (average 142,878 per sample, *Table A1* in the *Appendix*). The Good’s coverage values at 97% cutoff similarity were 98.6–99.6% (*Table A1*), indicating that the current numbers of sequence reads were sufficient to capture the *nosZ* clade I diversity in these samples. High-quality sequences were rarefied and subsampled to 24,000 reads per sample, leaving 6450 OTUs for alpha and beta diversity analysis.

Alpha diversity of *nosZ* clade I communities in relation to physicochemical properties

Physicochemical properties in sediments significantly differed (Table 1). Sediment $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, AP, TN, OM, and AK contents, and pH value in the last stage (V) decreased significantly ($P < 0.05$) compared to the ecological retention pond. These properties decreased from stage I to V. Richness (Chao and ACE indices, Fig. 1B and A, respectively) and diversity (Shannon index, Fig. 1D) of *nosZ* clade I communities in stage V were slightly, but non-significantly, higher than in the ecological retention pond and other stages. The pH value and NO_3^- content were negatively and significantly correlated with ACE and Chao ($P < 0.05$, Table 2).

Table 1. Physicochemical properties of sediments in Nansi Lake

Stage	pH	NO_3^- (mg/kg)	NH_4^+ (mg/kg)	AP (mg/kg)	AK (mg/kg)	TN (%)	OM (%)
0	7.7 ± 0.4cd	6.7 ± 0.1c	20 ± 0.1c	19.7 ± 0.1d	625.6 ± 0.4e	0.5 ± 0.02e	10.8 ± 0.4d
I	7.7 ± 0.3d	5.9 ± 0.1bc	13.3 ± 1.1b	13.6 ± 0.3c	327.9 ± 1.7d	0.3 ± 0.01d	6.4 ± 0.1c
II	7.6 ± 0.2bc	5.1 ± 0.1b	12.5 ± 1.5b	11.1 ± 0.2b	201 ± 1.3a	0.2 ± 0.01c	4.7 ± 0.2b
III	7.5 ± 0.1b	4.9 ± 0.1b	11.7 ± 0.5a	10.7 ± 0.5b	282.6 ± 2.8c	0.2 ± 0.03b	2.5 ± 0.3a
IV	7.5 ± 0.2b	4.5 ± 0.1b	9.4 ± 0.4a	7.5 ± 0.3a	273.1 ± 1.6b	0.2 ± 0.01ab	2.4 ± 0.1a
V	7.2 ± 0.1a	2.3 ± 0.4a	7.7 ± 1.3a	6.7 ± 1.0a	201 ± 1.3a	0.2 ± 0.01ab	2.2 ± 0.1a

Values are the mean ± standard deviation (n = 3). Values within the same column followed by a different letter indicate a significant difference ($P < 0.05$)

AP, available phosphorus; AK, available potassium; TN, total nitrogen; OM, organic matter

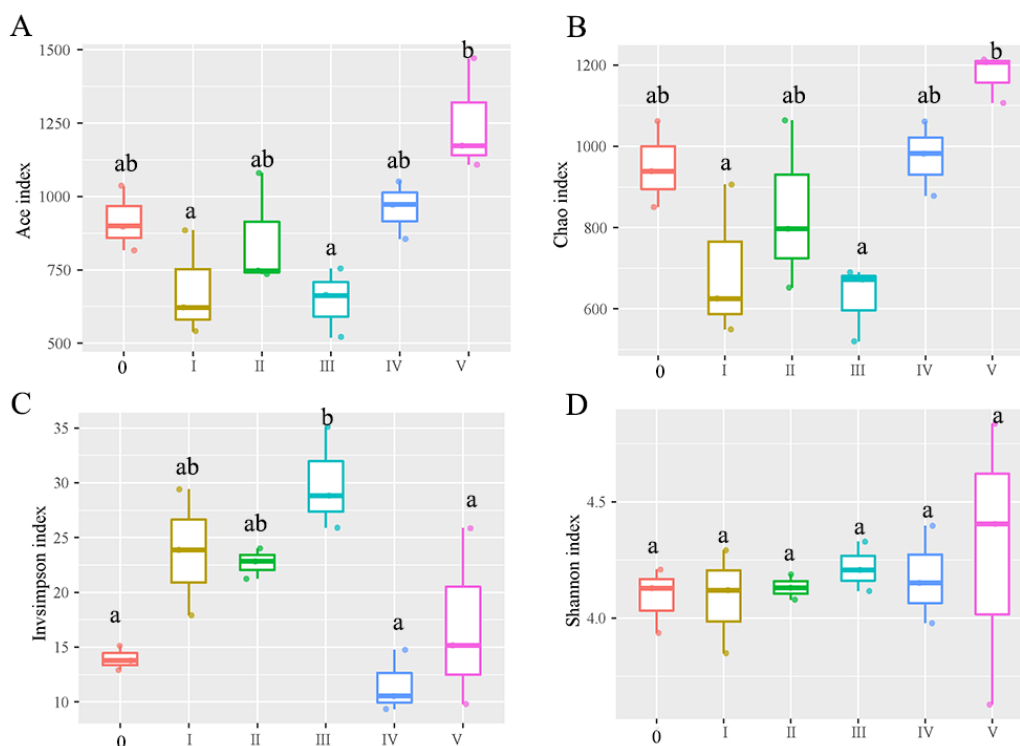


Figure 1. Alpha diversity indices of *nosZ* clade I communities

Table 2. Pearson correlation between physicochemical properties and alpha diversity of *nosZ* clade I communities

	pH	NO ₃ ⁻	NH ₄ ⁺	AP ¹	AK ²	TN ³	OM ⁴
Ace	-0.675**	-0.742**	-0.143	-0.327	-0.127	-0.124	-0.165
Invsimpson	0.239	0.358	-0.231	-0.028	-0.276	-0.209	-0.188
Shannon	-0.098	-0.137	-0.176	-0.180	-0.174	-0.191	-0.238
Chao	-0.563*	-0.708**	-0.018	-0.227	-0.027	-0.030	-0.077

* $P < 0.05$; ** $P < 0.01$

AP, available phosphorus; AK, available potassium; TN, total nitrogen; OM, organic matter

Beta diversity

Hierarchical clustering (at OTU level) revealed that *nosZ* clade I communities in IV and V samples were clearly distinguished from the other four samples, which were divided into two groups: I and III; and 0 and II (PERMANOVA, $P = 0.001$; Fig. 2A). Similarly, based on NMDS results, all 18 samples were separated into three groups: IV and V (red ellipse); 0 and II (green ellipse); and I and III (brown ellipse) ($P < 0.05$, Fig. 2B).

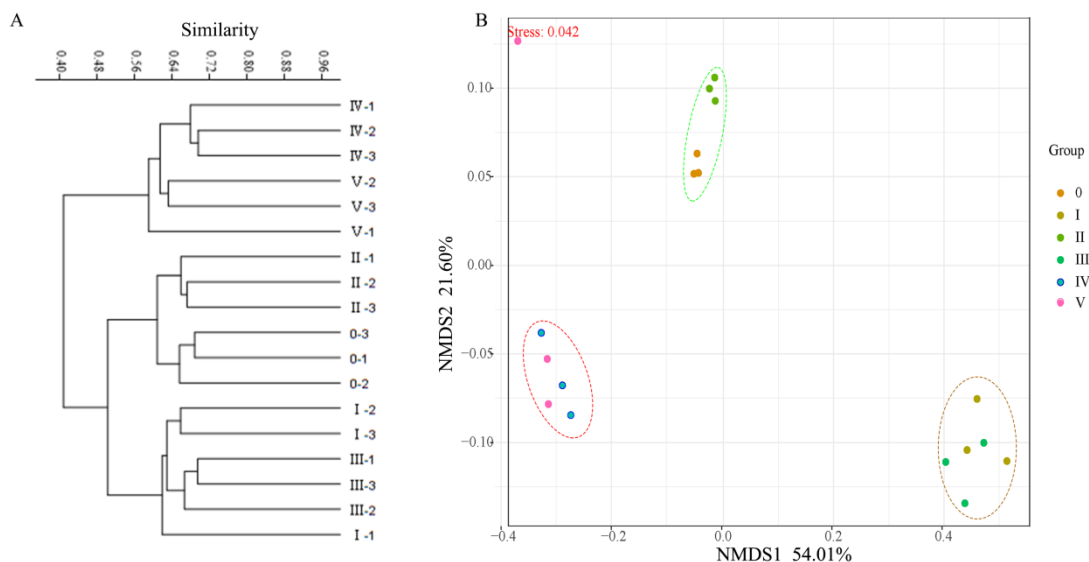


Figure 2. Hierarchical clustering and NMDS of *nosZ* clade I communities based on all samples

Taxa significantly different among samples

The sequence affiliated with the phylum Proteobacteria occupied the highest proportions (81.3–92.7%) of the *nosZ* sequences in wetland sediments, followed by Terrabacteria (0.02–0.5%) (Table A2). At the class level, different wetland stages had significantly different relative abundances of classes Alphaproteobacteria, Deltaproteobacteria, Actinobacteria, and Chloroflexi rather than Betaproteobacteria and Gammaproteobacteria. Notably, the proportion of Actinobacteria was lower in IV and V than in 0, I, II, and III. Similarly, the proportion of Chloroflexi was lower in III, IV, and V than in 0, I, and II (Table A2).

At the family level, Bradyrhizobiaceae, which was the most abundant group, was lower in all five stages than in the ecological retention pond (*Fig. 3A*). However, the percentages of Rhizobiaceae, Burkholderiaceae, Gemmatimonadales, Brucellaceae, and Cytophagia were higher in all stages than in the ecological retention pond (*Fig. 3B–F*, respectively).

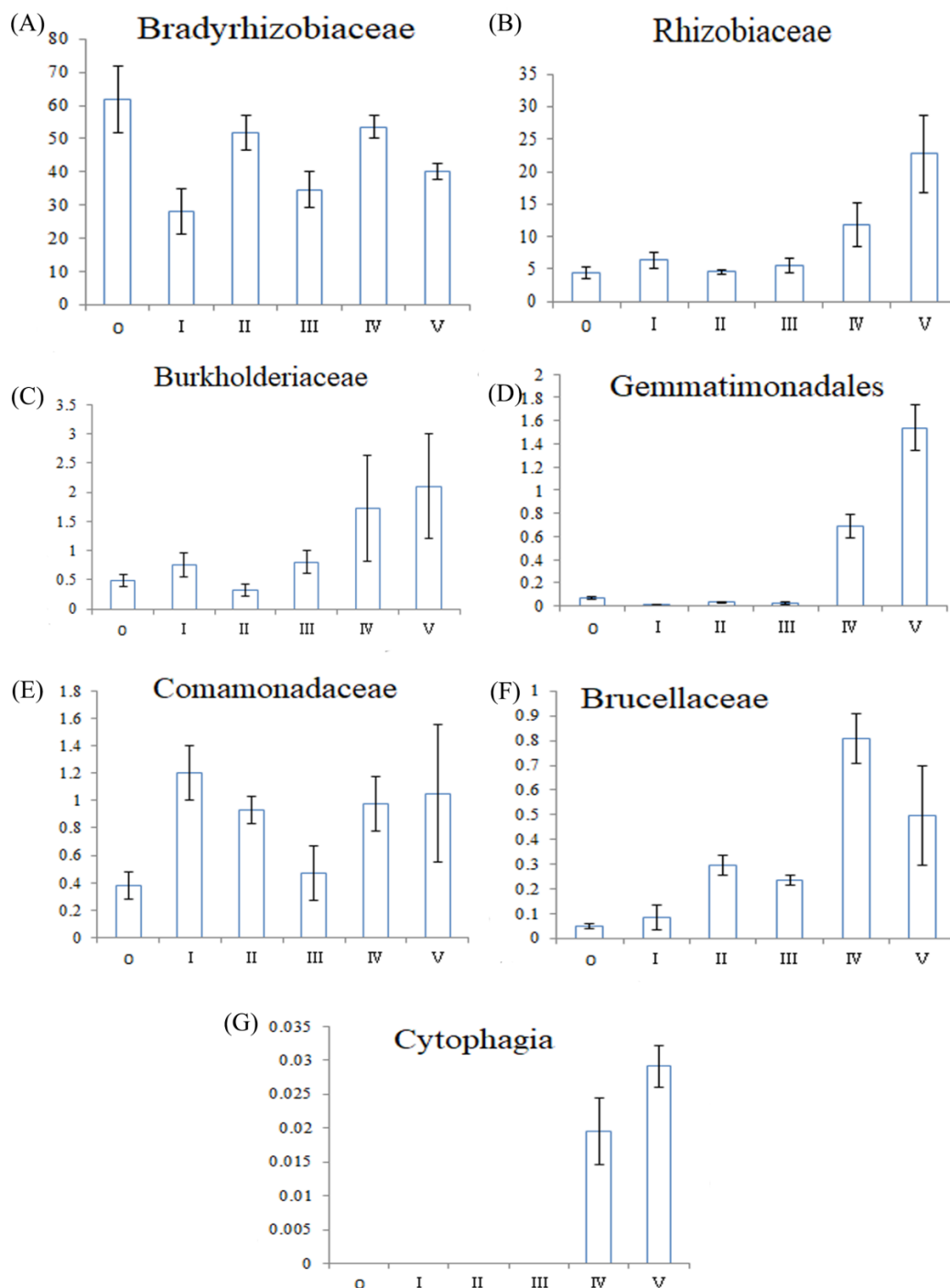


Figure 3. Relative average abundances of the six most abundant families for different wetland stages. Error bars indicate the standard deviation of relative abundance between three replicate samples. The numbers on the ordinate are relative average abundances (%) of microbial families

A total of 32 OTUs significantly differed among the six sediment samples (Fig. 4; Table A2, $P < 0.05$). Among them, 30 OTUs belong to Proteobacteria, with 19 classified as Alphaproteobacteria, eight as Betaproteobacteria, and four as Gammaproteobacteria. Only OTU80 was affiliated with Actinobacteria, and OTU200 with Gemmatimonadetes (Fig. 4). The top two most abundant *nosZ* clade I genera were *Bradyrhizobium* and *Mesorhizobium*, with proportion ranges of 17.94–44.74% and 1.72–17.30%, respectively (Table A3).

For the most abundant genus *Bradyrhizobium*, abundance was significantly ($P < 0.05$) lower in all five stages (17.9–37.8%) than in the ecological retention pond (44.7%) (Table A3). *Nitrospirillum*, *Paracoccus*, Nocardioideae, *Oligotropha*, and *Ralstonia* showed the same trend as *Bradyrhizobium*. However, we observed significantly ($P < 0.05$) higher proportions of genera *Rhizobium* and *Polyangium* in all five stages than in the ecological retention pond (Table A4).

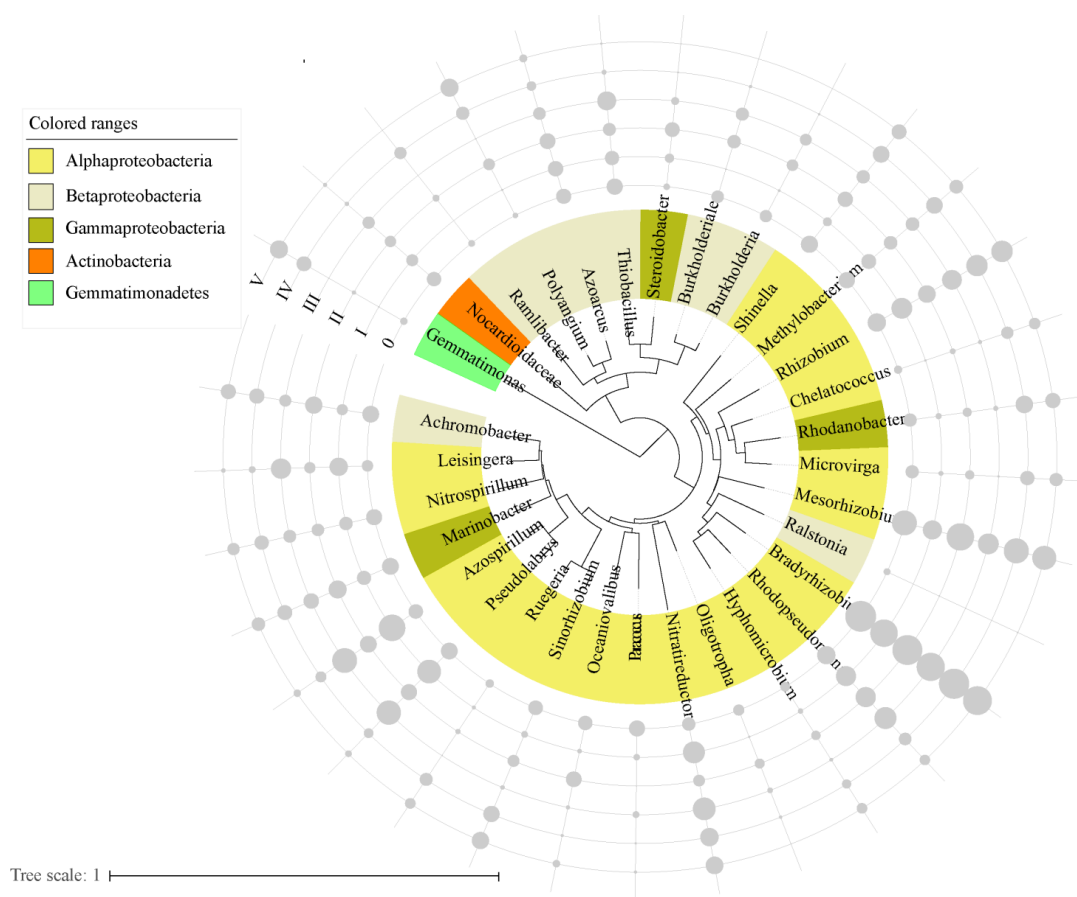


Figure 4. Top OTU members of the *nosZ I* community enriched in wetland stages and the ecological retention pond. Gray circles represent the relative abundance of each OTU. Taxonomic dendrogram shows the inferred evolutionary relationship of the enriched microbiota of each sample. Total relative abundances of all OTUs and significant effects across soil compartments are listed in Table A3

Correlation between physicochemical properties and *nosZ I* community

Of the 32 abundant OTUs, 22 were significantly correlated with more than at least one of the physicochemical properties (Fig. 5). *Ralstonia*, *Paracoccus*, *Oligotropha*,

Nocardioidaceae, and *Nitrospirillum* were positively and significantly related with sediment contents of NH_4^+ , AP, AK, TN, and OM. Of these 22 taxa, 10 had significant correlations with sediment content of NO_3^- , in which *Azospirillum*, *Rhodopseudomonas*, *Pseudolabrys*, *Nitratireductor*, and *Leisingera* were positively correlated and *Hyphomicrobium*, Polyangium, Gemmatimonadaceae, and *Sinorhizobium_Ensifer* were negatively correlated (Fig. 5; Table A5).

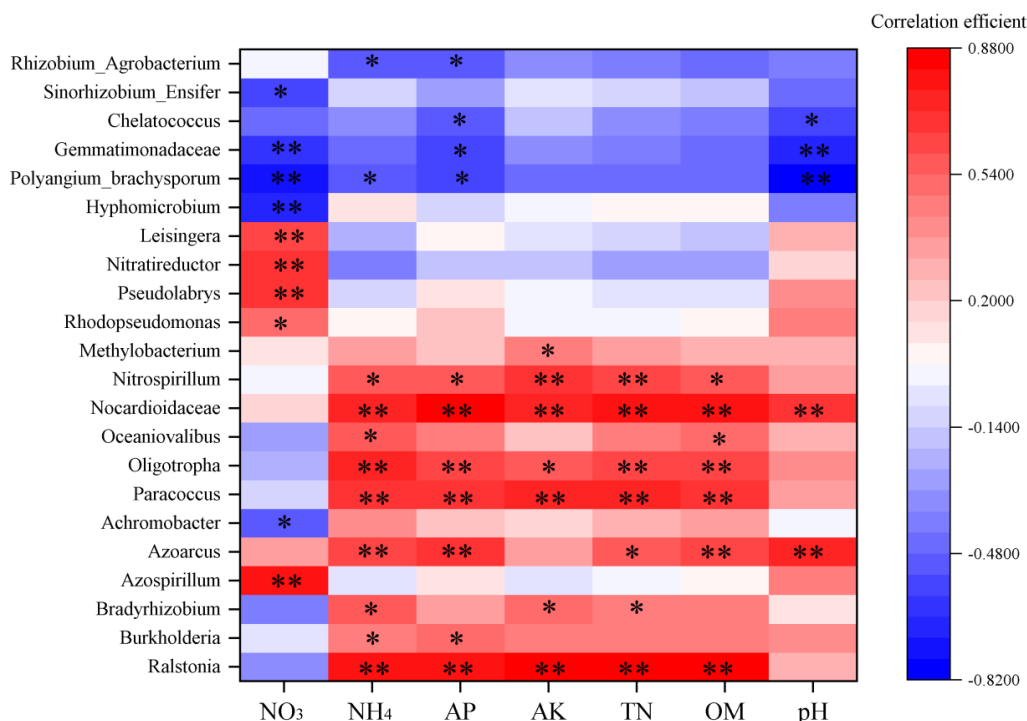


Figure 5. Pearson correlations between 'X-enriched taxon' and physicochemical properties.
* $P < 0.05$, ** $P < 0.01$

The RDA of physicochemical properties is shown in Figure 6. Seven properties explained 67.5% of the total variation of *nosZ I* communities. Sediment content of NO_3^- (explaining 24.2%, $P = 0.002$) was the most important factor shaping *nosZ I* communities, followed by pH (explaining 13.2%, $P = 0.004$) and NH_4^+ (explaining 9.6%, $P = 0.004$) (Table A5). All *nosZ I* communities in 18 samples were separated on the first axis into two groups: 0, I, II, and III (blue ellipse); and IV and V (green ellipse) (Fig. 6).

Discussion

Sediment physicochemical properties and adsorption

The obvious decrease in sediment content of NH_4^+ , NO_3^- , AP, TN, and OM can be attributed to the absorption of these substances as nutrients by the four plants in CWs. *Arundo donax*, which favors high absorption and a high level of macronutrient translocation, has the ability to remove a greater quantity of nutrients per unit area in the pilot system (Leto et al., 2013). *Phragmites australis* was reported to remove $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, TN, and total P (Tomoko et al., 2002). *Nymphoides peltatum* and *Potamogeton crispus* (Li et al., 2008) have the potential to absorb $\text{NH}_4^+\text{-N}$ and total P, respectively.

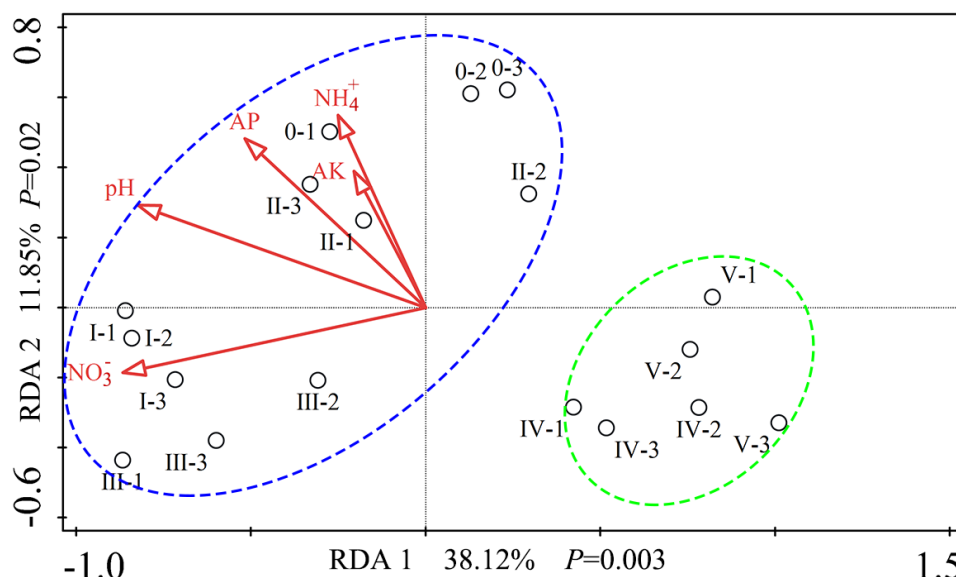


Figure 6. RDA results between physicochemical properties and *nosZ* I community at OTU level

The limited decline in pH value in wetland systems has been speculated to be due to the production of CO₂ resulting from decomposition of carbohydrates (i.e., plant residues), removal of various components of the wastewater retained in the plant root area (Leto et al., 2013), and nitrification of ammonia (Levinik-Hfferle et al., 2012). Studies showed that most horizontal subsurface-flow CW systems buffer wastewater, which is slightly acid or basic at the inflow and bring the pH values back to near neutral in an experimental site (Leto et al., 2013) and a pilot plant (Mitsch, 1997).

Richness and diversity of nosZ-denitrifier community

The slight but non-significant increase in alpha diversity indices of the *nosZ*-denitrifier community in the last stage of multi-stage CWs indicated linkage among the microbial community, treatment performance, and design of the CWs. Chen et al. (2020) stated that there was a negative relationship between the richness of denitrifying bacteria and the contents of NH₄⁺-N and TN, but no such relation was detected in our study. We observed that richness indices were significantly and negatively related to sediment pH, consistent with results of Szukics et al. (2010). In contrast, Li et al. (2020) showed that pH was positively correlated with richness of *nosZ*-encoding denitrifier in mangrove wetlands.

Jha (2015) reported that bacterial denitrifier gene abundance was correlated with NO₃⁻ content in soils. We found a negative correlation between *nosZ*-denitrifier richness and NO₃⁻, and soil NO₃⁻ was the most important single predictor of denitrification potential (Xiong et al., 2017). The above three results showed that NO₃⁻ has a very important effect on denitrifying processes.

nosZ-denitrifier community composition

The hierarchical clustering showed that the *nosZ*-denitrifying flora in sediments of stages IV and V significantly differed from the other four.

Proteobacteria was the predominant phylum in all sediment samples. The abundances of Deltaproteobacteria – which are mainly involved in sulfate reduction (Wang et al., 2012b), can fix atmospheric nitrogen, and many denitrify (Lower and Bazylinski, 2013) in anaerobic conditions – were higher in stages IV and V. The most abundant genus *Bradyrhizobium* (within order Rhizobiales and class Alphaproteobacteria), which can act as sinks or sources for N₂O (Tian et al., 2020), has been reported to denitrify. Many *Bradyrhizobium* strains have truncated denitrification pathways lacking one or more of the steps, and all N₂O-reducing *Bradyrhizobium* strains prefer N₂O to NO₃⁻ as an electron acceptor (Gao et al., 2021). The proportion of *Bradyrhizobium* decreased in sediments of CWs compared to those in the ecological retention pond (Fig. 4; Table A3), indicating that the lower levels of N (NO₃⁻, NH₄⁺, and TN) to be metabolized in the sediment reduced the demand for *Bradyrhizobium* after purification of the CAWs.

Brucellaceae are Gram-negative bacteria that cause brucellosis, one of the most distributed zoonoses worldwide, and are transmitted to humans by contact with either infected animals or their products (Casabuono et al., 2017). Rhizobiaceae strains are reported to utilize a wide range of organic substrates, such as aromatic constituents present in polyphenols and lignin (Rich, 2003). However, Brucellaceae and Rhizobiaceae in Rhizobiales showed increased relative abundance in CAWs.

The obvious decrease in relative abundance of Nocardioideae (in phylum Actinobacteria; Fig. 4) in stages IV and V, which are all active in the degradation of recalcitrant chemicals, may indicate a reduction in the content of chemical pollutants (Zhang et al., 2020) in the water and sediment after the depuration by CWs. Whether this assumption is correct requires further verification.

Linking soil physical and chemical properties to nosZ- denitrifier communities

Soil pH has been shown to be important in the denitrifying bacterial community in pristine forest (Szukics et al., 2010) and wetland (Bowen et al., 2020) soils. The sediment pH was also found to be closely correlated with *nosZ*-denitrifiers in CW in this study in the following four ways. First, we found that water in the last stage (i.e., V) was purified in this experiment, resulting in a lower pH in sediment than for the other five groups. This may be the reason for the obvious reduction of alkalinity in stage V that led to the non-significant increase in diversity of denitrifying bacteria (Fig. 2D). Second, the negative correlation between pH and richness indices (ACE and Chao; Table 2) also confirmed that the decrease of pH increased the abundance index of denitrifying bacteria. Third, pH was significantly related to Nocardioideae, *Azoarcus*, *Chelatococcus*, Gemmatimonadaceae, and *Polyangium_brachysporum* (Fig. 5). Fourth, pH was the second most important factor ($P = 0.004$; Table A5) shaping *nosZ* community structure.

Nitrate pollution is responsible for algal blooms and eutrophication, which also pose public health risks (Beman et al., 2005) and are recognized as vital environmental threats (Tilman et al., 2002) by researchers and agricultural policymakers (Kramer et al., 2006). Kramer et al. (2006) showed the important link between reduced NO₃⁻ leaching and enhanced denitrifier activity and efficiency. In this study, we also found that NO₃⁻ content was negatively related to *nosZ*-denitrifier communities including richness indices (ACE and Chao, Table 2) and community structure ($P = 0.002$, Table A5). Additionally, 10 out of 22 OTUs were correlated with NO₃⁻ content (Fig. 5). However, Li et al. (2015) concluded that NO₃⁻ positively affected the *nirK* and *nirS*

denitrifier community distribution in sediments of a *Myriophyllum elatinoides* purification system for treating swine wastewater.

Chen et al. (2020) confirmed that N salts (NH_4^+ , NO_2^- , NO_3^- , and TN) are important factors affecting the composition and distribution of *nosZ*-denitrifiers in samples. This study is consistent with our results, confirming that the reduced N content in water changed the composition of denitrifying bacteria, i.e., the adaptation mechanism of bacteria in different habitats was associated with different environmental factors.

Conclusion

In this study, we investigated the denitrifying community structure of the *nosZ* clade I in a multi-stage surface-flow CAWs to obtain a holistic view of how they respond to constructed wetland. The physical and chemical properties, diversity of denitrifying microorganisms, and community structure of the sediments were changed. The CAWs can remove suspended solids in river water by physical precipitation, and remove N, P, and some OM by the biological action of algae, microorganisms, and aquatic plants. It is necessary to study denitrification rate and denitrification process at RNA level in the future. Our study provides microbial community data supporting the idea that CAWs have positive effects on the environment and promote more sustainable wetland ecosystems.

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Availability of data and material. The data of Sequence Read Archive (SRA) that support the findings of this study are available in GenBank of NCBI at [<https://www.ncbi.nlm.nih.gov>] under Accession no. PRJNA734135.

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APPENDIX

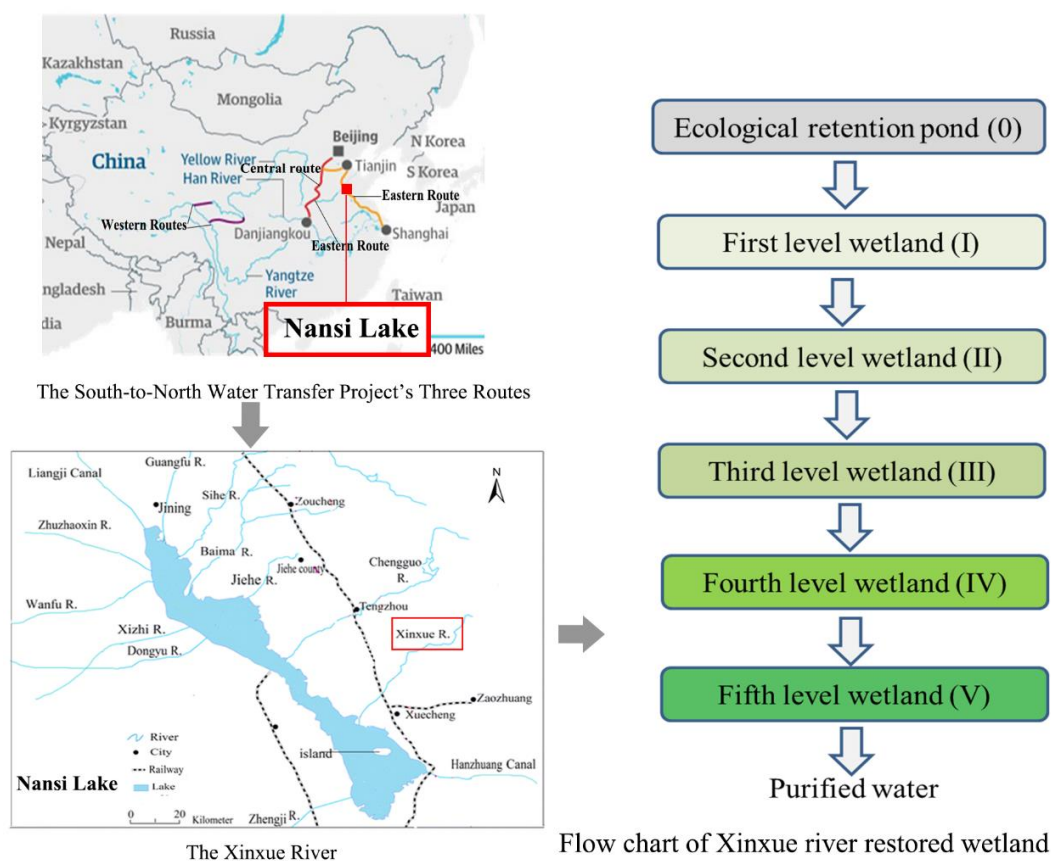


Figure A1. Study site and experimental design

Table A1. Sequence information of *nosZ* clade I gene

Sample	Rowdata	Final_clean	Percentm (%)	Goods coverage
0-1	164655	158355	96.17	99.2%
0-2	151354	146110	96.54	99.2%
0-3	220842	213293	96.58	99.0%
I-1	325573	307331	94.40	99.1%
I-2	28841	27879	96.66	99.6%
I-3	72431	68925	95.16	99.5%
II-1	149185	143253	96.02	99.4%
II-2	162141	154504	95.29	99.1%
II-3	240835	231836	96.26	99.3%
III-1	212982	206577	96.99	99.6%
III-2	69409	64853	93.44	99.4%
III-3	146448	140495	95.94	99.4%
IV-1	153800	145515	94.61	99.2%
IV-2	134196	126770	94.47	99.1%
IV-3	185788	176768	95.15	99.0%
V-1	54537	48365	88.68	99.1%
V-2	50561	47567	94.08	98.8%
V-3	48238	45685	94.71	98.6%

Table A2. The relative abundance (%) of different taxa in sediment groups

Phylum	Class	0	I	II	III	IV	V	P value
Proteobacteria	Alphaproteobacteria	83.19 ± 1.2c	69.24 ± 5.26a	82.78 ± 0.93b	74.55 ± 1.65a	84.77 ± 2.23c	78.06 ± 2.27b	0.02
	Betaproteobacteria	5.42 ± 0.29a	7.78 ± 3.08a	5.12 ± 0.69a	7.9 ± 1.31a	4.78 ± 1.02a	6.82 ± 1.03a	0.06
	Gammaproteobacteria	4.02 ± 0.65a	4.21 ± 0.75a	5.06 ± 0.58a	5.25 ± 3.39a	2.81 ± 0.26a	5.93 ± 3.7a	0.07
	Deltaproteobacteria	0.02 ± 0.01a	0 ± 0a	0.01 ± 0.01a	0 ± 0a	0.1 ± 0.03b	0.1 ± 0.02b	0.008
Terrabacteria	Actinobacteria	0.49 ± 0.15c	0.24 ± 0.1ab	0.22 ± 0.02ab	0.26 ± 0.08b	0.02 ± 0.01a	0.02 ± 0.01a	0.005
	Chloroflexi	0.03 ± 0.01ab	0.1 ± 0.1b	0.03 ± 0.03ab	0 ± 0a	0 ± 0a	0.01 ± 0a	0.005
Unclassified_Bacteria	Unclassified_Bacteria	6.7 ± 0.6a	18.35 ± 6.2c	6.69 ± 1.0a	11.79 ± 5.6b	6.74 ± 0.9a	7.33 ± 0.9a	0.001

Table A3. The proportion of enriched *nosZ* clade I taxa in each group

	0	I	II	III	IV	V	Phylum	Class	Order	Family	Genus
Otu1	0.447395	0.179402	0.346458	0.21754	0.378721	0.263073	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
Otu6	0.086991	0.01719	0.17302	0.027799	0.090421	0.068269	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
Otu26	0.014236	0.027837	0.0114	0.020881	0.032925	0.033827	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium_Agrobacterium_group
Otu16	0.014043	0.02252	0.023828	0.039114	0.002841	0.00151	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Rhodopseudomonas
Otu64	0.009985	0.000941	0.006041	0.003525	0.008886	0.005445	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
Otu45	0.009188	0.004384	0.012819	0.006407	0.005389	0.003643	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
Otu30	0.009073	0.001811	0.01048	0.002369	0.005876	0.005597	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter
Otu138	0.009017	0.063023	0.007391	0.074398	0.001198	0.000471	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys
Otu74	0.007795	0.004501	0.003076	0.016509	0.000251	6.93E-05	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Thiobacillaceae	Thiobacillus
Otu235	0.007754	0.00534	0.003702	0.003887	0.00879	0.003893	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
Otu153	0.007467	0.000427	2.78E-05	0.000488	6.97E-05	0.000111	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus
Otu105	0.00745	0.003516	0.00277	0.003023	0.00461	0.002563	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Nitrospirillum
Otu107	0.00572	0.006158	0.006806	0.002801	0.001156	0.000693	Proteobacteria	Betaproteobacteria	Rhodocyclales	Zoogloeaceae	Azoarcus
Otu286	0.005037	0.000472	0.002339	0.000209	0.007965	0.010442	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Sinorhizobium_Ensifer_group
Otu10	0.004858	0.139632	0.009159	0.085686	0.001769	0.000596	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Azospirillum
Otu67	0.004692	0.000222	0.00682	0.000181	0.000111	0.000623	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Oceaniovalibus
Otu80	0.004636	0.002335	0.001976	0.002563	0.000195	0.000194	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardiodaceae	Nocardiodaceae
Otu292	0.003466	0.037677	0.002158	0.047468	0.014356	0.011857	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Nitrateductor
Otu103	0.003453	0.000376	0.000988	0.000265	0.011073	0.005872	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Rhodanobacteraceae	Rhodanobacter
Otu85	0.00334	0.00025	0.001044	6.97E-05	0.004582	0.003297	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Microvirga
Otu87	0.002771	5.55E-05	0.003549	0.003273	0.000125	1.39E-05	Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified_Burkholderiales	Burkholderiales_Genera_incertae_sedis

Otu32383	0.002521	9.6E-05	0.002074	0.002188	6.96E-05	0	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia
Otu71	0.002005	0.014114	0.001921	0.024204	0.000488	0.000208	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Leisingera
Otu657	0.001852	6.92E-05	0.001308	0.000321	0.000125	2.77E-05	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Oligotropha
Otu91	0.001182	0.000292	0.0011	0.000153	0.001031	0.001593	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
Otu165	0.000807	0.000166	0.000278	4.18E-05	0.003147	0.00162	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Ruegeria
Otu157	0.000752	6.94E-05	0.000334	0.000181	0.002563	0.002022	Proteobacteria	Alphaproteobacteria	Rhizobiales	Chelatococcaceae	Chelatococcus
Otu200	0.000571	5.57E-05	0.000195	2.78E-05	0.005403	0.012992	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas
Otu710	0.000501	0	5.57E-05	0	0	5.54E-05	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia
Otu245	0.000473	0	0.000501	0	0.002548	0	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter
Otu120	0.000445	0.001054	0.005164	0.000683	5.57E-05	2.77E-05	Proteobacteria	Gammaproteobacteria	Nevskiales	Sinobacteraceae	Steroidobacter
Otu63	0.000306	6.92E-05	0.002186	4.18E-05	0.003442	0.012898	Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified_Burkholderiales	Polyangium_brachysporum

Table A4. The Pearson correlation between 'X-enriched taxon' and physicochemical parameters

	NO ₃ ⁻	NH ₄ ⁺	AP	AK	TN	OM	pH
Sinorhizobium_Ensifer_group	-0.549*	-0.129	-0.282	-0.076	-0.131	-0.183	-0.438
Rhodospseudomonas	0.494*	0.036	0.227	0.000	0.029	0.058	0.454
Rhizobium_Agrobacterium_group	0.040	-0.481*	-0.488*	-0.316	-0.416	-0.462	-0.368
Ralstonia	-0.331	0.790**	0.784**	0.858**	0.877**	0.827**	0.292
Pseudolabrys	0.707**	-0.138	0.115	-0.011	-0.055	-0.040	0.390
Polyangium_brachysporum	-0.728**	-0.484*	-0.574*	-0.440	-0.426	-0.456	-0.819**
Paracoccus	-0.139	0.680**	0.667**	0.758**	0.731**	0.669**	0.360
Oligotropha	-0.204	0.740**	0.639**	0.560*	0.648**	0.650**	0.382
Oceaniovalibus	-0.302	0.559*	0.463	0.235	0.435	0.491*	0.292
Nocardiodaceae	0.197	0.749**	0.867**	0.763**	0.811**	0.794**	0.686**
Nitrospirillum	0.014	0.581*	0.547*	0.678**	0.609**	0.557*	0.367
Nitratireductor	0.657**	-0.386	-0.149	-0.171	-0.285	-0.301	0.188
Methylobacterium	0.141	0.348	0.246	0.472*	0.334	0.293	0.293
Leisingera	0.614**	-0.221	0.045	-0.049	-0.124	-0.143	0.308
Hyphomicrobium	-0.698**	0.101	-0.091	-0.080	0.054	0.037	-0.396
Gemmatimonadaceae	-0.604**	-0.436	-0.540*	-0.347	-0.395	-0.444	-0.70**
Chelatococcus	-0.424	-0.328	-0.498*	-0.184	-0.324	-0.383	-0.548*
Burkholderia	-0.059	0.481*	0.523*	0.434	0.455	0.427	0.418
Bradyrhizobium	-0.387	0.555*	0.369	0.502*	0.471*	0.432	0.115
Azospirillum	0.790**	-0.054	0.142	-0.031	-0.017	0.045	0.450
Azoarcus	0.327	0.616**	0.665**	0.335	0.553*	0.646**	0.732**
Achromobacter	-0.485*	0.375	0.247	0.173	0.301	0.314	-0.018

* $P < 0.05$, ** $P < 0.01$

Table A5. Forward selection results of RDA

Name	Explains (%)	Contribution (%)	pseudo-F	P value
NO ₃ ⁻	24.2	35.6	5.1	0.002
pH	13.2	19.4	3.2	0.004
NH ₄ ⁺	9.6	14.2	3	0.004
AP	9.3	13.6	2.5	0.014
AK	5.5	8	1.3	0.202
TN	3.2	4.1	0.5	0.341
OM	2.5	1.6	0.7	0.732