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RESEARCH ARTICLE

Submerged plants alleviated the impacts of increased ammonium pollution on anammox bacteria and *nirS* denitrifiers in the rhizosphere

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Abstract

Excess nitrogen input into water bodies can cause eutrophication and affect the community structure and abundance of the nitrogen-transforming microorganisms; thus, it is essential to remove nitrogen from eutrophic water bodies. Aquatic plants can facilitate the growth of rhizosphere microorganisms. This study investigated the impact of ammonium pollution on the anammox and denitrifying bacteria in the rhizosphere of a cultivated submerged macrophyte, *Potamogeton crispus* (*P. crispus*) by adding three different concentrations of slow-release urea (0, 400, 600 mg per kg sediment) to the sediment to simulate different levels of nitrogen pollution in the lake. Results showed that the ammonium concentrations in the interstitial water under three pollution treatments were significantly different, but the nitrate concentration remained stable. The abundance of anammox 16S rRNA and nitrite reductase (*nirS*) gene in rhizosphere sediments exhibited no significant differences under the three pollution conditions. The increase in the nitrogen pollution levels did not significantly affect the growth of anammox bacteria and *nirS* denitrifying bacteria (denitrifiers). The change trend of the abundance ratio of (anammox 16S rRNA)/*nirS* in different nitrogen treatment groups on the same sampling date was very close, indicating that this ratio was not affected by ammonium pollution levels when *P. crispus* existed. The redundancy analysis showed that there was a positive correlation between the abundance of anammox 16S rRNA and *nirS* gene and that the abundance of these bacteria was significantly affected by the mole ratio of NH_4^+/NO_3^-. This study reveals that submerged plants weaken the environmental changes caused by ammonia pollution in the rhizosphere, thereby avoiding strong fluctuation of anammox bacteria and *nirS* denitrifiers.

Keywords Rhizosphere · Nitrogen · Anammox · Denitrifier · Abundance

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Introduction

The removal of nitrogen in aquatic ecosystems is mainly accomplished by plant absorption and nitrogen-transforming microorganisms capable of nitrogen conversion processes such as ammonia oxidation, nitrite oxidation, and nitrate reduction. However, excessive accumulation of nitrogen will destroy the stability of the community structure and abundance of these nitrogen-transforming microorganisms, thereby breaking the balance of nitrogen input and output in water bodies (Le et al. 2010), finally causing environmental problems such as water quality decline and eutrophication.

Submerged plants are an important part of the aquatic ecosystem. They can provide habitat and food for aquatic animal, absorb pollutant (Brisson and Chazarenc 2009), inhibit algae growth (Zhao et al. 2021), and reduce sediment resuspension (Tang et al. 2018). Anammox bacteria and denitrifying bacteria are two important nitrogen-transforming microorganisms.

They can convert the ammonium (NH_4^+) and nitrite (NO_2^-), nitrate (NO_3^-) into nitrogen gas, respectively, thereby removing the excessive nitrogen from lakes and maintaining the nitrogen balance in water bodies (Pajares et al. 2017). These two microorganisms are both cooperative and competitive. The denitrification process consumes organic matter, thus slowing down the inhibitory effect of organic matter on the growth of anammox bacteria (Yang et al. 2019). Moreover, in the denitrification process of converting NO_3^- to nitrite (NO_2^-), NO_2^- produced by denitrifying bacteria can be used by anammox bacteria as a reaction substrate, thus facilitating the growth of anammox bacteria (Wang et al. 2017; Koop-Jakobsen and Giblin 2009). However, in the denitrification process of converting NO_2^- to NO (nitric oxide), *nirS* denitrifiers with Cu-containing enzymes and *nirK* denitrifiers with cytochrome cd1 enzymes may compete with anammox bacteria for NO_2^- (Wang et al. 2020).

In shallow lakes, aquatic plants can reduce the nitrogen level in the lake by directly absorbing NH_4^+ and NO_3^- either from water bodies or sediments (Zhou et al. 2016; Zhao et al. 2014). Aquatic plants can also facilitate the growth of rhizosphere microorganisms by providing them nutrients through the root system, leading to a much higher abundance of rhizosphere microorganisms, compared to the non-rhizosphere environment, thus showing the rhizosphere effect (Christensen et al. 1994). Plant roots can release oxygen to the rhizosphere and form diverse micro-environments such as aerobic, anoxic, and anaerobic conditions in the rhizosphere (Niu et al. 2015). This provides different living environment for the coexistence of anaerobic microorganisms such as anammox and denitrifying bacteria in the rhizosphere (Wang et al. 2020) and more possibilities for combining denitrification with anammox (Kumar and Lin 2010). It has been reported that the activity and the abundance of anammox bacteria in the rhizosphere were higher than those in the non-rhizosphere (Nie et al. 2015). The coexistence of anaerobic and aerobic environments in the rhizosphere was also considered to be beneficial to the growth of anammox bacteria (Wang et al. 2015; Vazquez-Padin et al. 2010).

The growth of anammox and denitrifying bacteria in sediments can be affected by the changes in water pollutants. Some studies have shown that the abundance of *nirS* gene in sediments was decreased with the increase in lake nutrients (Wan et al. 2019; Guo et al. 2014), while eutrophication accelerated the anammox bacteria growth (Zhao et al. 2019b). However, there is still a lack of understanding of how these two bacteria respond to the increased nitrogen content in the presence of submerged plants in lakes. Plants have been reported to be able to maintain their growth environment, especially the stability of the physical and chemical properties of rhizosphere environment (Hussain et al. 2011). Based on it, we hypothesized that submerged plants could provide a stable environment for anammox and denitrifying bacteria in the rhizosphere, thus alleviating the rapid impact of increased pollution on these two bacteria in the rhizosphere. To test this hypothesis, the abundance of anammox and denitrifying bacteria in the rhizosphere of a submerged macrophyte, *Potamogeton crispus* (*P. crispus*), under three ammonium pollution levels in the lake sediment were investigated. The most popular nitrogen fertilizer today is urea, which can dissolve in water within a few seconds, and the nitrite produced during the oxidation process of the ammonia produced by its hydrolysis can become the substrate of the anammox reaction. Therefore, we choose water-dissolved slow-release urea as ammonium pollution levels.

Materials and methods

Experiment design

The sediment used to cultivate *P. crispus* in the experiment was sampled from Lake Liangzi (114° 38′ 23″ N, 30° 14′ 28″ E), a mesoeutrophic lake located in Yangtze River catchment, Hubei Province, China. The total nitrogen content in the sediment was 0.50 g kg^{-1}, and the concentrations of NH_4^+-N and NO_3^--N in the fresh sediment interstitial water were 4.39 mg L^{-1} and 0.22 mg L^{-1}, respectively (Wang et al. 2018). The submerged plant *P. crispus* was selected from Liangzi Lake as a cultivated plant which is one of the dominant plants in the middle reaches of the Yangtze River. It is characterized by rapid growth and well-developed root systems. A three-compartment with multiple interlayers rhizobox design was used to conduct rhizosphere experiments in the present study (Wang et al. 2018). The size of the rhizobox was 175×175×115 (length × width × height) mm (Fig. 1). The 20-mm width in the center of the rhizobox chamber was the central root chamber. Submerged plants were planted only in this part, and the 1–5-mm area on both sides of the central root chamber was the rhizosphere. Six sheets of nylon mesh (pore size <25 μm, 1 mm each) were inserted on rhizosphere to restrain the plant root to grow within the central root compartment only. The rhizobox was, therefore, divided into three main compartments and a total of seven layers, i.e. the central root compartment (20 mm in width, 1 layer), rhizosphere (1–5 mm from the root compartment, 5 layers), and non-rhizosphere (>5 mm from the root compartment, 1 layer) compartments. The rhizobox were filled with air-dried and sieved (1 mm) Lake Liangzi sediments in all layers.

In November 2014, six *P. crispus* turions with similar growth characteristics were transplanted into each central root compartment to start the cultivation. After transplanting, the whole rhizobox and *P. crispus* were immersed under the distilled water to simulate the growth conditions of submerged plants. After 5 months, the central root compartment was filled with the root system in April 2015. Three concentrations of

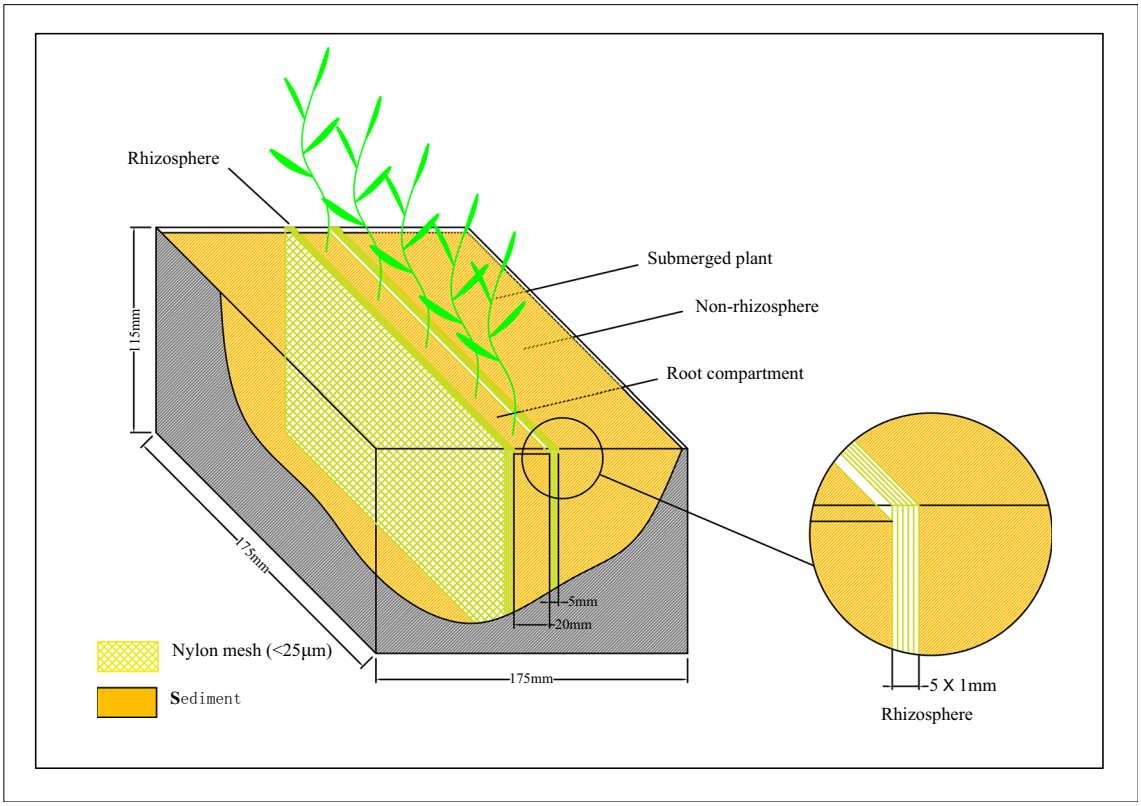

Fig. 1 The schematic diagram of the rhizobox (modified from Wang et al. 2018)

water-dissolved slow-release urea (Luxi Chemical Co., Ltd., Liaocheng, Shandong, China) were injected into sediment to simulate three nitrogen-contaminated sediment conditions (oligotrophic, mesoeutrophic, and hypereutrophic) in freshwater lakes. Before injection, the urea was dissolved in ultrapure water, and then the urea solution was injected into the sediment with a sterile syringe. Each rhizobox was arranged with 10 injection points, and the injection depth interval was 3 cm at each point to ensure that the urea solution could be evenly distributed in the sediment. After injection, the urea contents in the sediment were 0 mg kg^{-1} (N0), 400 mg kg^{-1} (N400), and 600 mg kg^{-1} (N600), respectively. On the 14, 28, and 42 days after urea injection, sediment samples from three replicate rhizobox were collected by destructive sampling. At each rhizobox, a total of seven layers of sediments were collected from three compartments: root compartment (R), rhizosphere compartment (N1-N5), and non-rhizosphere compartment (Non). Plant cultivation and sediment collection followed the procedures described by Wang et al. (2018).

Sediment chemical properties analysis

Before the samples were collected, the dissolved oxygen (DO) and pH of the interstitial water in each layer of the rhizobox were measured by using a microelectrode system (Unisense, Aarhus, Denmark). Each sediment was centrifuged at 4000 rpm for 15 min to obtain interstitial water, and then the interstitial water was filtered with a 0.45-μm pore filter membrane. The contents of NH_4^+-N and NO_3^--N in the interstitial water were measured by using a flow injection analyzer (SEAL Analytical AA3; SEAL Analytical, Norderstedt, Germany). All samples were individually tested for physical and chemical indicators, as well as anammox 16S rRNA and *nirS* gene abundance.

DNA extraction and PCR amplification

Genomic DNA of each sediment sample was extracted using Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. The purity and concentration of the extracted DNA samples were checked by super differential spectrophotometer (NanoPhotometer-N60; Implen, Munich, Germany), and then the qualified DNA samples were used for PCR amplification. The amplification primers for *nirS* gene were nirS1F/nirS6R (Braker et al. 1998). Anammox 16S rRNA amplification consisted of two steps with the amplification of primers pla46f/630r as the first step (Juretschko et al. 1998; Neef et al. 1998) and that of primers Amx368f/Amx820r as the second step (Schmid et al. 2005). PCR amplification of anammox 16S rRNA and *nirS* gene was performed in a total volume of 25 mL system containing 1 mL of each primer (10

mM), 12.5 mL of I-5™ 2X High-Fidelity Master Mix, and 9.5 mL of double-distilled water (ddH_2O), with 1 mL of DNA as templates (20–50 ng). The anammox 16S rRNA and *nirS* gene amplification process is shown in Table 1. PCR products were tested by using agarose gel (1.0%) electrophoresis.

Cloning, sequencing, and phylogenetic analysis

The DNA samples of the same layer were mixed in equal amounts and used for sequencing. Then, the PCR products of anammox 16S rRNA and *nirS* gene were purified with magnetic bead purification kits (GeneOn BioTech, China). After purification, these products were cloned with the pClone 007 Vector Linker Kit (TSING KE, Beijing, China) and transformed into competent *Escherichia coli* cells according to the manufacturer's instructions. Approximately 59 anammox 16S rRNA and 61 *nirS* positive clones were randomly picked and checked by agarose gel. The re-amplified PCR conditions of the anammox 16S rRNA and *nirS* gene in every clone are shown in Table 1. A 20-μL aliquot of the PCR product was digested with restriction endonuclease Msp I, separated by 110 mV electrophoresis on a 15% polyacrylamide gel for about 1 h and then stained with ethidium bromide (0.5 μg mL^{-1}). Images of the gel were taken using a Kodak Gel Logic 100 system (Eastman Kodak, Rochester, NY, USA) to store their fingerprint of each clone. For each clone library, anammox 16S rRNA and *nirS* gene were sequenced by ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences with 97% identity were classified into same OTUs with anammox 16S rRNA and *nirS* gene using MOTHUR software (Wise et al. 2020; Schloss et al. 2009). Alignment of DNA sequences was carried out using the ClustalW 1.6, and a neighbor joining phylogenetic tree was constructed using MEGA 6.0 software. Bootstrap values for each branch were determined using 1000 iterations.

The sequences obtained in this study were deposited in the GenBank database with accession numbers of MK641594-MK641650 for anammox 16S rRNA and MK783879-MK783936 for *nirS*, respectively.

Real-time quantitative PCR (qPCR)

Quantitative analysis of anammox 16S rRNA and *nirS* genes was performed by real-time quantitative PCR by using the SYBR Green method with the QuantStudio™ 6 Flex quantitative PCR instrument (Thermo Fisher Scientific, Singapore). The anammox 16S rRNA and *nirS* genes qPCR primers (Tsushima et al. 2007; Throback et al. 2004) and amplification procedures are shown in Table 1. Each qPCR reaction system contained 10 μL mixture as follows: 5 μL of SybrGreen qPCR Master Mix, 0.5 μL of each primer (10 mM), 3 mL of ddH_2O, and 1 μL of DNA template. The ddH_2O was set as negative control, and the known copy number of plasmid DNA subjected to ten-fold serial dilution was set as positive control. Six standard plasmid DNA samples were obtained for quantitative amplification with three replicates each to generate a standard curve. The amplification efficiency of anammox 16S rRNA and *nirS* genes was 92.4% and 94.7%, respectively. The correlation coefficient (r^2) was greater than 0.99, and the melting curve showed a single peak.

Table 1 Primers used in this study

Gene name	Primer name		Primer sequences (5′–3′)	Thermal profile for PCR	Reference
Primers used for PCR amplification					
nirS	nirS1F nirS6R		CCTAYTGGCCGCCRCART CGTTGAACTTRCCGGT	95 °C for 8 min; 35 cycles of 10 s at 98 °C, 10 s at 56 °C, 20 s at 72 °C; 5 min at 72 °C	(Braker et al. 1998)
Anammox 16S rRNA	First step	pla46f 630r	GGATTAGGCATGCAAGTC CAKAAAGGAGGTGATCC	95 °C for 8 min; 35 cycles of 10 s at 98 °C, 10 s at 59 °C, 20 s at 72 °C; 5 min at 72 °C	(Juretschko et al. 1998; Neef et al. 1998)
	Second step	Amx368f Amx820r	TTCGCAATGCCCGAAAGGTT CGCAATGCCCGAAAGG AAAACCCCTCTACTTAGTGC CC	95 °C for 8 min; 35 cycles of 10 s at 98 °C, 10 s at 59 °C, 20 s at 72 °C; 5 min at 72 °C	(Schmid et al. 2005)
Primers used for real-time quantitative PCR					
nirS	nirSCd3aF nirSR3cd		AACGYSAAGGARACSGG GASTTCGG RTGSGTCTTSAYGAA	95 °C for 15 min; 40 cycles of 10 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C	(Throback et al. 2004)
Anammox 16S rRNA	AMX809F AMX1066R		GCCGTAAACGATGGGCACT AACGTCTCACGACACGAGCT G	95 °C for 15 min; 40 cycles of 10 s at 95 °C, 30 s at 59 °C, 30 s at 72 °C	(Tsushima et al. 2007)

Statistical analysis

The physicochemical properties (pH, and NH_4^+-N and NO_3^--N concentrations) of the interstitial water and the abundance of anammox 16S rRNA and *nirS* gene were investigated under three nitrogen pollution concentrations through one-way ANOVA and Tukey's test ($P<0.05$) with SPSS 20.0 software. Detrended correspondence analysis (DCA) (Canoco 4.5 software) was used to examine the correlation between microbial abundance and its environmental factors. Since the length of the gradient value obtained by DCA in this study was less than three, the redundancy analysis (RDA) method was selected from the Canoco software. The significance test of Monte Carlo permutations (999) was used to explore the environmental factors related to the abundance of anammox 16S rRNA and *nirS* gene.

Results

Physicochemical properties of interstitial water in the rhizosphere

The concentration of NH_4^+-N in the sediment interstitial water differed significantly with the average concentration of 1.87, 16.42, and 20.16 mg L^{-1} for the three nitrogen treatments (N0, N400, and N600), respectively ($P<0.05$). The NH_4^+-N concentration in the urea addition treatment (N400 and N600) was significantly higher than that in the non-addition treatment (N0) during the entire experiment of up to 42 days (Fig. 2A). On day 28, the average of NH_4^+-N concentrations in urea addition treatments (N400: 20.94 mg L^{-1}, N600: 28.01 mg L^{-1}) was significantly higher than that on day 14 (N400: 15.54 mg L^{-1}, N600: 16.20 mg L^{-1}) and day 42 (N400: 12.79 mg L^{-1}, N600: 16.28 mg L^{-1}) after the urea addition.

The NO_3^--N concentration in the root compartment and non-rhizosphere showed no significant difference. During the entire experiment, the NO_3^--N concentration in the rhizosphere interstitial water reached the peak at 14 d after the urea addition and then was gradually decreased with the extended time of urea addition (Fig. 2B). In contrast, the NO_3^--N concentrations in the root compartment and non-rhizosphere interstitial water remained stable with the extended time of urea addition. The average interstitial water NO_3^--N concentrations were 1.18, 1.21, and 1.34 mg L^{-1}, respectively, for three urea addition treatments of N0, N400, and N600, and there was no significant difference between different treatments ($P>0.05$). The average pH values in the rhizosphere (1–5 mm) for three treatments of N0, N400, and N600 ranged from 7.46 to 7.63, 7.60 to 7.73, and 7.60 to 7.64, respectively. There was no significant difference in oxygen concentration at the sediment-water interface under each treatment, and the average value was 11.86 μmol L^{-1}.

Nitrogen-transforming bacterial abundance in the rhizosphere of *P. crispus* under ammonium pollution

The gene abundance of anammox 16S rRNA in the rhizosphere was generally higher than that in the root compartment and non-rhizosphere, but there was no significant difference between them ($P>0.05$) (Fig. 3). The anammox 16S rRNA gene abundance in the rhizosphere for each treatment group (0, 400, 600 mg kg^{-1}) was 1.96×10^7, 1.82×10^7, and 1.83×10^7 copies g^{-1} ($P>0.05$) (Fig. 3), respectively. The gene abundance of anammox 16S rRNA (2.01×10^7 copies g^{-1}) on day 14 was significantly higher than that on day 28 (1.69×10^7 copies g^{-1}), but it was closer to that on day 42 (1.89×10^7 copies g^{-}). During the entire experiment, the anammox 16S rRNA abundance for each treatment group (0, 400, 600 mg kg^{-1}) was 1.96×10^7, 1.82×10^7, and 1.83×10^7 copies g^{-1}, respectively. And there was no significant difference between different groups ($P>0.05$, Fig. 4).

Under N0 treatment, the abundance of *nirS* gene in rhizosphere showed significant difference on days 14 and 28 ($P<0.05$) (Fig. 3). The *nirS* gene abundance in the rhizosphere for each treatment group (0, 400, 600 mg kg^{-1}) was 2.25×10^5, 2.12×10^5, and 1.99×10^5 copies g^{-1} ($P>0.05$) (Fig. 3), respectively. Under N400 and N600 treatments, the abundance of *nirS* gene in the rhizosphere, root compartment, and non-rhizosphere showed no significant difference ($P>0.05$). Throughout the entire experiment, the average abundance of *nirS* gene under three nitrogen treatments (0, 400, 600 mg kg^{-1}) was 2.25×10^5, 2.12×10^5, and 1.99×10^5 copies g^{-1}, respectively, and no significant difference was observed between them ($P>0.05$, Fig. 4). The fluctuation trend of (anammox 16S rRNA)/*nirS* ratio was very close on the same sampling date under three nitrogen treatments.

Impact of key physicochemical factors on rhizosphere bacterial abundance

The first two RDA axes together explained 62.2% of the variance (Fig. 5). RDA analysis showed that the abundance of the anammox 16S rRNA and *nirS* gene was significantly correlated with the concentration of NO_3^--N ($P=0.001$, $F=14.725$, 999 Monte Carlo permutations) and the mole ratio of NH_4^+-N/NO_3^--N in the interstitial water ($P=0.001$, $F=7.655$, 999 Monte Carlo permutations). The abundance of the anammox 16S rRNA gene was positively correlated with the abundance of the *nirS* gene. The abundance of the anammox 16S rRNA gene was negatively correlated with the concentration of NO_3^--N and pH in the interstitial water. The abundance of *nirS* gene was negatively correlated with pH and the concentrations of NH_4^+-N and NO_3^--N in the interstitial water. The

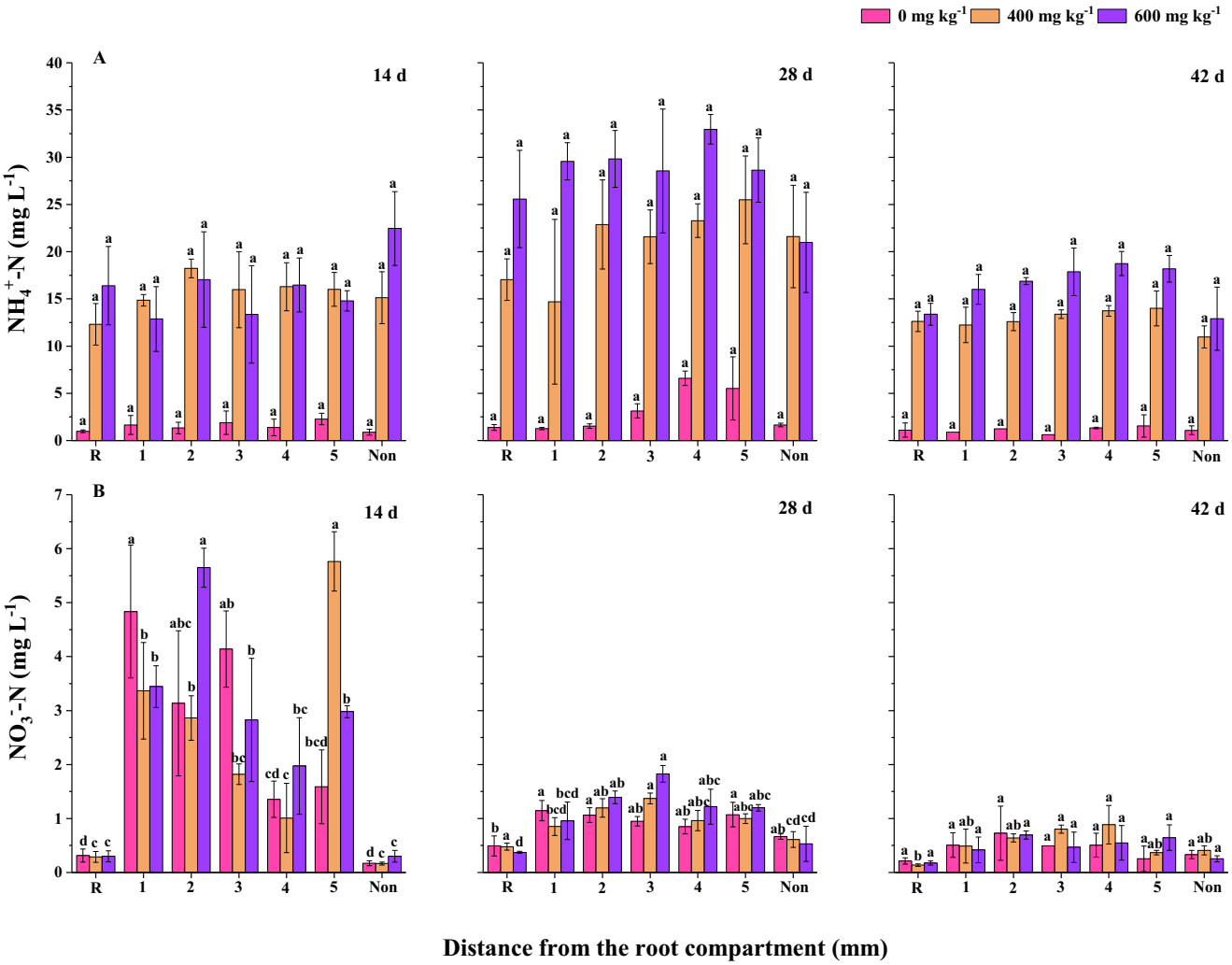

Fig. 2 NH_4^+-N concentration (**A**) and NO_3^--N concentration (**B**) in sediment interstitial water under different nitrogen pollution conditions. Different letters for the same gene indicated the significant difference between different distances from root at $P < 0.05$ (Tukey's test). Error bars indicated standard deviation

abundance of anammox 16S rRNA and *nirS* gene was positively correlated with the ratio of NH_4^+-N/NO_3^--N in the interstitial water.

Diversity of anammox and *nirS* denitrifiers in the rhizosphere

A total of 57 anammox 16S rRNA gene sequences were obtained from the constructed clone library. A total of five OTUs were obtained with a similarity of 97% (Fig. 6). The sequences of OTU1 and OTU2 accounted for 61.4% and 17.5% of the cloned library, respectively. Anammox 16S rRNA gene sequences in the sediments of phylogenetic trees were mainly clustered to the genus *Brocadia* and *Kuenenia*. OTU1, OTU2, OTU3, and OTU5 were all assigned to the genus *Brocadia*. The sequences of this genus accounted for 93% of all the sequences of anammox 16S rRNA. Therefore, genus *Brocadia* was the dominant species of anammox in this study, and the similar sequences were from freshwater lake sediments, dehydrated aluminum sludge, suspended sediments of the Yellow River, and rhizosphere sediment of *Potamogeton*. OTU4 belonged to the genus *Kuenenia* and had a high similarity with the sequences from freshwater sediment, but its sequences accounted for only 7% of the total number of sequences.

Among the 58 *nirS* sequences, 84.5% belonged to Betaproteobacteria (β-Proteobacteria) class, 5.2% belonged to Gammaproteobacteria (γ-Proteobacteria) class, and 10.3% belonged to Bacilli class (Fig. 7). The β-Proteobacteria class was the most dominant group in the *nirS* gene clone library with 24 OTUs belonging to this class. The β-Proteobacteria class was divided into three clusters, namely uncultured cluster, *Thauera* cluster, and *Cupriavidus* cluster, respectively. Uncultured cluster (24 sequences) contained more sequences than *Thauera* cluster (13 sequences) and *Cupriavidus* cluster (12 sequences) in the clone library. OTU1 exhibited the

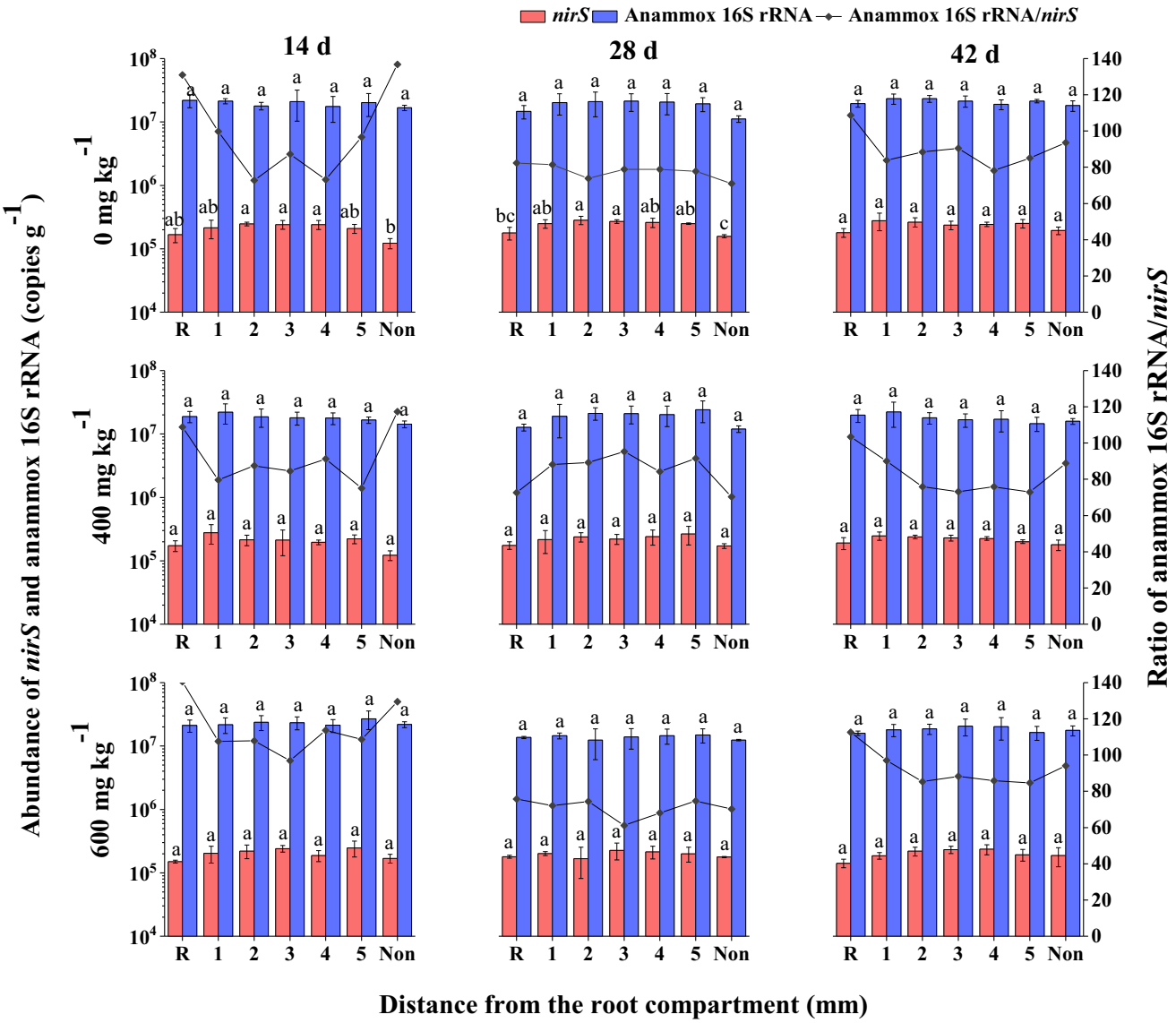

Fig. 3 Abundance of anammox 16S rRNA and *nirS* gene in rhizosphere under different nitrogen pollution conditions. Different letters for the same gene indicated the significant difference between different distances from root at $P < 0.05$ (Tukey's test). Error bars indicated standard deviation

highest abundance, which belonged to *Cupriavidus* cluster including 9 sequences, and these 9 sequences exhibited high similarity with those in the sediments of submerged plants. Three OTUs (OTU23, OTU24, OTU28) belonging to *Thiothrix* cluster, γ-Proteobacteria class, exhibited the similar sequences with those from Yangtze lake sediment and Jiulong river estuary sediment. OTU4 and OTU6 belonged to *Bacillus* cluster, Bacilli class.

Discussion

Many studies have shown that changes in nitrogen content in the environment will significantly change the abundance of anammox and denitrifying bacteria in sediments (Zhao et al. 2020; Fu et al. 2019; Kim et al. 2016). Guo et al. (2014) found that compared with those in mesoeutrophic lakes, denitrifying bacteria in hypereutrophic lakes exhibited the lowest abundance and evenness. However, the eutrotrophic Dianchi Lake had greater anammox bacterial abundance than mesotrophic Erhai Lake (Yang et al. 2017). The similar phenomenon was also found in the sediment of Yangcheng Lake, that is, the maximum abundance of anammox bacteria was in months with high ammonia concentration (Zhang et al. 2016). Therefore, a high nitrogen input was likely to be able to stimulate the growth of anammox bacteria (Nie et al. 2019). The content of ammonium also affected the composition of anammox bacteria and the types of denitrifying bacteria. Some studies found that in eutrophic Taihu Lake and Kitaura Lake, community structure analysis showed that

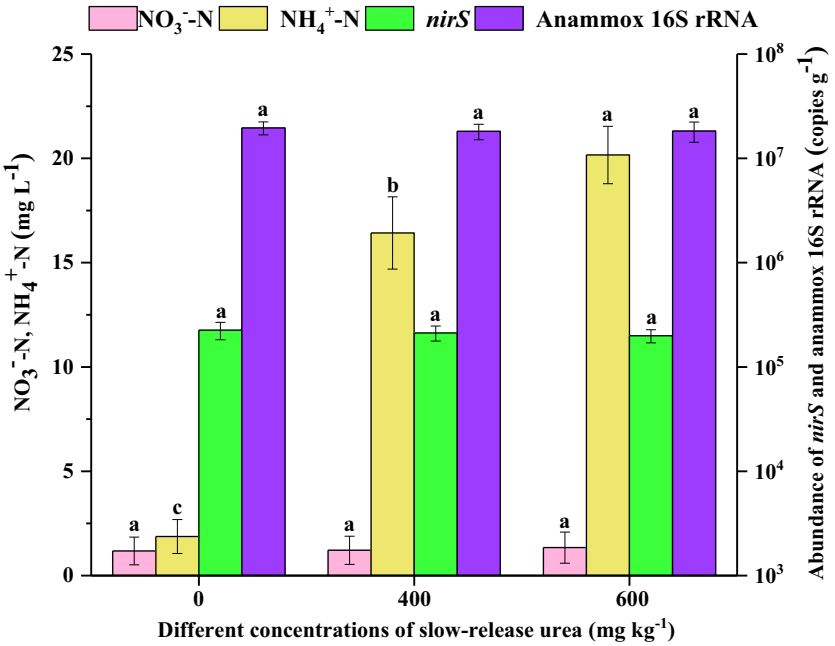

Fig. 4 Average concentrations of NH_4^+-N and NO_3^--N in interstitial water and the average abundance of anammox 16S rRNA and *nirS* gene in sediment at different slow-release urea concentrations during the whole experiment. Different letters above the bars indicated significant differences ($P<0.05$) (Tukey's test) between different distances from root. Error bars indicated standard deviation

anammox bacteria *Ca. Brocadia* sp. were the dominant genus (Qin et al. 2018; Zhu et al. 2015), and the species richness of denitrifying bacteria decreased with the decrease of ammonium concentrations from surface flow wetland to ditch wetland (Chen et al. 2020a). In the present study, the NH_4^+-N concentrations in the sediment interstitial water for each nitrogen pollution treatment were significantly different during the whole experiment (Fig. 4) in the presence of *P. crispus*. Especially, the maximum NH_4^+-N concentration of 600 mg kg^{-1} urea treatment was 20.2 mg L^{-1}, while the minimum NH_4^+-N concentration of 0 mg kg^{-1} urea treatment was 0.9 mg L^{-1}. However, the abundance of anammox 16S rRNA and *nirS* gene showed no significant difference under the three nitrogen pollution conditions. Therefore, the presence of submerged plants provided a stable environment for anammox bacteria and *nirS* denitrifiers to avoid rapid fluctuations in the abundance of these two bacteria in the rhizosphere.

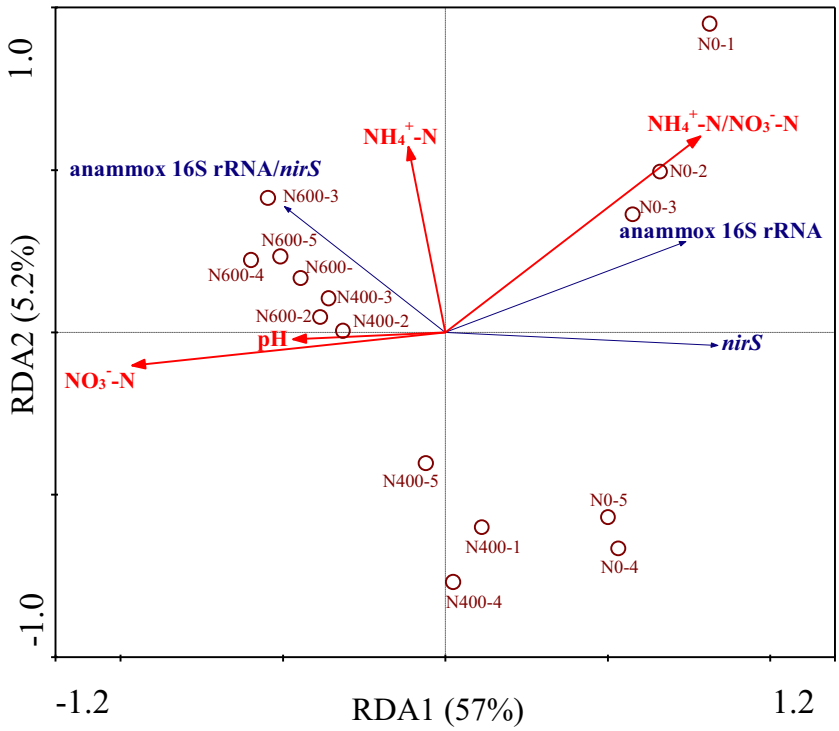

Fig. 5 Redundancy analysis of the abundance of microbial functional gene and environmental factors. N0, N400, and N600 represent three concentrations of added slow-release urea. The number followed the urea concentration (1~5) indicates the distance from rhizosphere within 1~5 mm

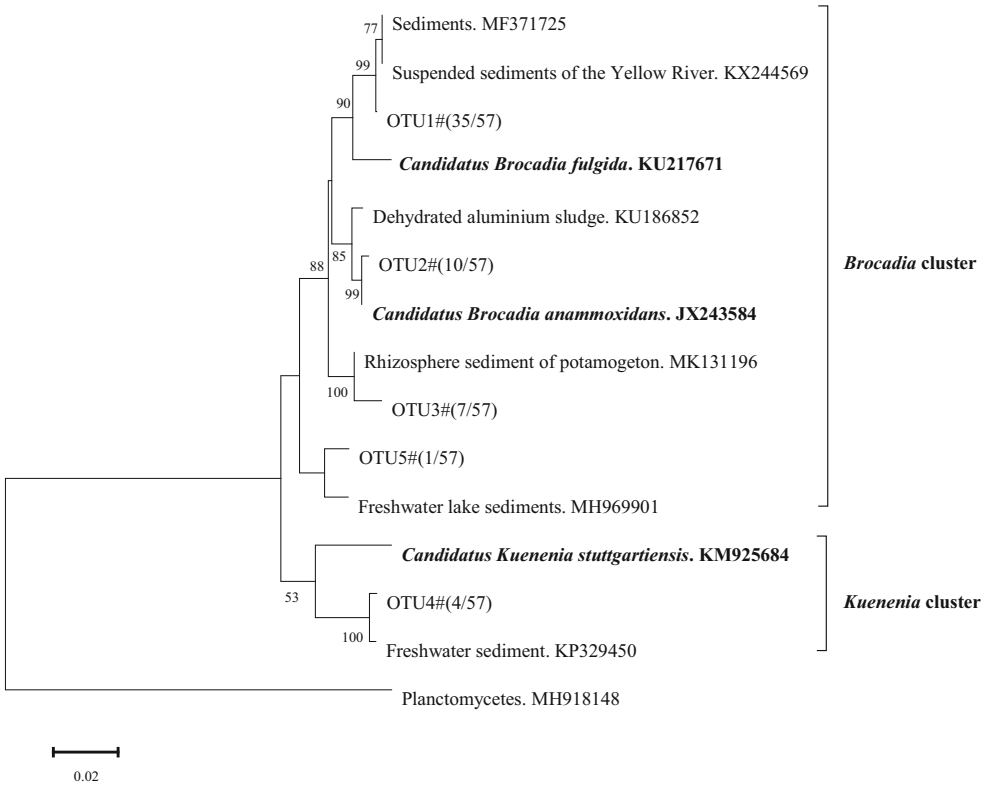

Fig. 6 Phylogenetic tree of representative anammox 16S rRNA sequences and reference sequences from GenBank. The number in parentheses indicates that the sequence in the OTU out of the total number of sequences. The numbers at the nodes represent percentages that indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets (bootstrap values greater than 50% are shown). Branch lengths represent sequence differences indicated by the scale bar

The ratio of the abundance of two microorganisms can reflect which microorganism has better adaptability to the environment (Kent et al. 2019; Liu et al. 2015). Our previous study found that the ratio of the abundance of anammox 16S rRNA to *nirS* gene in the rhizosphere could quickly respond to the damage of *P. crispus* leaves at day 10 after the damage treatment (Hu et al. 2020). However, in the present study, during the 42 days of pollution treatment, no significant changes in the gene abundance ratio were found between different nitrogen pollution treatments, although NH_4^+-N concentrations differed significantly (Fig. 3). Our results indicated that the ecological relationship between these two bacteria was not impacted by different pollution levels in the presence of submerged plant. This further confirms that submerged plants provided a stable environment for anammox bacteria and *nirS* denitrifiers to avoid the rapid fluctuations in the abundance of these two bacteria in the rhizosphere.

One reason why plants can provide a stable environment might be the radial oxygen loss (ROL) from plants. When there were no plants in the lake, the sediment-water interface gradually becomes anoxic, and nitrification can only occur in the oxygen-containing area of the surface sediments (Satoh et al. 2007). However, oxygen secretion from plant roots can provide oxygen to ammonium-oxidizing microorganisms in deep rhizosphere sediments (Okabe et al. 2012). Therefore, the amount of oxygen secreted from the root system is an important rate-limiting factor for ammonia oxidation in rhizosphere sediments (Wang et al. 2012; Lam et al. 2007). Different macrophytes may show different ROL (Tian et al. 2015); however, there might be a certain limit on the amount of oxygen loss. For example, the ROL values of *Juncus effusus* L. and *Juncus inflexus* L. were $(9.5 \pm 1)\times10^{-7}$ and $(4.5 \pm 0.5)\times 10^{-7}$ mol O_2 h^{-1} $root^{-1}$ (Sorrell 1999), respectively. The limited oxygen loss of the root system prevents the ammonium around the rhizosphere from being rapidly oxidized to nitrate, thus avoiding the impact of the rapid rise in nitrate concentrations on anammox and denitrifying bacteria activities. Our results also showed that although the ammonium concentration under each pollution treatment was significantly different, the nitrate concentration was relatively stable (Fig. 4). However, the concentration of nitrate only increased significantly in the rhizosphere on 14 days and remained stable in the subsequent 28 and 42 days (Fig. 2). This indicated that the presence of plants can stabilize the concentration of nitrate in the rhizosphere and provide a more stable growth environment for anammox bacteria and denitrifying bacteria.

Another reason why plants can provide a stable environment wasthat plants may also increase their NH_4^+-N absorption capacity when NH_4^+-N content increases in the sediment, thereby alleviating the ammonium pollution. Most macrophytes have been confirmed to prefer ammonium rather than nitrate due to their energy-saving strategies (Fang et al. 2007; Tylova-Munzarova et al. 2005). Therefore, plants can absorb ammonium in the interstitial water faster than nitrate (Zhao

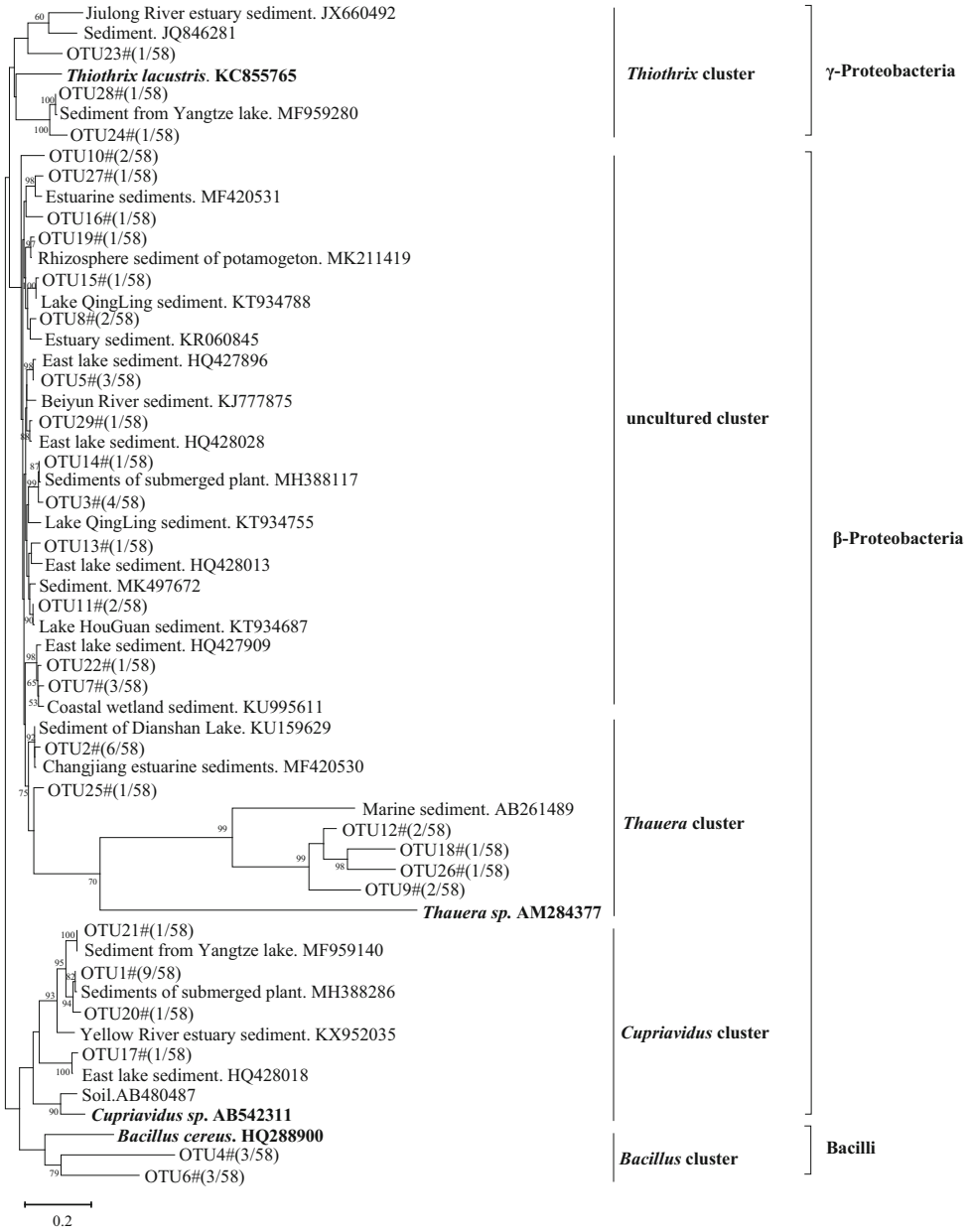

Fig. 7 Phylogenetic tree of representative *nirS* sequences and reference sequences from GenBank. The number in parentheses indicates that the sequences in the OTU out of the total number of sequences. The numbers at the nodes represent percentages that indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets (bootstrap values greater than 50% are shown). Branch lengths represent sequence differences indicated by the scale bar

et al. 2019a). The ammonium concentration in sediment has been detected to be significantly reduced after planting *Phragmites australis* (Toyama et al. 2016). In the present experiment, the concentration of urea added in N600 treatment was 1.5 times as much as that in N400 treatment, but the average ammonium concentration in N600 treatment was detected to be only 1.2 times as much as that in N400 treatment (Fig. 4), indicating that when the ammonium content in the interstitial water was increased, *P. crispus* also increased its capacity of ammonium absorption to avoid the damage to the micro-habitat caused by the rapid rise of ammonium in the rhizosphere to a certain extent.

The third reason why plants can provide a stable environment may be that plant roots increase the absorption of nitrate in interstitial water. On 14 days, nitrate in rhizosphere interstitial water showed an upward trend (Fig. 2). It may be that ammonia-oxidizing archaea and ammonia-oxidizing bacteria (AOB) in the rhizosphere oxidized the ammonia released by urea, which caused the concentration of nitrate to rise. Studies also found that the application of urea and fertilizer stimulated the enrichment of AOB (Chen et al. 2020b), and it also significantly increased the abundance and diversity of AOB genes (Tao et al. 2021). The enrichment of AOB accelerates ammonia oxidation speed. However, on 28 and 42 days, the concentration of nitrate in rhizosphere interstitial water remained relatively low when the ammonia concentration did not decrease significantly. This indicated that when the abundance of two nitrate consumers, anammox bacteria and

denitrifying bacteria, did not increase significantly, plant roots were likely to increase the absorption of nitrate after short-term adaptation.

Nie et al. (2019) found that the ratio of NH_4^+/NO_3^- in the soil was a key factor regulating the anammox process. In the present study, the gene abundance of anammox 16S rRNA was negatively correlated with NO_3^--N content in the interstitial water, but it was positively correlated with the ratio of NH_4^+/NO_3^-, which might be due to the fact that at high ammonium concentrations, anammox bacteria might be more inclined to use NO_2^- produced by nitrification as reaction substrate compared to NO_3^- or NH_4^+ (Nie et al. 2019). In the present study, the abundance of *nirS* gene was also negatively correlated with NO_3^-, which was consistent well with the results of our previous study (Hu et al. 2020). The research on neutralized used acid biofilter and reeds rhizosphere sediment also found that the abundance of *nirS* gene was not positively correlated with NO_3^- (Xu et al. 2020; Wang et al. 2017). This might be attributed to the interference of the anaerobic ammonia oxidation process, since the intermediate products from nitrification and denitrification were used as reaction substrates, these intermediate products might interfere with the second step of the denitrification process dominated by *nirS* denitrifiers (Wang et al. 2017).

Five genera of anammox bacteria have been identified (Zhou et al. 2018). The present study found that *Candidatus Brocadia* and *Candidatus Kuenenia* of anammox bacteria could coexist in sediments, and these two genera were reported to be more adapted to the growth in freshwater systems (Sun et al. 2014; Zhu et al. 2013). In our study, *Ca. Brocadia* was dominant in anammox bacteria, and this genus was also found to be dominant in the sediments of rivers and eutrophic lakes in previous studies (Hu et al. 2012; Yoshinaga et al. 2011; Zhang et al. 2007). *Candidatus Brocadia* prefers a rich ammonium environment (Oshiki et al. 2016). The high ammonium content in the present study might be the reason for *Ca. Brocadia* predominance. *Candidatus Brocadia* was also reported to coexist with other genera of anammox bacteria (Sonthiphand et al. 2014). Our study also found that a small amount of *Ca. Kuenenia* coexisted with *Ca. Brocadia*. *Candidatus Kuenenia* showed lower affinity constants for NH_4^+ and NO_2^- which might make it more suitable for a low ammonium environment (Oshiki et al. 2016). The rich ammonium environment in the present study might have an inhibitory effect on the growth of these bacteria.

This study indicated that β-Proteobacteria and γ-Proteobacteria accounted for 84.5% and 5.2% of the total number of *nirS* sequences, respectively. Both the β-Proteobacteria class and the γ-Proteobacteria class belonged to the Proteobacteria phylum. Previous study has found that the Proteobacteria phylum has an advantage in the river environment (Zwart et al. 2002), which is in line with our findings for this phylum. The present study found that *Thauera* cluster contained 13 sequences, which has already been confirmed to be more suitable for survival in an eutrophic environment in previous study (Yang et al. 2013). Some studies have also reported that the nitrate addition will increase the denitrification activity, but this nitrate addition cannot change the community composition of the denitrifying bacteria (Wallenstein et al. 2006).

Conclusions

In this study, three different concentrations of slow-release urea were added to the rhizosphere sediments of *P. crispus* to investigate their impacts on anammox and denitrifying bacteria. Our results showed that the ammonium concentrations in the interstitial water were significantly increased after the addition of the slow-release urea, but the concentrations of nitrate remained stable. The abundance of the anammox 16S rRNA and *nirS* gene showed no significant difference among three nitrogen pollution treatments. And the change trend of the ratio of (anammox 16S rRNA)/*nirS* in different nitrogen treatment groups on the same sampling date was very close. These results support our hypothesis that under increased nitrogen pollution conditions, submerged plants weaken the environmental changes caused by ammonia pollution in the rhizosphere, thereby avoiding strong fluctuations anammox bacteria and *nirS* denitrifiers.

Author contribution Yangfan Xu: Methodology, investigation, formal analysis, data curation, and writing-original draft. Jing Lu: Writing-review and editing. Shanshan Huang: Investigation, resources, and data curation. Jianwei Zhao: Conceptualization, visualization, supervision, and writing-review and editing, project administration, and funding acquisition.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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