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The abundance of *nirS*-type denitrifiers and anammox bacteria in rhizospheres were affected by the organic acids secreted from roots of submerged macrophytes

Abstract： Excessive nitrogen has been a global concern to cause lake eutrophication. The denitrification and anammox processes are considered to be effective biological pathways for nitrogen removal. Submerged macrophytes also play a key role in the nitrogen cycle of lakes. However, the mechanism of submerged macrophytes on regulating biological nitrogen removal pathways has not been well quantified. Therefore, this study investigated the impacts of submerged macrophytes on the community structures and abundance of the *nirS*-type denitrifiers and anammox bacteria in the rhizospheres. The qPCR results indicated that the abundance of two bacteria in the near-rhizospheres of submerged macrophytes were significantly lower than the root compartments and non-rhizospheres, while the concentrations of organic acids in the near-rhizospheres were higher than those of the root compartments and non-rhizospheres. The RDA results illustrated that concentrations of NO_3^--N, NO_2^--N, citric acid and oxalic acid were the key environmental indicators which had the significant impact on the microbial community. The concentrations of citric acid and oxalic acid were negatively correlated with the *nirS*-type denitrifiers abundance, and the oxalic acid concentrations were negatively correlated with the anammox bacteria abundance. These results indicated that submerged macrophytes could reduce the abundance of *nirS*-type denitrifiers and anammox bacteria by releasing organic acids. In addition, the highest diversity of denitrifier community were found in the rhizosphere of the *Hydrilla verticillata*, while the highest diversity of anammox community were found in the *Potamogeton maackianus* rhizosphere. These results indicate that the impacts of submerged macrophytes on the biological nitrogen removal pathways were species-dependent.

Keywords： Submerged macrophytes; Rhizosphere organic acids; Anammox bacteria; Denitrifier; Lake

sediments

1 Introduction

Excessive nitrogen inputs into the aquatic ecosystems have caused severe eutrophication problems such as algal blooms and biodiversity degradation. Denitrification and anammox are two important permanent biological nitrogen removal processes, which has received much attention in the past decades. Under anaerobic conditions, denitrifying bacteria can reduce nitrate (NO_3^-) through nitrite (NO_2^-) to nitric oxide (NO) and nitrous oxide (N_2O) successively, and finally convert N_2O to dinitrogen gas (N_2). Anammox bacteria can produce N_2 via the reduction of $NO_2^-$and the oxidation of ammonia (NH_4^+). Some studies have shown high abundance of denitrifiers and anammox bacteria were found in the plant rhizospheres (Hamonts et al., 2013; Hou et al., 2018; Li et al., 2016).

The microorganisms are likely to colonize the rhizospheres by utilizing the root-derived sugars, amino acids, fatty acids, and organic acids as their carbon and nitrogen sources, or as their signal stimulus (Bais et al., 2006). Therefore, the abundance and species of rhizosphere microorganisms were generally higher than that of the non-rhizosphere, which has been known as the “rhizosphere effect” (Christensen et al., 1994; Egamberdieva et al., 2008). It has been reported that root exudates of plants can regulate the structure of the microbial community in the rhizosphere (Gschwendtner et al., 2011; Li et al., 2014). A study of anammox in the rice paddy showed that rice could secrete abundant organic acids during its growth, and the concentrations of the succinic acid were significantly positively correlated with the abundance of anammox bacteria (Li et al., 2016). Due to the “rhizosphere effect”, the abundance, diversity and activity of denitrifiers and anammox bacteria in the rhizosphere and non-rhizosphere environment can vary significantly. Nie et al. (2015) found that the contribution of anammox to nitrogen

loss in the rhizospheres of paddy fields was 31-41%, while that of non-rhizosphere was only 2-3%. Ruiz-Rueda et al. (2009) studied the community structure and the potential activity of the denitrifiers in the rhizospheres of *Typha latifolia* and *Phragmites australis* in a constructed wetland, and found that the community structure of the denitrifiers in the rhizospheres differed from that in the bulk sediment. The *nirS* gene is the marker gene for denitrification that encodes the cytochrome cd_1 nitrite reductase, which catalyzes nitrite reduction to nitric oxide (Zumft, 1997), and this gene is often used to determine changes in the diversity, abundance and activity of denitrifiers (Lipsewers et al., 2016). Since NO_2^- is the common substrate for both denitrification and anammox, the *nirS*-type denitrifiers and anammox bacteria can also compete for nitrite, especially under conditions with low NO_2^- concentrations (Kumar and Lin, 2010).

The microbial community in the plant rhizospheres can vary with the plant species, seasons and waterbody or soil types (Dewedar et al., 2009; Herrmann et al., 2009; Hou et al., 2015; Yin et al., 2018; Zhao et al., 2017). There are some studies showing that root exudates could inhibit the activities of microorganisms. Subbarao et al. (2006) found that the root exudates of *Brachiaria humidicola* can restrain the activities of the ammonia monooxygenase and hydroxylamine reductase in the pathway of ammonia-oxidation in *Nitrosomonas*, resulting in the inhibition of nitrification. Therefore, the mechanism of how plant root exudates affect nitrogen removal microbe communities has not been well quantified.

Lake ecosystems are important biological nitrogen removal systems, which often have higher denitrification rates than that of the estuaries and oceans (Mulholland et al., 2008; Pina-Ochoa and Alvarez-Cobelas, 2006). As the main ecological group in shallow lakes, submerged macrophytes play an important role in alleviating eutrophication by assimilating the excessive nitrogen (Dhote and Dixit, 2009; Engelhardt and Ritchie, 2001; Xie et al., 2013) and more importantly by stimulating the nitrification and

denitrification processes through changing the micro-environments for relevant microbes (Forshay and Dodson, 2011; Søndergaard et al., 2010; Soana et al., 2015). The submerged macrophytes can also affect the rhizosphere denitrifier abundance by secreting roots exudates, such as organic acids during the growth and change the rhizosphere micro-environments (Reilly et al., 2000). However, the impacts of the root exudates secreted by submerged macrophytes on the abundance of *nirS*-type denitrifiers and anammox bacteria in lakes as well as on the ecological relationship between these two microbe groups remain unclear (Wang et al., 2017). Therefore, the purpose of this study is to explore the relationship between organic acids secreted by submerged macrophytes and the abundance of *nirS*-type denitrifiers and anammox bacteria in the rhizospheres, and microcosm experiments were set up to investigate 1) if submerged macrophytes have impacts on the denitrifiers and anammox bacteria's communities in their rhizospheres, 2) if different macrophyte species have different impacts during their growth, and 3) the key drivers causing these impacts.

2 Materials and methods

2.1 The experiment design

Submerged macrophytes were cultivated using a three compartment rhizobox with multiple interlayers to separate the root compartment, near-rhizosphere, and non-rhizosphere. The design of the rhizobox was referred to that of Wang et al. (2018). The size of rhizobox was 175×175×115 (length×width×height, mm) filled with lake sediment. Six nylon meshes (pore size < 25 μm) were inserted into the rhizobox near the plant roots (5 mm apart from the main root) to prevent the root growing to the adjacent sediment interlayers, and thus ensure separation of the root exudates and microbe in the sediment interlayers. Therefore, the rhizobox was separated into seven interlayers: the root compartment

(20 mm in width, R), the near-rhizosphere (5 mm in width, five sub-compartments, N1–N5), and the non-rhizosphere (> 5 mm, Non). The rhizoboxes were submerged into the water tank (height of 350 mm) during cultivation to simulate the submersion condition for submerged macrophytes.

The test sediments were taken from Lake Liangzi in Ezhou City, Hubei Province, China, where the water quality characteristics were suitable for the growth of submerged macrophytes. The nylon meshes were filled with sediment and fixed in the rhizobox, then the root compartment and non-rhizosphere were filled with sediment. Three dominant submerged macrophytes in Lake Liangzi were selected to conduct the experiment: *Hydrilla verticillata, Potamogeton maackianus,* and *Vallisneria natans*. In April 2015, five apical shoots of *H. verticillata* (about 10 cm in height), and five seedlings of *P. maackianus* and *V. natans* (about 10 cm in height) were planted separately in the root compartment of different rhizoboxes with 3 replicates for each species. When the roots filled with the root compartment (sample day 0), the sediment samples for each plant were started to collected from seven interlayers (R, N1-N5, and Non). One-third of the plant leaves turned to yellow at day 40, so the previous four sampling periods (day 0, 10, 20 and 30) were considered to be the mature stage, while day 40 was considered to be the decline stage. One portion of the sediment sample was stored at 4 ºC for physical and chemical analysis (concentrations of NH_4^+-N、NO_3^--N、NO_2^--N and organic acids). Another portion of the sediment samples was air-dried for pH determination, and the rest portion was stored at -80 ºC to analyze the community structure and abundance for the *nirS*-type denitrifiers and anammox bacteria. The sediment samples collected from the seven interlayers (R, N1-N5, and Non) were used for microbial quantitative analysis. All samples for each plant in one rhizobox were combined for cloning and sequencing the community structures.

2.2 Physical and chemical indicators in rhizosphere sediments

Sediment pore-water was extracted by centrifuging the moist sediments at 4000 rpm for 10 min, and the supernatants were filtered through a 0.45 μm syringe filter. The obtained pore-water were used to measure the concentrations of NH_4^+-N, NO_3^--N and NO_2^--N using a continuous flow analyzer (SEAL Analytical Inc., Germany) in the laboratory. The air-dried sediments were sieved through a 0.85 mm sieve, and 10 g sediment was well-mixed with 25 mL distilled water, the settled for 30 mins. The pH value of the water above the settled sediment was read directly by a pH meter (METTLER TOLEDO, Switzerland).

2.3 Concentrations of organic acids in rhizosphere sediments

The moist sediments were centrifuged at 12000 rpm at 5 °C for 30 mins, and the collected supernatants were filtered through a 0.22 μm syringe filter. These filtrates were used to analyze the organic acid concentrations by Triple TOF^{TM} 4600 LC/MS/MS (AB SCIEX, USA). Main reagents used were: standard products of tartaric acid, malic acid, succinic acid, malonic acid, citric acid, acetic acid and oxalic acid which purchased from Aladdin Reagent.

The LC/MS/MS analysis conditions: Chromatographic column was Agilent ZORBAX SB-Aq (4.6 mm×150 mm, 5 μm). Mobile phase A (100% methanol): B (0.1% formic acid solution) = 5: 95, injection volume 5 μL, flow velocity 0.4 mL min^{-1}. ESI was negative ion mode, and the operating parameters were as follows: capillary voltage 2000 V, 60 psi spray pressure, dry gas velocity of 11 L min^{-1}, dry gas temperature of 350 °C.

2.4 Genomic DNA extraction, cloning, and sequencing

The genomic DNA of the sediment samples were extracted using the Fast DNA Spin Kit for Soil (Qbiogene Inc., USA). The primer sequences for the PCR amplification are listed in Table S1. The reaction mixtures were: I-5™ 2X High-Fidelity Master Mix 12.5μL, primer F (10 mmol L^{-1}) 1 μL, primer R (10 mmol L^{-1}) 1 μL, DNA template (20–50 ng) 1 μL, and double-distilled water (ddH_2O) 9.5 μL. The PCR procedures were pre-denaturation for 5 mins at 98 °C, followed by 35 cycles of denaturation for 10 s at 98 °C, then annealing for 10 s at 56 °C for *nirS* gene and 59 °C for anammox 16S rRNA gene, followed by 20 s elongation at 72 °C, and a final elongation step at 72 °C for 5 mins. The agarose gel (1.0%) electrophoresis was used to selected the PCR products. The PCR products were purified immediately using the SK8131 Pure Kit (Tsingke, Wuhan, China), cloned into the vectors (Tsingke, Wuhan, China) and transformed into competent Escherichia coli cells (Tsingke, Wuhan, China). The vector-specific primer was used to verify positive transformants, and the positive clones were randomly selected for sequencing for each sample.

The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers MH388043-MH388236 for *nirS* gene and MK112279-MK112452 for anammox 16S rRNA gene.

2.5 Real-time quantitative PCR (qPCR)

In this study, the primer sequences for the qPCR are listed in Table S1. The reaction mixtures were: SybrGreen qPCR Master Mix 5 μL, primer F (10 mM) 0.5 μL, primer R (10 mM) 0.5 μL, DNA template 1 μL, and ddH_2O 3 μL. The PCR condition was 95 °C pre-denaturation for 15 min, followed by 40 cycles of 95 °C denaturation for 10 s, 56 °C for *nirS* gene and 59 °C for anammox 16S rRNA gene annealing

for 30 s, 72 °C elongation for 30 s. Negative controls with no template DNA but all other reaction mixtures were added in parallel to exclude the possibility of contamination. Standard curve coefficients of variation and efficiencies were as follows: *nirS* (R^2 = 0.992, efficiency = 94.7%) and anammox 16S rRNA (R^2 = 0.992, efficiency = 92.4%), and the dissolution curves were single peak.

2.6 Statistical analysis

All data were tested for normality before the statistical analysis by SPSS 20.0 (SPSS Inc., USA). The significant differences (Tukey's test, $P < 0.05$) for environmental data amongst all sediment interlayers of different submerged plants were also detected by the SPSS. All sequences were classified into operational taxonomic units (OTUs) with 97% sequence similarity cut-off using Mothur software (Version 1.36.1). One representative sequence was then selected from each OTU for the phylogenetic analysis. Phylogenetic analyses were conducted with Mega software (Version 6.0). Colinear analysis was used to remove the non-significant environmental factors to affect the functional gene abundance. The impacts of the selected significant environmental factors on functional gene abundance were analyzed using Redundancy analysis (RDA) (R Core Team, Version 3.5.0).

3 Results and discussion

3.1 Physical and chemical properties of rhizosphere sediments

For all three submerged species in this study, the pH values of the root compartments and non-rhizospheres were higher than those of the near-rhizospheres, and the pH values of the near-rhizospheres decreased with the longer distance to the roots. However, these pH differences between the root

compartment, non-rhizosphere, and near-rhizosphere were only significant for *H. verticillata* sediments ($P < 0.05$; Table S2 a), rather than that of *P. maackianus* and *V. natans* ($P > 0.05$; Table S2b, c).

In the rhizosphere sediments, the concentrations of NH_4^+-N was the highest, followed by the concentration of NO_3^--N, and concentrations of NO_2^--N were the lowest. With the distance increasing from the roots, the concentrations of NH_4^+-N in the *V. natans* rhizospheres increased significantly ($P < 0.05$), and there was no significant difference for that of *H. verticillata* and *P. maackianus* ($P > 0.05$). The concentrations of NO_3^--N and NO_2^--N in the rhizosphere sediments of the three species showed no significant difference ($P > 0.05$).

3.2 Temporal variations of organic acid concentrations

There were seven organic acids found in the rhizosphere sediments of the studied submerged macrophytes, and acetic acid, oxalic acid, succinic acid and malic acid were the dominant ones with higher concentrations (Fig. 1).

In the mature stage (day 0 to 20) of three submerged macrophytes, the concentrations of total organic acid in the rhizosphere sediments showed a tendency to increase first and then decrease with the increase of the distance from the roots. However, at day 30 the concentrations of these acidic substances in seven sediment interlayers were similar for every plant. In addition, the concentrations of these substances fluctuated greatly in the *P. maackianus* rhizospheres when entered to the decline stage after 40 days, while those of the other two plants fluctuated much less.

In this study, the increase of malic acid concentration was the most obvious one compared to other organic acids. Previous studies have shown that the concentrations and composition of root exudates could change at different growth stages of plants (Neumann et al., 1999). Roots could adjust the secretion

of organic acids to the rhizosphere to cope with certain stress conditions (Henry et al., 2007). With increased period of salt stress, the malic acid releasing potential of *Acacia ampliceps* was increased (Abbas et al., 2016). He et al. (2016) found that enhanced UV-B radiation increased both the oxalic acid and the succinic acid content at maturity stages in rice root exudates, however, the contents of tartaric acid and the malic acid were reduced. In this study, the leaves of submerged macrophytes turned yellow and photosynthesis might be weakened, and the plants would adjust the secretion of organic acids to adapt to this change. Hence, there were some changes in the content of organic acid.

(Fig. 1)

3.3 Phylogenetic analysis

3.3.1 The *nirS*-type denitrifiers community structure

After the establishment of clonal libraries for the sediment samples of the three plants, 68, 62 and 64 *nirS* gene sequences were obtained for plant *H. verticillata, P. maackianus,* and *V. natans*, respectively. All sequences were classified into representative OTUs with 97% sequence similarity cutoff, and 43 OTUs were obtained. These representative OTUs were chosen to construct the phylogenetic tree (Fig. 2).

To facilitate the analysis, the phylogenetic tree was divided into 4 clusters: Cluster 1 contained 38 OTUs, which was *Pseudomonas aeruginosa*; Cluster 2 contained 1 OTU, which was *Sulfuricaulis limicola*; Cluster 3 contained 2 OTUs, which was *Herbaspirillum sp.*; Cluster 4 contained 2 OTUs, which was *Azospirillum brasilens*. Cluster 1 contained the largest number of sequences compared to other Clusters, and 91.24% of sequences were clustered into this group. In the phylogenetic tree (Fig. 2), these sequences were highly homologous to the known sequences in lake sediments and estuarine sediments in China, and the denitrification sequences were mainly distributed in the Yangtze river basin.

The number of *nirS* gene OTUs in the rhizosphere were 32, 9 and 10 for plant *H. verticillata, P. maackianus,* and *V. natans*, respectively. The number of *nirS* gene OTUs distributed in the *H. verticillata* rhizosphere was much higher than that in *P. maackianus* and *V. natans* rhizospheres, which reflected the highest diversity of *nirS* gene in the rhizosphere of *H. verticillata*, indicating that plant species would have an impact on microbial diversities.

(Fig. 2)

3.3.2 The anammox bacterial community structure

After the establishment of clonal libraries for the sediment samples of the three plants, 63, 54 and 57 anammox 16S rRNA gene sequences were obtained, respectively, for plant *H. verticillata, P. maackianus,* and *V. natans*, respectively. All sequences were classified into representative OTUs with 97% sequence similarity cutoff, and 18 OTUs were obtained. The phylogenetic tree of anammox bacterial community shown in Fig. 3.

To facilitate the analysis, the phylogenetic tree was divided into 6 clusters: Cluster 1 was *Candidatus Jettenia sp.*; Cluster 2 was *Candidatus Brocadia sp.*, Cluster 3 and Cluster 4 were *Candidatus Kuenenia sp.*; Cluster 5 was *Deferrisoma camini*; Cluster 6 was *Pelobacter acetylenicus*. *Candidatus Kuenenia* and *Candidatus Brocadia* have been known as the two most common genera in anammox bacteria, and *Candidatus Jettenia* was mainly distributed in freshwater lakes (Oshiki et al., 2016). The above three common genera were all detected in this study. Another common anammox genus is *Candidatus Scalindua*, which was not detected in this study. This is likely due to *Candidatus Scalindua* is mainly distributed in the marine environment (Awata et al., 2015).

The OTU numbers of *H. verticillata*, *P. maackianus* and *V. natans* were 5, 11 and 9, respectively.

The number of anammox gene OTUs distributed in the rhizosphere of *P. maackianus* was much higher than that of *H. verticillata* and *V. natans*, indicating that the diversity of anammox gene in the rhizosphere sediments of *P. maackianus* was the highest. *H. verticillata* and *P. maackianus* were more likely to exist in the same OTU, while *V. natans* were mostly in a separate OTU, indicating that the anammox bacterial community of *H. verticillata* and *P. maackianus* were similar, which was quite different from the *V. natans*. By comparing the sequences in the known environments, it was found that the sequences in the rhizosphere sediments of the three plant species were mainly highly homologous with those in shallow lakes and estuaries. It was worth noting that most of these lakes and estuaries came from the Yangtze river basin, indicating that the anammox sequences obtained in this study widely existed in the ecological environments of the Yangtze river basin.

(Fig. 3)

3.4 Temporal variations of *nirS*-type denitrifier and anammox bacteria abundance

As shown in Fig. 4, the abundance of *nirS*-type denitrifiers in *H. verticillata, P. maackianus* and *V. natans* rhizobox were 2.6×10^7-7.3×10^9, 1.7×10^7-1.5×10^9 and 2.8×10^7-3.6×10^{10} copies g^{-1} dry sediments, respectively. With the increase of the distance from the roots, the abundance of *nirS*-type denitrifiers in the rhizosphere sediments showed a trend of decrease then increase for each sampling period.

The abundance of anammox bacteria were 8.5×10^7-4.5×10^9, 4.4×10^7-5.1×10^9, and 5.2×10^7-2.2×10^9 copies g^{-1} dry sediments in *H. verticillata*, *P. maackianus*, and *V. natans* rhizobox, respectively. With the increase of the distance from the roots, the abundance of anammox bacteria in the rhizosphere sediments of three submerged macrophytes also showed the trend of decrease then increase for each sampling period.

When submerged macrophytes entered into the decline stage (40 d), the abundance of the anammox bacteria in the rhizospheres showed an overall upward trend compared to the mature stage, while *nirS*-type denitrifiers decreased to a certain extent. This result indicates that anammox bacteria can adapt to the rhizosphere environment of the decline plants more quickly than the *nirS*-type denitrifiers, which enables them to obtain a dominant position in the competition between the two bacteria when plants decline.

The average abundance of *nirS*-type denitrifiers were 1.8×10^9, 2.7×10^9, and 7.6×10^9 copies g^{-1} dry sediments in *H. verticillata, P. maackianus*, and *V. natans* rhizobox, respectively. The lowest average abundance of *nirS*-type denitrifiers was in *H. verticillata* rhizosphere, indicating that *H. verticillata* had the strongest inhibitory effect on the denitrification process. The average abundance of anammox bacteria in *H. verticillata, P. maackianus*, and *V. natans* rhizobox were 9.4×10^8, 8.8×10^8, and 1.2×10^9 copies g^{-1} dry sediments, respectively. The average abundance of anammox bacteria in the *P. maackianus* rhizosphere was the lowest, indicating that *P. maackianus* had the strongest inhibitory effect on the anammox process. Compared the three plants in this study, *V. natans* was the most conducive to nitrogen removal in lake sediments. Therefore, in order to evaluate the nitrogen removal effect of submerged macrophytes in lakes more accurately, not only the presence of aquatic plants but also the types of plants should be considered in the future.

Many studies had shown that plant root exudates, such as amino acids, organic acids, sugars, contributed to microbial growth, thus increasing the microbial abundance (Haichar et al., 2014; Somenahally et al., 2011). However, the concentrations of organic acids were lower in the root compartments and non-rhizospheres which showed higher microbial abundance in this study. By analyzing the correlation between the total organic acid concentrations and microbial abundance, it was

found that the total organic acid concentrations were negatively correlated with the abundance of *nirS*-type denitrifiers (r = -0.292, $P < 0.01$) and anammox bacteria (r = -0.203, $P < 0.05$). Organic acids were the most common carbon substrates, and they were important carbon source of microorganisms. This negative impact of total organic acids for *nirS*-type denitrifiers in this study was more likely that organic acids promoted the rapid growth of other types of heterotrophic bacteria, and denitrifying bacteria were in a weak position in the process of competing for reaction substrates (Shi et al., 2013). Anammox bacteria are chemoautotrophic microorganisms, which generally take inorganic carbon as the only carbon source, and have no demand for organic carbon source (Strous et al., 1999). The presence of organic matters often leaded to the inhibition of anammox process (Jin et al., 2012), or even the loss of anammox function (Tang et al., 2010; Chamchoi et al., 2008). In addition, the organic matter was beneficial to the growth of heterotrophic bacteria, and the reproduction of heterotrophic bacteria will make anammox bacteria at a disadvantage in the process of competing for substrates (Zhao et al., 2013; Lisa et al., 2015). Therefore, these adverse effects of organic matter may be the reasons for the results that the negative correlations between anammox bacteria abundance and total organic acids in this study. Another reason may be that the release of higher organic acid concentrations in the near-rhizosphere sediments decreased the pH compared to the root compartment and non-rhizosphere sediments, and the lower pH inhibited the growth of *nirS*-type denitrifiers and anammox bacteria. This assumption was supported by the fact that the pH of near-rhizosphere was lower than that of the root compartment and non-rhizosphere (Table S2).

(Fig. 4)

3.5 Correlations between functional gene abundance and environmental factors

Through the collinearity analysis on the environmental factors, 4 environmental factors,

concentrations of NO_3^--N, NO_2^--N, citric acid and oxalic acid were found to have significant influence on the functional gene abundance. Concentrations of citric acid and oxalic acid were negatively related to the *nirS* gene abundance, and anammox 16S rRNA gene abundance was negatively related to the oxalic acid concentration (Fig. 5). Concentrations of citric acid and oxalic acid were negatively correlated with the microbial abundance, which was consistent with the result for total organic acids. The same reason may be that the decrease of pH due to increased organic acid concentrations in the sediments inhibited the growth of bacteria.

Concentrations of NO_2^--N was positively related to *nirS* gene abundance, which is consistent with the findings from Li et al. (2018) in constructed wetlands. The *nirS* gene involved in the process of reducing NO_2^- to NO, and NO_2^-, as the substrate for *nirS* gene activities, whose concentration is often positively correlated with *nirS* abundance. Concentrations of NO_3^--N was positively correlated with anammox 16S rRNA gene abundance, which was consistent with the result in Chesapeake Bay (Daigger, 2014). Concentrations of NO_3^--N may affect the production of NO_2^--N through reduction or other unknown reactions, thus affecting the abundance of anammox bacteria (Sun et al., 2014). Concentrations of NH_4^+ is an important factor affecting the abundance of *nirS* gene and anammox 16S rRNA gene in lake sediments (Dang et al., 2010). However, the role of $NH_4^+$was not obvious in this study, probably because the content of NH_4^+ in the rhizobox was relatively high and was not a limiting factor for the related gene activities. This study showed that the NO_2^--N concentration was significantly positively correlated with the ratio of *nirS*/anammox 16S rRNA, indicating that NO_2^--N was beneficial to *nirS* gene in the competition between the *nirS* and anammox 16S rRNA.

(Fig. 5)

4 Conclusion

The dominant organic acids in the rhizosphere sediments of submerged macrophytes were acetic acid, oxalic acid, succinic acid and malic acid. Concentrations of the total organic acids in the root compartment and non-rhizosphere sediments were lower than that of the near-rhizosphere sediments. Conversely, the abundance of *nirS*-type denitrifiers and anammox bacteria in the root compartment and non-rhizosphere sediments were higher than that of the near-rhizosphere sediments. This study also found the abundance of *nirS*-type denitrifiers and anammox bacteria were negatively correlated with total organic acids concentrations. More specifically, the abundance of *nirS*-type denitrifiers and anammox bacteria in this study were mainly negatively affected by the citric acid and oxalic acid concentrations in the rhizospheres, and positively related to the concentrations of NO_3^--N and NO_2^--N in the rhizospheres. The highest community diversity of *nirS*-type denitrifiers amongst the three plants was found in the *H. verticillata* rhizosphere sediments, while the highest community diversity of anammox bacteria was in *P. maackianus* rhizosphere sediments. The results showed that the species of submerged macrophytes can affect the community structure and abundance of *nirS*-type denitrifiers and anammox bacteria. The results of this study are conducive to evaluate the nitrogen removal processes more accurately with the presence of submerged macrophytes in nature. Our results are also helpful to select submerged macrophytes which have less inhibition on the nitrogen removal process of microorganisms in the phytoremediation of lakes, thereby increasing the nitrogen removal effect.

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