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Nitrogen-loss and associated microbial communities in sediments from the Yangtze Estuary and adjacent sea

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Introduction: Denitrification and anammox play the crucial role for N removal processes in coastal ecosystems, but the ecological distribution of denitrifying and anammox microorganisms and their N removal rates in the Yangtze Estuary and its adjacent sea are required in-depth analysis.

Methods: Here, we utilized high-throughput sequencing, qPCR, and ¹⁵N isotope to reveal the community structure and function of denitrifying and anammox microorganisms in the surface sediments from Yangtze Estuary and adjacent sea.

Results: The results suggested that the gene abundances of *nirS* and *nirK* for denitrifiers were higher than AMX 16S rRNA for anammox bacteria. The genera composition of *nirS*- and *nirK*-encoding denitrifiers communities showed different distribution patterns. Furthermore, *Candidatus Anammoximicrobium* dominated the anammox community, implying the anammox oxidation capacity of the other genera should be noted in marine sediments.

Discussion: Compared to anammox, denitrification was the dominant contributor of nitrogen removal process and contributed 73.5% on average. The sediment Chl_a was the key factor to regulate denitrification and anammox rates, indicating the fresh organic matter was more labile and easier to be utilized by NO_x⁻ removal processes.

KEYWORDS

denitrification, anammox, *nirS/nirK*, AMX 16S rRNA, ¹⁵N isotope

Introduction

Nitrogen is a necessary nutrient element for all kinds of life. However, due to the rapid expansion of various anthropogenic N input, numerous estuaries and adjacent seas have high nitrogen loading, causing serious environmental problems such as eutrophication globally (Howarth, 2008), increased harmful algal blooms (Gobler, 2020), biodiversity loss (Worm et al., 2006), and increasing nitrous oxide (N₂O) emission (Murray et al., 2015; Lin and Lin, 2022). Excess anthropogenic N inputs have severely affected N-balance in coastal ecosystems (Zhao et al., 2020b), thus, N pollution has been widely concerned in global.

Both denitrification and anammox are the critical pathways in microbially-mediated N removal processes, which can remove excess nitrogen in estuarine and coastal ecosystems. Under hypoxic or anaerobic conditions, denitrification convert nitrate (NO₃⁻) to N₂O or N₂ into the atmosphere, while anammox can oxidize ammonium (NH₄⁺) with nitrite (NO₂⁻) to N₂ (Thamdrup and Dalsgaard, 2002; Medhi et al., 2017). It has been reported that ~45% of reactive N can be removed by denitrification in estuarine sediments (Seitzinger et al., 2006), which was considered as the main N removal process in aquatic ecosystems before the discovery of anammox (Trimmer et al., 2003; Fernandes et al., 2012). In 1995, the anammox process was found in a wastewater treatment plant (Mulder et al., 1995). Additionally, it has also been detected in marine sediments, freshwater sediments, and anaerobic water column (Thamdrup and Dalsgaard, 2002; Trimmer and Nicholls, 2009; Lisa et al., 2014). Some studies showed that anammox was the main N removal process in the marine environments, contribution up to 67% of N₂ production (Dalsgaard and Thamdrup, 2002; Kuypers et al., 2003). Hou et al. (2015) reported that 20.7% of inorganic nitrogen can be removed by anammox in coastal wetland ecosystems in China, reflecting a non-negligible role of anammox to N removal in marine ecosystems. However, denitrification and anammox have varied in their contribution to N removal in marine ecosystems, low anammox rates and even lower denitrification rates were found in most sites, while high denitrification rates were detected in a few isolated places (Babbin et al., 2014). C/N ratio was the crucial environmental factor to regulate the balance between denitrification and anammox, resulting in the different ratios of denitrification to anammox (Babbin et al., 2014). In addition, the two nitrogen removal processes have different effects on N₂O emissions, as well on C mineralization (Ravishankara et al., 2009; Babbin et al., 2020). Therefore, further explorations about denitrification and anammox are critical for maintaining and conserving the health of coastal ecosystems.

Different molecular markers have been broadly utilized to understand and identify denitrifying and anammox microorganisms. Two nitrite reductases (cytochrome cd 1 and copper-containing nitrite reductases) catalyze NO₂⁻ to form a gaseous product NO, which are the key enzymes in the

denitrification process (Beman, 2014). The genes (*nirS* or *nirK*) encoding nitrite reductase are widespread and efficient biomarker for precise and direct identification of denitrifying communities in numerous environments, such as the aquatic ecosystems and sediments (Abell et al., 2010; Mosier and Francis, 2010; Xie et al., 2020). The 16S rRNA gene and the other functional genes (*hzo* and *hzs*) are both used to investigate and detect the community structure and abundances of anammox bacteria (Harhangi et al., 2012; Yang et al., 2020). Due to high specificity and good amplification efficiency, 16S rRNA gene was broadly employed to identify anammox bacteria in diverse environments and enrichment cultures (Li et al., 2010; Yang et al., 2020). In addition, the abundance, community composition and rates of denitrification and anammox can be regulated by multiple environmental parameters, for instance, salinity (Bernhard et al., 2005), pH (Nicol et al., 2008), nitrogen availability (Di et al., 2010), and sediment total organic carbon (TOC) (Zheng et al., 2019). Thus, it is essential to common research of the diversity, abundance, and activity of denitrification and anammox under different geographic environmental factors, to better understand the contribution and role of N removal *via* denitrification and anammox, and further reveal the niche of denitrifiers and anammox bacteria in coastal ecosystems.

The Yangtze Estuary and its adjacent sea, the East China Sea (ECS), was chosen as the research region to investigate the characteristics of denitrifiers and anammox bacteria. Yangtze River, the greatest river in Asian regions, can transport amounts of anthropogenic nitrogen *via* estuary region to adjacent ECS (Chen et al., 2016). Increasing nitrogen has resulted in significant eutrophication and the appearance of toxic algal blooms in coastal ecosystem (Smith and Schindler, 2009; Anderson et al., 2012). In addition, the Yangtze River also transports most terrestrial sediments through currents into the ECS (Lim et al., 2007). The benthic sediments serve as the significant sink for reactive nitrogen and play the vital role in the N biogeochemical cycle (Lin et al., 2017). Meanwhile, the complex benthic environment favors the growth and survival of microorganisms (Basha et al., 2018). Liu and Wang demonstrated that the diversity of *nirK*- gene-encoded encoding denitrifiers was lower than that of *nirS*-encoding denitrifiers by high-throughput sequencing within sediments from northern ECS (Liu et al., 2018; Wang et al., 2019). Lin et al. (2017) confirmed that denitrification rates were higher than anammox *via* ¹⁵N isotope tracer incubation experiment in surface sediment from Yangtze Estuary and its adjacent sea. Currently, molecular detection is used to understand the microbial community, and ¹⁵N isotope tracer is used to study the denitrification and anammox rates. However, few studies have focused on common research of the community structure, abundance, and activity of anammox and denitrifiers bacteria in coastal ecosystems, to better understand the role of denitrifiers and anammox bacteria in N removal processes.

Herein, we studied the characteristics of denitrifiers and anammox bacteria and environmental parameters in surface

sediments (0–5 cm) from the Yangtze Estuary and its adjacent sea. The community structures and abundances were analyzed using the high-throughput sequencing and qPCR, as well as the rates of denitrification and anammox were tested by ^{15}N isotope tracer technology. The main objective of this work is to reveal possible roles of denitrifiers and anammox bacteria in nitrogen removal processes as well as how these microorganisms respond to various environmental factors in coastal ecosystems.

Materials and methods

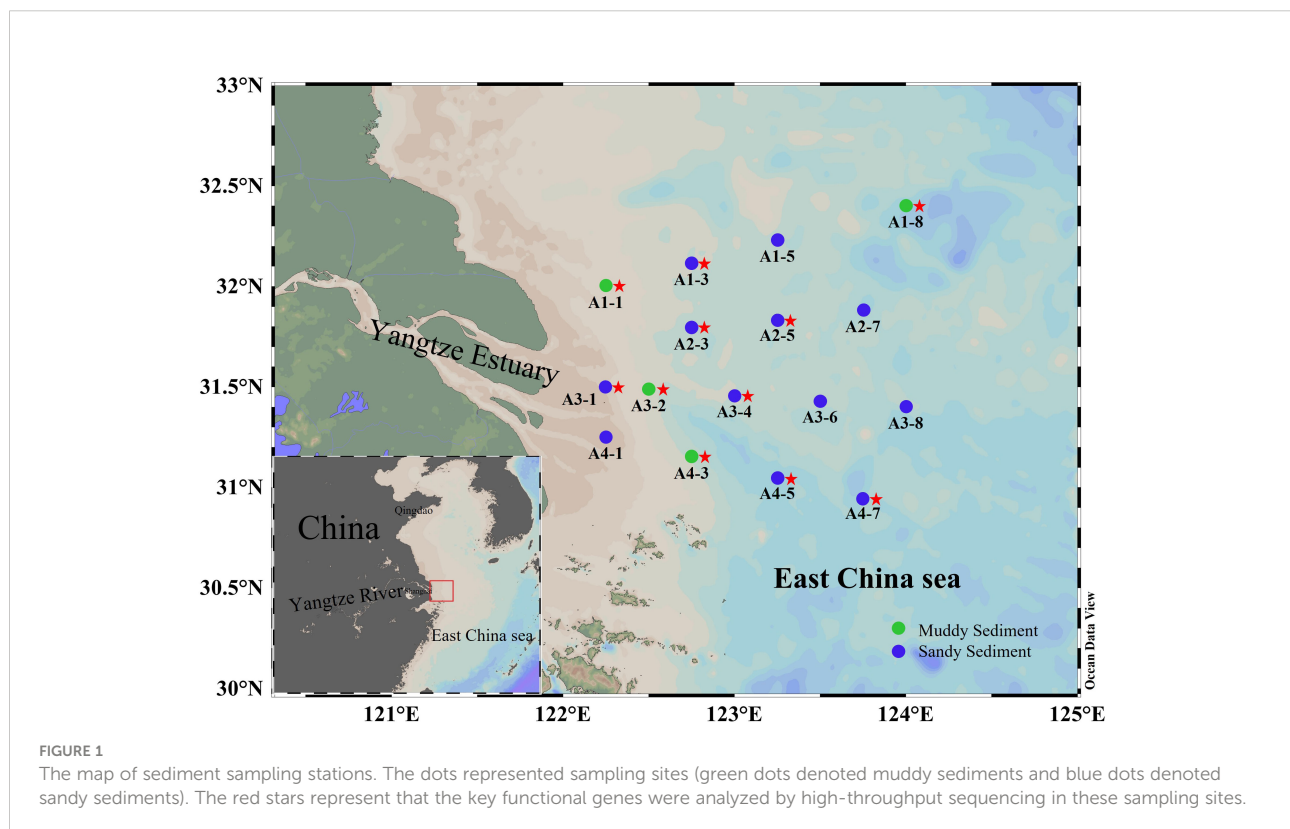
Sampling collection and environmental factor analysis

The bottom water and surface sediments have been gathered from 16 stations in the Yangtze Estuary and adjacent sea from 1 to 6 March 2022 (Figure 1). At each station, surface sediments (0–5 cm) was extracted from the box cores, mixed homogeneously, and placed into air-tight sterile plastics bags. The sediment samples were then subdivided into two parts for different analyses. One part immediately stored at 4°C for denitrification and anammox rates incubation. Another was stored on board (-20°C) and then transferred to laboratory storage (-80°C) for DNA extraction and molecular analysis. The bottom water was filtered through a filter (0.45 μm) and

stored in polyethylene bottles (-20°C). The salinity was determined onsite by HQ40d Water Analyzer (Hach, Loveland, USA). The sediment water content was calculated using wet-dry weight method (Klute, 1986). The NH_4^+ , NO_3^- and PO_4^{3-} in bottom water were analyzed using QuAAtro nutrient autoanalyzer (Seal Analytical, Germany). 2 M KCl was used to extract the exchangeable NH_4^+ , NO_2^- and NO_3^- from the sediments and then determined by QuAAtro nutrient autoanalyzer (Seal Analytical, Germany). Sediment TN and TOC were detected by the elemental analyzer (Vario EL cube, Germany). A laser particle sizer analyzer (LS 13320, Beckman, USA) was used to measure sediment grain size ($M\Phi$). The sediment Fe(II) and Fe(III) contents were measured by phenanthroline spectrophotometric method (Lovley and Phillips, 1987). Under the dark condition of 4°C for 24 hours, Sediment Chl a can be extracted with 80% (v/v) acetone. A UV-5200 visible spectrophotometer was used to measure the absorbance of Chl a after centrifuging these extracts at 2200 g for 10 min.

Measurement of denitrification and anammox rates

The potential denitrification and anammox rates were measured by ^{15}N isotope-tracing technique (Lin et al., 2017).



Briefly, the laboratory slurry incubation experiments were made with the *in situ* benthic sterilized seawater and the sediments at a seawater/sediment mass ratio of 7:1, and the homogeneous mixture was fed with helium gas for 30 min before transferring into 12-mL vials (Labco Exetainers, UK). Subsequently, these 12-mL vials were in dark preincubated for 24 h to eliminate residual dissolved oxygen, nitrite, and nitrate. After the preincubation, three treatments were given to these vials as follows: (1) $^{15}\text{NH}_4^+$ ($100\ \mu\text{mol N L}^{-1}$), (2) $^{15}\text{NO}_3^-$ ($100\ \mu\text{mol N L}^{-1}$), and (3) $^{15}\text{NH}_4^+ + ^{14}\text{NO}_3^-$ ($100\ \mu\text{mol N L}^{-1}$). After 0 h and 8 h incubation, these vials were spiked with ZnCl_2 solution (200- μL 50%) to stop microbial activity. Produced $^{29}\text{N}_2$ and $^{30}\text{N}_2$ concentrations during incubation were determined by the membrane inlet mass spectrometry (MIMS, Hiden Analytical Ltd, UK), and denitrification and anammox rates were calculated according to previous methods (Thamdrup and Dalsgaard, 2002; Song et al., 2016).

DNA extraction and quantification for denitrifiers and anammox bacteria

Total DNA extraction was performed from 0.5 g of homogenized sediment samples by PowerSoil[®] DNA Isolation Kit (MoBio, USA). The obtained DNA was used as the template for real-time qPCR analysis with the primer pairs *cad3A*/*R3cd* for the *nirS* gene, and *FlaCu*/*R3Cu* for *nirK* gene, and *Amx-808-F*/*Amx-1040-R* for Anammox 16S rRNA gene (Supplementary Table 1). Reactions were carried out using ABI PRISM[®]7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The 20- μL qPCR mixture reaction system contained FastStart Universal SYBR Green Master (ROX) (10 μL , Roche, Germany), ddH_2O (6.6 μL), each primer (0.6 μL , 10 μM), bovine serum albumin (0.2 μL , 20 $\text{mg}\cdot\text{mL}^{-1}$), and template (2.0 μL). The qPCR reactions of *nirS*, *nirK*, and Anammox 16S rRNA gene were performed as the following: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, and 58°C for 40 seconds, 72°C for 60 seconds (Throbäck et al., 2004; Hamersley et al., 2007). The significant linear relationships ($R^2 > 0.996$) were produced with a 10-fold dilution series (10^2 – 10^9 copies) of standard plasmids containing the key functional genes, and the qPCR efficiency was 84%, 110%, and 92%, respectively. The abundance of the key functional genes can be calculated by the standard curves. The melting curves contained a single peak, and non-specific amplification was not found by agarose gel electrophoresis, demonstrating amplification specificity.

High-throughput sequencing

11 sampling stations have been picked from 16 stations according to the class of muddy and sandy sediments (the value of median grain size ($> 64\ \mu\text{m}$) was classified to sandy sediment).

The PCR amplicons of key functional genes were from the same primer pairs with qPCR (*cd3A*/*R3cd* and *FlaCu*/*R3Cu* for denitrifiers, and *Amx-808-F*/*Amx-1040-R* for anammox bacteria). The PCR conditions were as follows: initial denaturation was performed at 98°C for 2 min, followed by control at 98°C for 15 s, 55°C for 30 s and 72°C for 30 s (25–30 cycles), and finally extended at 72°C for 5 min. The Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) were used for the purification and quantification of PCR amplicons, respectively. The Illumina novaSeq PE250 platform (Shanghai, China) was used for the paired-ended sequenced of high-throughput sequencing of *nirS*/*nirK* and AMX 16S rRNA genes. The QIIME2 (2019.4) was used to incorporate, quality-filter, and dereplicate the sequences. High quality sequences with 97% similarity level were clustered into OTUs based on Vsearch (v2.13.4_linux_x86_64), and then representative OTUs were output. The raw sequences obtained in this study for *nirS*, *nirK* and AMX 16S rRNA was kept in NCBI under the accession numbers of PRJNA891470, PRJNA891480, and PRJNA891491.

Statistical analyses

The map of the sampling stations and the distribution of environmental parameters were drawn using Ocean Data View (ODV 5.2.1). The differences of *nirS*, *nirK* and AMX 16S rRNA gene abundances in the same station were studied using one-way analysis of variance (ANOVA). Correlation between the three microbial communities and environmental factors were identified with CCA/RDA analyses with Monte Carlo tests by Canoco 5 software. The Pearson and Spearman correlation analysis of denitrifiers and anammox bacteria with environmental parameters was created by IBM SPSS statistic 26 and R (version 4.2.1, R package corrplot) (Wei and Simko, 2021). MEGA11, TBtools and Origin 2022b software were used for graphics. Finally, the phylogenetic tree was visualized and beautified by Evolview online tool (<http://www.evolgenius.info/evolview-v2/>).

Results

Station characteristics

The physicochemical parameters of bottom water and surface sediments were given in Figure 2. The salinity ranged from 27.3 to 37.1 (Figure 2A). Bottom water NH_4^+ (0.1–3.15 μM), NO_3^- (3.55–23.93 μM), PO_4^{3-} (0.21–0.71 μM) values varied across different sampling sites (Figures 2B–D). The average concentrations of NH_4^+ and NO_3^- in the sediments varied from 0.32–1.20 $\mu\text{g N g}^{-1}$, 0.04–0.86 $\mu\text{g N g}^{-1}$, respectively

(Figure 2E, F). The sediment Fe(II)/Fe(III) ratios were in a range of 0.29–6.19 (Figure 2G). The contents of sediment TN and TOC ranged from 0.23 to 1.19 mg N g⁻¹ with an average of 0.51 ± 0.25 mg N g⁻¹ and from 1.05 to 6.97 mg C g⁻¹ with an average of 2.62 ± 1.65 mg C g⁻¹, respectively (Figures 2H, I). The sediment median grain size showed a greater change. The sampling stations of A1-1, A1-8, A3-2, and A4-3 were muddy sediments (8.83–44.27 μm), while other stations were sandy sediments (93.32–333.41 μm) (Figure 2L). Meanwhile, the C/N ratios (TOC/TN) were higher in muddy sediments (A1-1, A1-8, A3-2, and A4-3) than in sandy sediments (Figure 2J). The average content of Chla in sediment was in a range of 0.12–1.75 μg g⁻¹, with obvious spatial pattern (Figure 2K).

Abundances of denitrifiers and anammox bacteria and their influencing factors

The *nirS/nirK* gene and AMX 16S rRNA gene were utilized to quantify denitrifiers and anammox bacteria abundances in surface sediments, respectively. The abundances of *nirS* gene were ranging between 9.87 × 10⁵ to 2.71 × 10⁷ copies g⁻¹ with the mean value of (1.28 ± 0.93) × 10⁷ copies g⁻¹ (Figure 3A, B). The *nirS* gene abundance had negative correlation with NO₃⁻ concentration in the bottom water ($r = -0.576$, $p < 0.05$) (Figure 4). The *nirK* gene abundances ranged from 4.64 × 10⁵ to 1.26 × 10⁷ copies g⁻¹ with the mean value of (4.52 ± 3.34) × 10⁶ copies g⁻¹ (Figure 3A, B). Compared to *nirS/nirK* gene, the abundances of AMX 16S rRNA gene were obtained between 1.03 × 10⁵ to 2.97 × 10⁶ copies g⁻¹ with the mean value of (1.40 ± 0.84) × 10⁶ copies g⁻¹ (Figure 3A, B). In all surface sediment samples, *nirS* abundances were the highest, followed by *nirK* abundances and then AMX 16S rRNA gene.

Potential rates of denitrification and anammox and their influencing factors

The potential rates of denitrification and anammox were analyzed in all sampling site of the surface sediments (n = 16), which varied considerably, ranging from 0–0.56 nmol N g⁻¹ h⁻¹ and 0–0.091 nmol N g⁻¹ h⁻¹, respectively (Figure 3C, D). The highest denitrification rate was detected at A1-1, whereas the lowest denitrification rate was observed at A3-8. The potential denitrification rates had positive association with sediment water content, NH₄⁺, TN, TOC, C/N, and Chla, while negatively regulated by sediment NO₃⁻ and sediment grain size (Figure 4, Supplementary Table 5). Compared to denitrification rates, the highest anammox rate was detected at A2-3, whereas the lowest anammox rate was observed at A3-4. Anammox was positively correlated with NH₄⁺ and Chla, and negatively correlated with NO₃⁻ (Figure 4, Supplementary Table 5). Moreover, Pearson's correlation analyses showed that

anammox was significantly related to denitrification ($r = 0.710$, $p < 0.01$) (Figure 4, Figure 5A; Supplementary Table 5). The contribution of denitrification to N removal (DEN%) was higher than anammox in most sampling sites except A3-4, A3-6, A3-8, and A4-1 (Figure 5B), with the contribution of 73.5% on average. In addition, DEN% was positively related to water content, NH₄⁺, Fe(II)/Fe(III), TN, TOC, C/N, and Chla, and negatively regulated by NO₂⁻.

Community diversity and composition of *nirS*- and *nirK*-encoding denitrifiers

Totally, 1,219,102 filtered, high-quality *nirS* sequences were acquired from 11 surface sediments samples, ranging from 73,783–172,218 for each sample (Supplementary Table 2). The effective sequences were clustered into 7,536 OTUs based on the 97% similarity level. Additionally, Good's coverage (99.73–99.91%) indicated that most of the *nirS*-encoding denitrifiers had been obtained. The highest OTU numbers (1,186) and Chao1 richness estimators (1267.16) were observed in the samples A4-7. Higher Shannon diversity index and Simpson indices were found in samples A2-3, A4-5 and A4-7 (Supplementary Table 2). Moreover, the diversity of *nirS* gene decreased with the increasing the NH₄⁺ concentration in the bottom water (Shannon: $r = -0.732$, $p < 0.05$; Simpson: $r = -0.897$, $p < 0.01$) (Supplementary Table 6). Compared to the *nirS* gene, 763,468 high-quality reads of *nirK* gene sequences were acquired and 3,151 OTUs were identified (Supplementary Table 3). The diversity and richness of *nirK*-encoding were lower than *nirS*-encoding denitrifiers in all samples. In additionally, the richness of *nirK* gene declined with the increasing NO₃⁻ concentrations in the bottom water (Chao1: $r = -0.606$, $p < 0.05$) and increased with the sediment total nitrogen concentration (Chao1: $r = 0.603$, $p < 0.05$) (Supplementary Table 6). The rarefaction curves showed that the majority of the *nirS*- and *nirK*-encoding denitrifiers was captured in the current study (Supplementary Figures 1A, B).

A total of 50 dominant OTUs of the *nirS* gene were phylogenetically classified into 10 different clusters (Figures 6A, B; Supplementary Figures 2, 3). Within these 10 clusters, only C9 belonged to α-proteobacteria and C5, C6, and C7 affiliated to β-Proteobacteria, whereas the other 6 clusters were belonged to γ-Proteobacteria. C3 only contained OTU01, which was existed exclusively in the A3-2 station, closely affiliating to *Pseudomonas* (Figures 6A, B, Supplementary Figure 3). C8 (belonging to *Marinobacter*), C9 (belonging to *Paracoccus*) and C10 (belonging to *Chromohalobacter*) were observed almost in muddy sediments (Figures 6A, B). According to a redundancy analysis (RDA) with the dominant OTUs, the first two axes (RDA1 and RDA2) explained 79.97% of the cumulative variance in the *nirS*-encoding denitrifiers communities (Figure 6C). The bottom water NH₄⁺

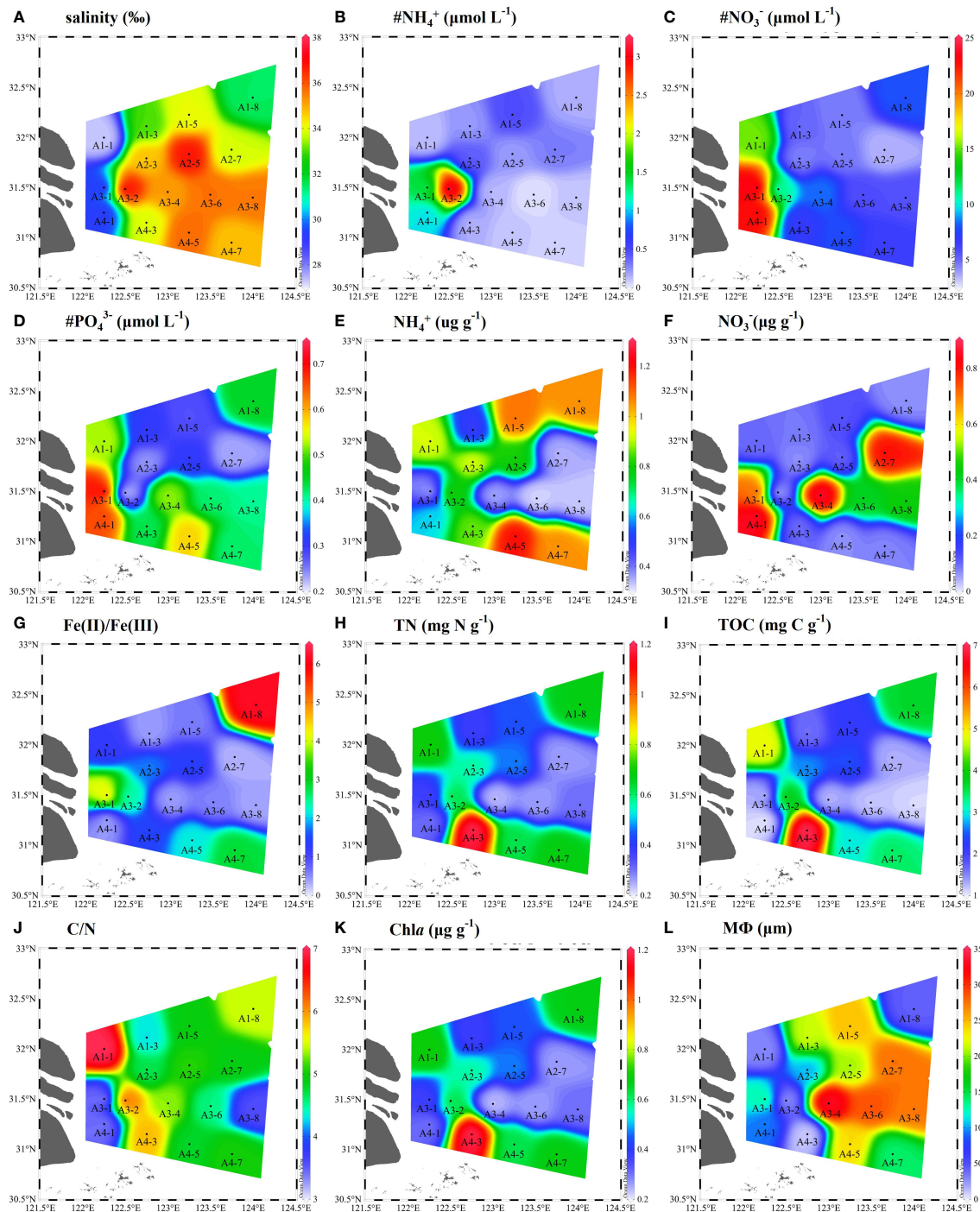
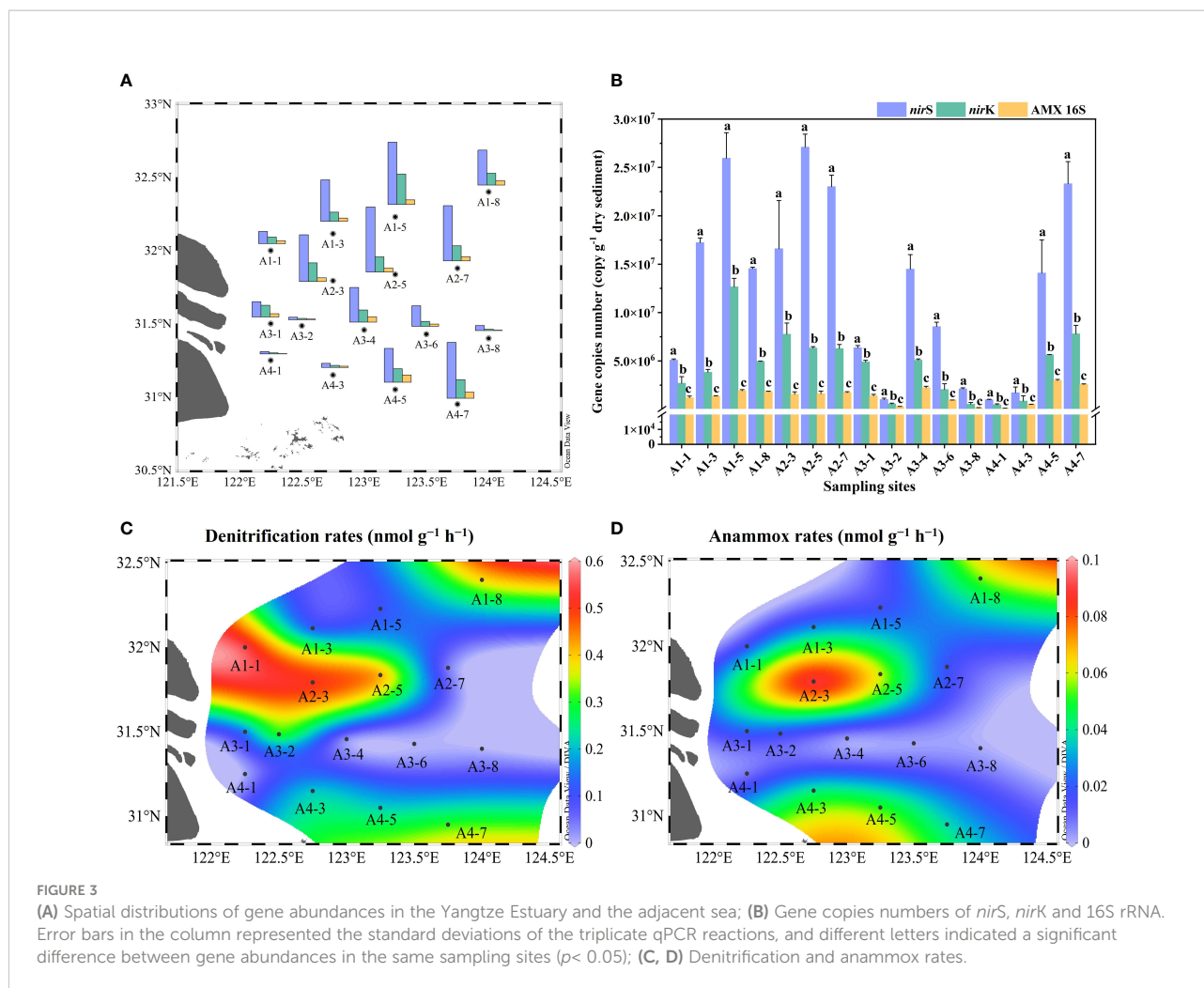


FIGURE 2

Spatial distributions of physicochemical parameters in bottom water and surface sediments of the Yangtze Estuary and East China Sea (ECS) (A–L). # represents the bottom water properties ($\# \text{NH}_4^+$, $\# \text{NO}_3^-$, $\# \text{PO}_4^{3-}$) and $\text{M}\Phi$ denote median grain size.

(pseudo- $F = 3.1$ and $p < 0.01$), NO_3^- (pseudo- $F = 2.7$ and $p < 0.01$), and C/N (pseudo- $F = 2.6$ and $p < 0.05$) were the major factors influencing the *nirS* gene community. At genus levels, *Pseudomonas* was dominant genera only in A3-2, while

Marinobacter was dominant genera and observed in all sampling stations (Supplementary Figure 4B). Spearman's correlation analysis showed that *Pseudomonas* was positively related with in the bottom water ($p < 0.01$) (Supplementary Table 7), and



Marinobacter was positively correlated with sediment NH_4^+ ($p < 0.05$).

Dominant OTUs (top 50 OTUs) of *nirK* gene were classified into 9 clusters, all belonging to the Proteobacteria (Figures 7A, B, Supplementary Figures 5, 6). Among the 9 clusters, C2, C5, and C7 belonged to the β -Proteobacteria, whereas the other 6 clusters were intimately related to the α -Proteobacteria (Figure 7B, Supplementary Figure 6). Moreover, C7 (with the lowest abundances of top 50 OTUs) only contained OTU32 which was detected in the A3-2 and A3-4 sample, showing some similarities with the sediment denitrifying microorganisms in the Yangtze lakes (Jiang et al., 2017). There were no obvious differences in the relative abundance of OTUs between muddy sediments and sandy sediments (Figure 7A). From the CCA (Figure 7C), the first two axes explained 36.10% of the cumulative variance, and NH_4^+ in the bottom water (pseudo-F = 2.0 and $p < 0.01$), PO_4^{3-} (pseudo-F = 2.1 and $p < 0.05$), sediment NO_3^- (pseudo-F = 2.2 and $p < 0.05$), and MΦ (pseudo-F = 2.0 and $p < 0.05$) had significant influences on the *nirK*-encoding denitrifiers community. At genus level,

Mesorhizobium was the dominant genera in A2-5 and A3-1 sample with no other genera observed, however, *Bradyrhizobium* was detected in most sampling stations except A2-5 and A3-1 (Supplementary Figure 7B). Spearman's correlation analysis showed that *Mesorhizobium* was positively related with sediment Fe(II)/Fe(III) ($p < 0.05$) (Supplementary Table 7).

Community diversity and composition of anammox bacteria

For the anammox 16S rRNA gene (AMX 16S), a total of 479,390 high-quality reads were gained, which ranged from 40,134–47,445 for each sample (Supplementary Table 4). Based on the 0.03 genetic distance (97% as cutoff), 23,950 OTUs (2177 ± 440 OTUs per samples, $n=11$) were detected. The highest OTU numbers, Chao1 and Shannon index were found in station A4-7, showing the higher diversity and richness of the anammox 16S rRNA gene in surface sediment of station

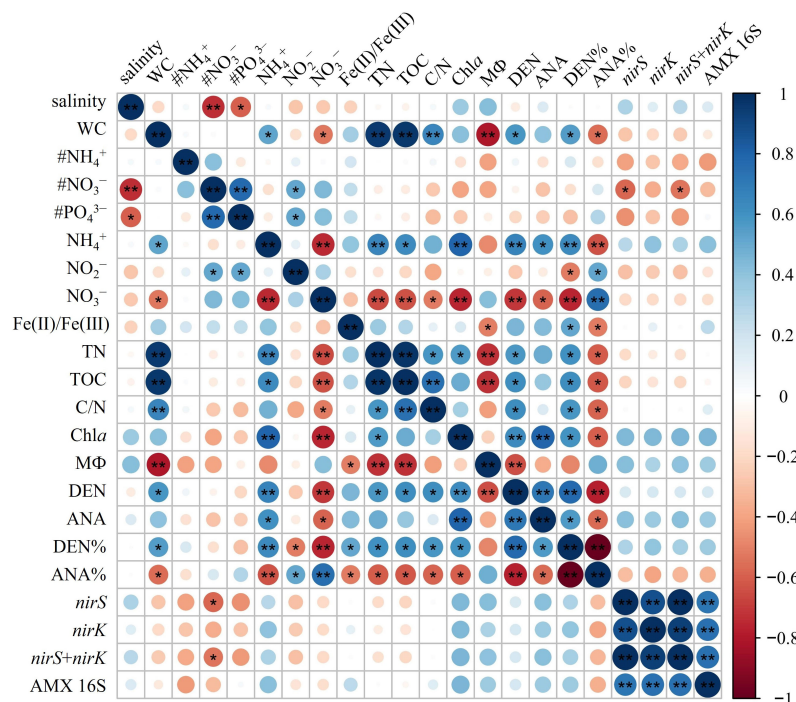


FIGURE 4

Pearson's correlations of environmental factors with the denitrification/anammox and related gene abundances. * significant at $p < 0.05$, ** significant at $p < 0.01$. # represents bottom water parameter ($\# \text{NH}_4^+$, $\# \text{NO}_3^-$, $\# \text{PO}_4^{3-}$). WC, water content; Chla, Chlorophyll a; DEN, denitrification rates; ANA, anammox rates; DEN%, the contribution of denitrification to N removal; ANA%, the contribution of anammox to N removal.

A4-7 than the other stations. In addition, Pielou's evenness (from 0.76 to 0.84) and Good's coverage values (from 98% to 99%) can be observed from each sample, demonstrating the dependability of the sequencing accuracy. The community diversity of the AMX 16S rRNA gene decreased with the increasing concentration in the bottom water (Chao1: $r = -0.681$, $p < 0.05$; Observed species: $r = -0.710$, $p < 0.05$), decreasing sediment NH_4^+ (Chao1: $r = 0.677$, $p < 0.05$; Observed species: $r = 0.678$, $p < 0.05$), and decreasing Chla concentration (Chao1: $r = 0.730$, $p < 0.05$; Observed species: $r = 0.722$, $p < 0.05$) (Supplementary Table 6). The rarefaction curves indicated that the majority of the anammox bacteria was captured in the current study (Supplementary Figure 1C).

The dominant OTUs (top 10 OTUs) of AMX 16S rRNA gene and their related sequences from the GenBank were phylogenetically analyzed to 4 clusters, including *Ca. Anammoximicrobium moscowii* and the other uncultured clusters (Figures 8A, B; Supplementary Figures 8, 9). The OTUs (OTU173, OTU159, and OTU65) were closely related to *Ca. Anammoximicrobium moscowii*, and the OTU abundances were higher in muddy sediment than those in sandy sediment. The other clusters were related to uncultured anammox bacteria, showing a very wide range and matched with marine habitats and activated

sludge (Figure 8B). According to a redundancy analysis (RDA), the link between AMX 16S communities and environmental factors may be described by the first two axes (65.75% of the total variance) (Figure 8C). The sediment MΦ (pseudo-F = 6.1 and $p < 0.01$), Chla (pseudo-F = 2.3 and $p < 0.05$), and bottom water NO_3^- (pseudo-F = 3.4 and $p < 0.05$), had huge impact on the anammox community. At genus level, *Candidatus Anammoximicrobium* was the dominant genera in all sampling stations except A3-2, while the dominant genera in A3-2 was *Rhodoplanes* (Supplementary Figure 10B). Spearman's correlation analysis revealed that *Candidatus Anammoximicrobium* was negatively related with sediment water content ($p < 0.05$), TOC ($p < 0.05$), and positively correlated with sediment grain size (Supplementary Table 7).

Discussion

Community characteristics of *nirS/nirK*-encoding denitrifiers

In coastal aquatic ecosystems, denitrification in sediments can reduce bioavailable nitrogen from anthropogenic and natural inputs, thus, sediments are recognized as hotspot

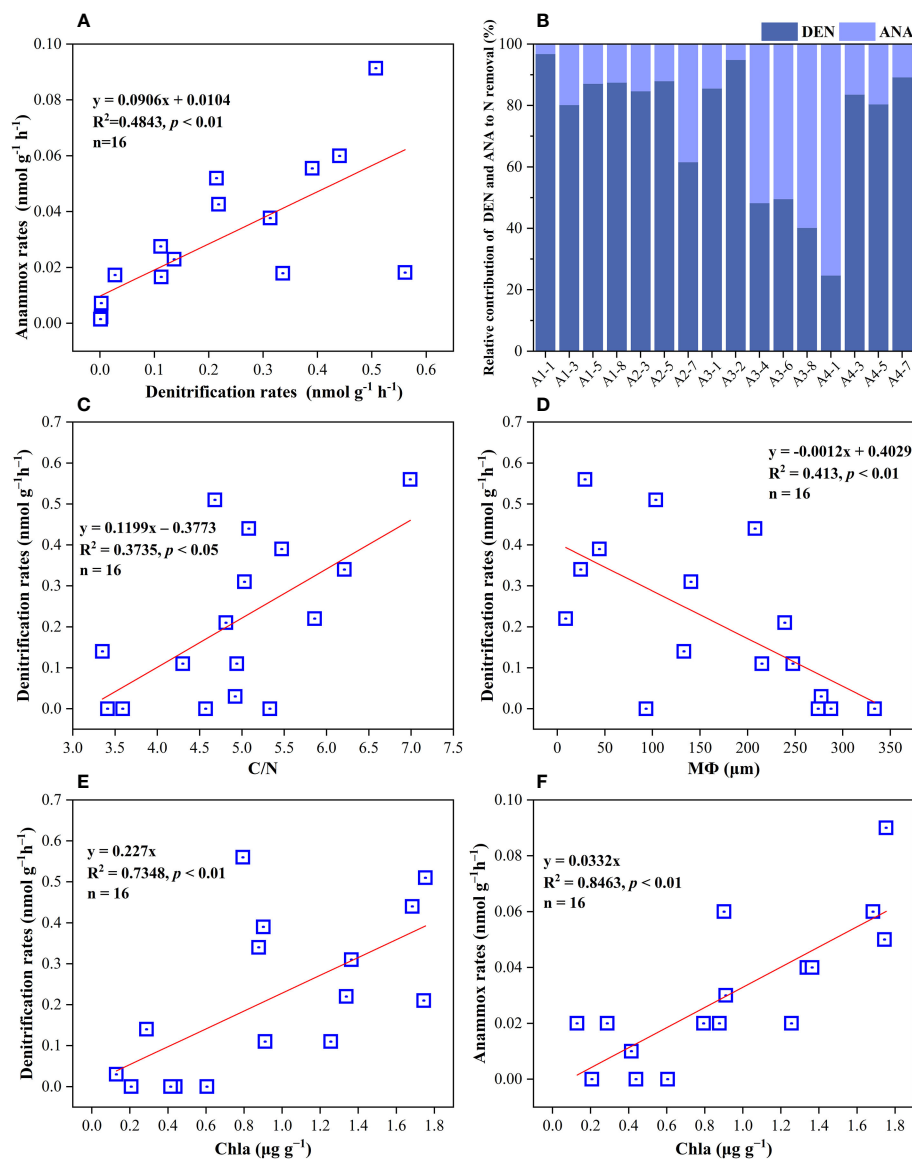


FIGURE 5

(A) Correlations analysis of anammox rates with denitrification rates in surface sediments. (B) The percentage of denitrification and anammox to NO_x^- removal in all sampling sites. (C–F) their regulating factors in sediments of the Yangtze Estuary and adjacent sea.

regions of N loss (Seitzinger et al., 2006). Lin et al. (2017) reported that the contribution of denitrification to N loss (2.2×10^6 t N yr⁻¹) was higher than anammox (4.6×10^5 t N yr⁻¹) in the surface sediments from the East China Sea. The biogeochemical cycle of nitrogen is driven by microorganisms, and the microbial communities are constantly changing to adapt to the complex coastal environments (Dang and Lovell, 2016; Hutchins and Fu, 2017). Thus, a further understanding of the microbial processes of N loss is still highly significant. Here, potential rates of denitrification and anammox, and their related gene abundances and community composition, along with

influencing environmental factors were explored in the surface sediments from the Yangtze Estuary and the adjacent sea.

In the present study, the average abundance of *nirS* gene (1.28×10^7 copies g⁻¹) in the surface sediment was greater than that of *nirK* gene (4.52×10^6 copies g⁻¹), which were similar to the observations in the San Francisco Bay (1.50×10^7 copies g⁻¹ for *nirS* and 3.20×10^6 copies g⁻¹ for *nirK*) (Mosier and Francis, 2010). Some previous studies showed that the mRNA abundance of *nirS* was also higher compared with *nirK*, suggesting a main role in N removal processes of *nirS*-encoding denitrifiers (Graham et al., 2010; Liu et al., 2021). The differential abundance between *nirS* and *nirK* may be due to the

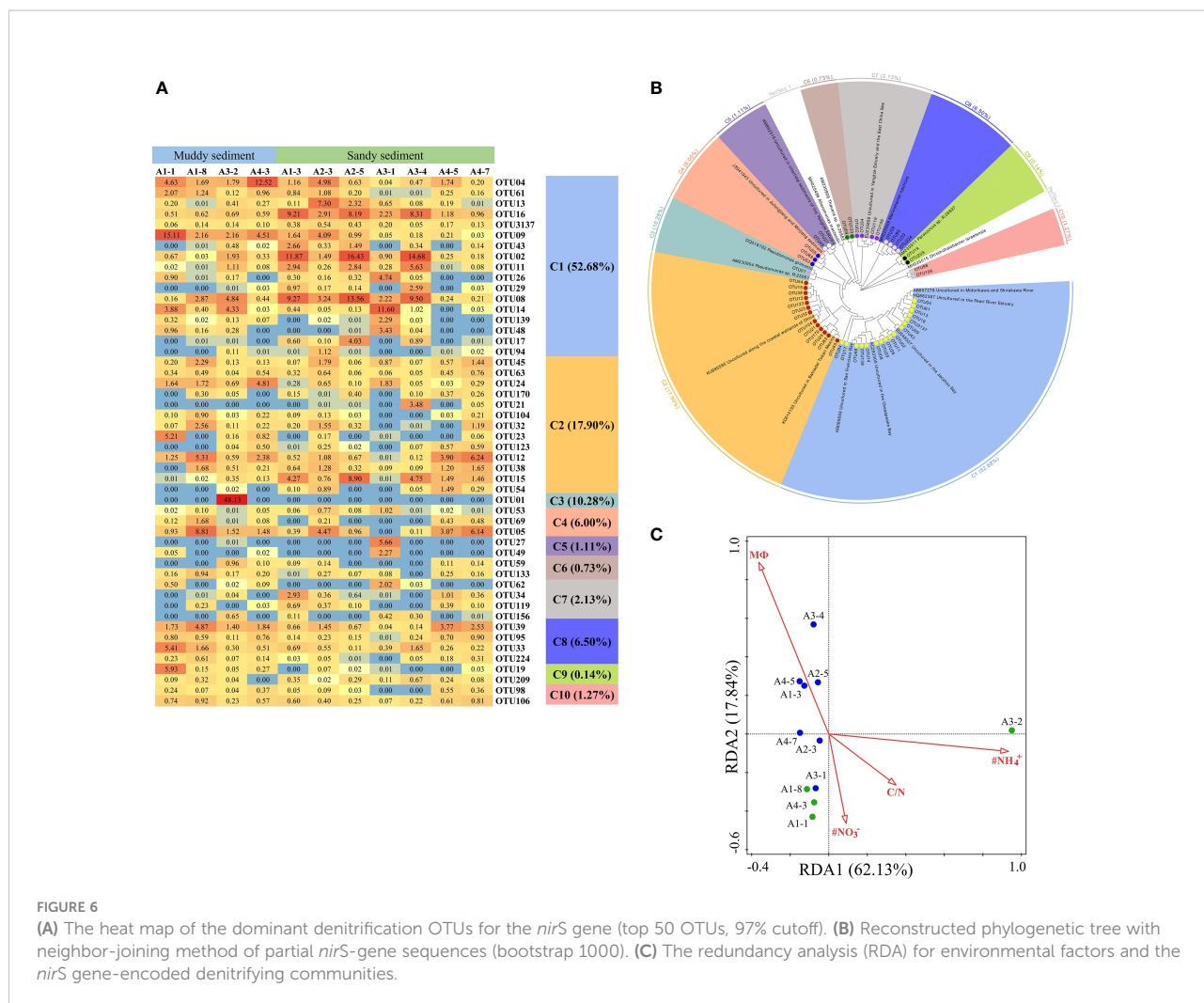


FIGURE 6

(A) The heat map of the dominant denitrification OTUs for the *nirS* gene (top 50 OTUs, 97% cutoff). (B) Reconstructed phylogenetic tree with neighbor-joining method of partial *nirS*-gene sequences (bootstrap 1000). (C) The redundancy analysis (RDA) for environmental factors and the *nirS* gene-encoded denitrifying communities.

competition for the substrates required by different enzyme synthesis (Huang et al., 2011). Moreover, compared to *nirK*-encoding denitrifiers, the variety of *nirS*-encoding denitrifiers was also greater, which is consistent with previous studies (Shi et al., 2019b; Ming et al., 2021). In general, *nirS*- and *nirK*-encoding denitrifiers had different respond to environmental parameters, and *nirS* denitrifiers had better adaptation to the coastal environments (Jones and Hallin, 2010; Likhitrattanasapal et al., 2021).

In consistent to our study (Figure 3), the abundance of *nirS* and *nirK* have the same change trends across the sampling sites (Zhang et al., 2019), which was affected by both the microbial interactions between *nirS* and *nirK*-denitrifiers, as well as the different preferences in structure components of corresponding nitrite reductases (Cutruzzola et al., 2001). Nitrite reduction is the key step in denitrification, and both the *nirS* and *nirK*-encoded nitrite reductase are functionally identical while being evolutionarily distinct (Shrewsbury et al., 2016). The previous studies reported that both enzymes are often absent in the same bacterium, and the estimated number of denitrifiers in the soil

sites was determined using the summation of *nirS* and *nirK* copy counts (Philippot et al., 2011; Zhang et al., 2019). However, *Pseudomonas* was found in *nirS*- and *nirK*-encoding denitrifiers at genus level in our study (Supplementary Figures 4B, 7B). Furthermore, there other different genera were detected in *nirS*- and *nirK*-encoding denitrifiers, which have capabilities of denitrification (Kim et al., 2008; Borrero-De Acuña et al., 2017; Gao et al., 2021). Thus, marker genes of *nirS* and *nirK* should be used to detect denitrifiers together. In addition, there are another four different genes involved in the denitrification pathway, nitric oxide reduction (*norB* and *qnorB*), and nitrous oxide reduction (*nosZI* and *nosZII*). A single gene does not capture the entire denitrifying microbial community, due to several marker genes involved in denitrification pathway (Shrewsbury et al., 2016). Furthermore, Zhang et al. (2019) found that the contribution of denitrification to N removal was positively related to the relative abundance of *nosZ/nirS*, implying that marker genes from different denitrification steps should be considered simultaneously.

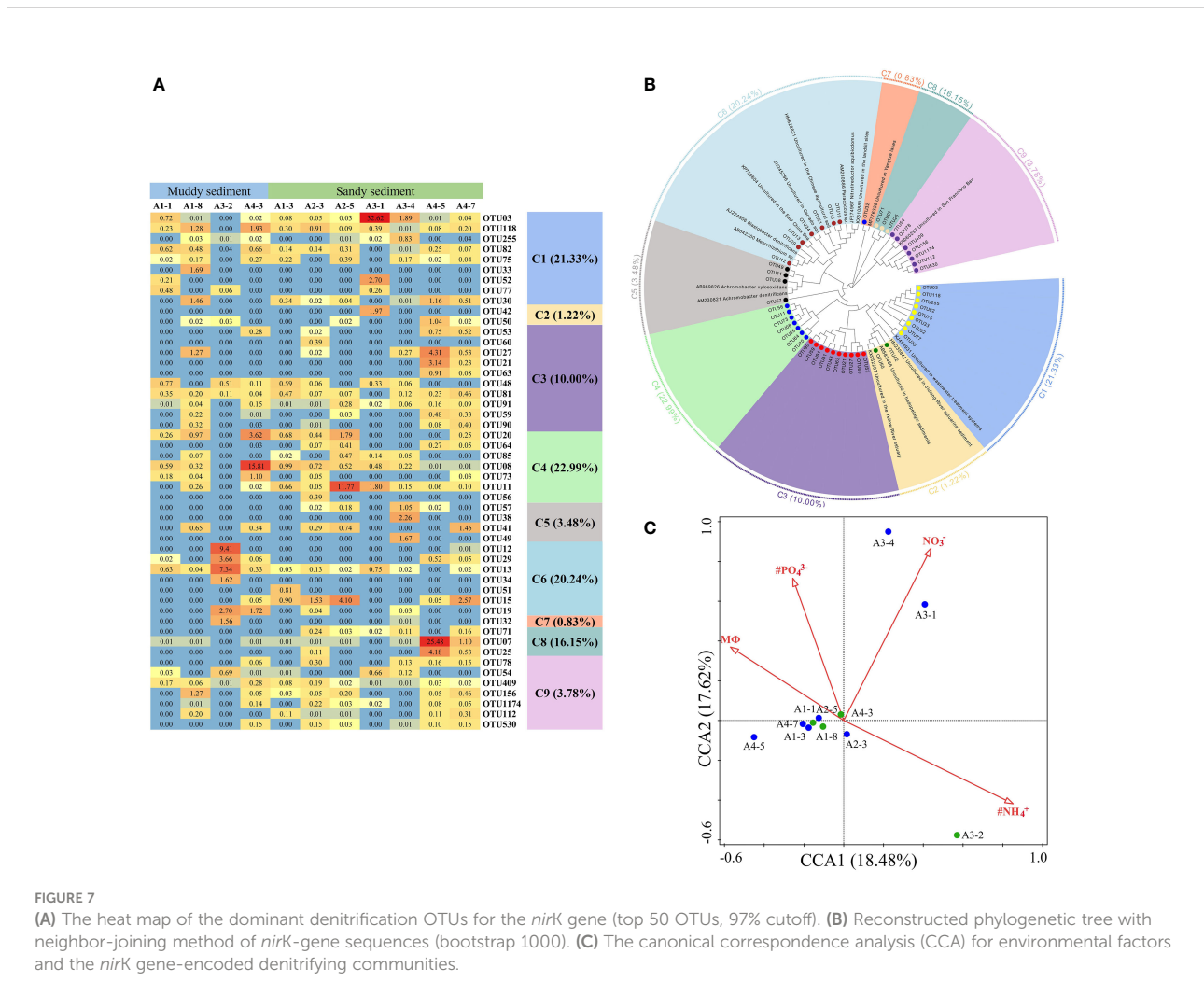


FIGURE 7

(A) The heat map of the dominant denitrification OTUs for the *nirK* gene (top 50 OTUs, 97% cutoff). (B) Reconstructed phylogenetic tree with neighbor-joining method of *nirK*-gene sequences (bootstrap 1000). (C) The canonical correspondence analysis (CCA) for environmental factors and the *nirK* gene-encoded denitrifying communities.

According to phylogenetic analysis, the dominant OTUs of *nirS* showed some similarities to multiple estuarine and marine sediments (Dang et al., 2008; Hong et al., 2014; Zheng et al., 2015; Gao et al., 2016). In contrast to *nirS*, the *nirK*-encoding denitrifiers were found to originate from several habitats, such as marine sediments, fresh sediments, agricultural soil, and wastewater treatment systems (Figure 7B, Supplementary Figure 6). Numerous studies have revealed that *nirS* and *nirK*-denitrifiers occupy different niches or have distinct habitat preferences in the environment (Smith and Ogram, 2008; Hallin et al., 2009; Knapp et al., 2009). The communities of *nirS* and *nirK* gene were observed to clearly separate in saline environments, and *nirS*-encoding denitrifiers predominated in marine environment (Jones and Hallin, 2010), in consistent with our study. Furthermore, *nirK* denitrifiers might exhibit more habitat selection, showing positive correlations with organic matter content (Smith and Ogram, 2008). In the present study, RDA/CCA demonstrated that C/N ratio and MΦ was the key factor affecting the community of *nirS* and *nirK*,

respectively (Figure 6C, Figure 7C). High level of C/N ratio may promote the growth of denitrification bacteria, as previous studies showed that the denitrifiers OTUs and Shannon index decreased with decreasing organic matter and N contents (Chen et al., 2018; Wu et al., 2021). Meanwhile, the dominant OTUs of *nirS*- and *nirK*-encoding denitrifiers were affiliated with Proteobacteria (Figures 6B, 7B, Supplementary Figures 3, 6), which was consistent with previous reports (Kim et al., 2016; Xie et al., 2020). Furthermore, γ - Proteobacteria and α -Proteobacteria was the dominant class of *nirS*- and *nirK*-encoding denitrifiers, respectively. Some studies showed that denitrification can be performed by phylogenetically different microbes, and the most of denitrifiers early reported are belonging to subphyla of the Proteobacteria (Peralta et al., 2010; Wu et al., 2021). This phylum of Proteobacteria are widely distributed in aquatic environments because of high phylogenetic and phenotypic diversities (Liu et al., 2015; He and Zhang, 2016; Shi et al., 2019a). With the Proteobacteria community, *Pseudomonas* and *Mesorhizobium* were the

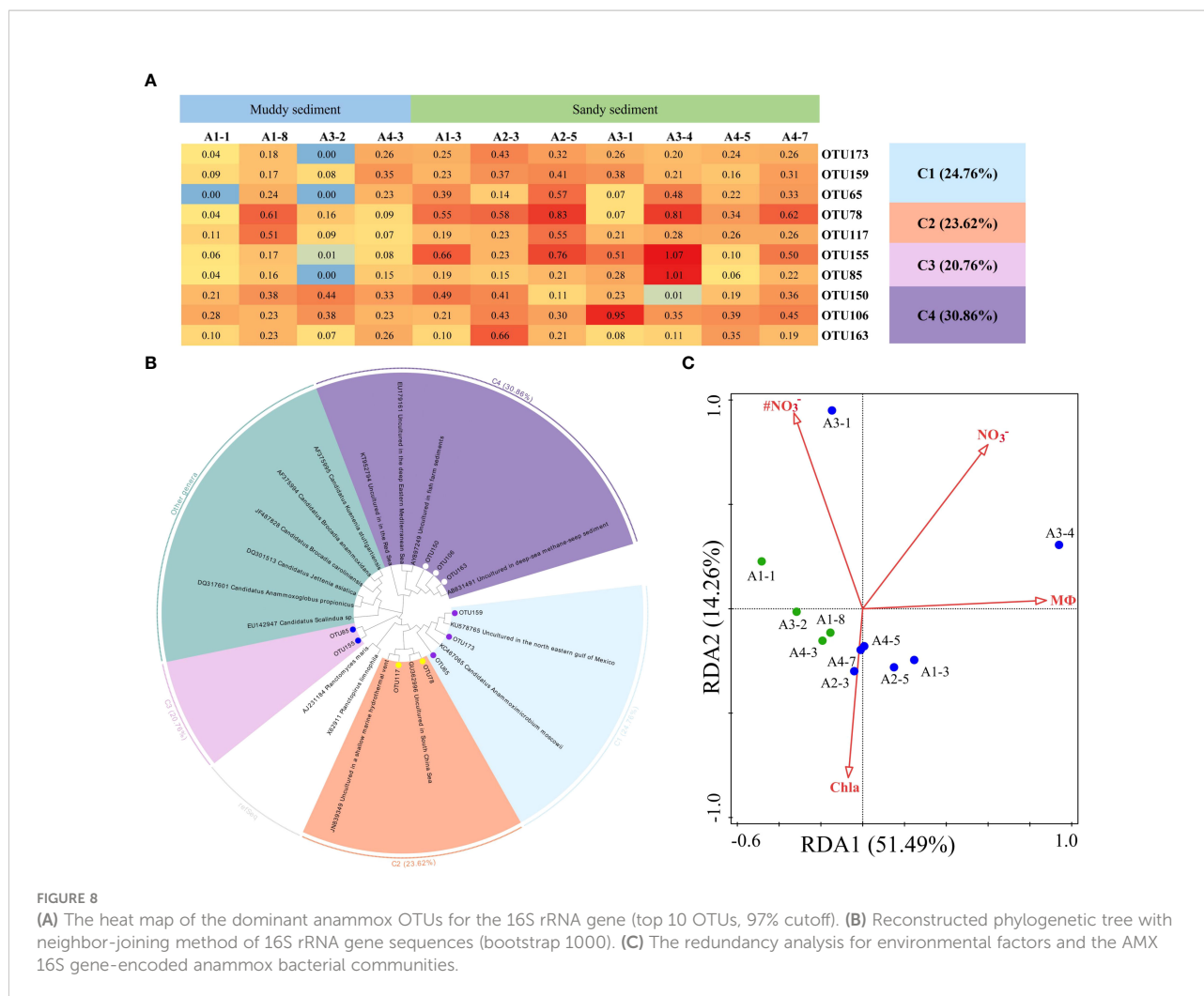


FIGURE 8 (A) The heat map of the dominant anammox OTUs for the 16S rRNA gene (top 10 OTUs, 97% cutoff). (B) Reconstructed phylogenetic tree with neighbor-joining method of 16S rRNA gene sequences (bootstrap 1000). (C) The redundancy analysis for environmental factors and the AMX 16S gene-encoded anammox bacterial communities.

dominant genera of *nirS*- and *nirK*-encoding denitrifiers, respectively. *Pseudomonas stutzeri* strain XL-2 and strain XL-2 were identified as the main denitrifiers which can remove 97.9% of nitrate (Dang and Lovell, 2016). In *nirS* bacterial community, *Pseudomonas* was mainly affected by NH_4^+ in the bottom water; while in *nirK* bacterial community, *Mesorhizobium* was significantly influenced by with sediment Fe(II)/Fe(III). In addition, it has been reported that the higher abundance of *Mesorhizobium* was observed in sandy soil (Li et al., 2019), in consistent to our study that *Mesorhizobium* was the dominant genera in A2-5 and A3-1 with high median grain size.

Community characteristics of anammox bacteria

In the present study, the abundance of AMX 16S rRNA gene was ranging from 1.03×10^5 to 2.97×10^6 copies g^{-1} (Figures 3A, B), which was lower than that of *nirS* and *nirK* gene in all sampling sites. The gene abundances of AMX 16S rRNA gene

were like to the values in the Pearl River Estuary (Wu et al., 2020), but 1-2 orders magnitude lower than those in paddy soil (Zhu et al., 2011) and the coastal wetland sediments of China (Hou et al., 2015). Furthermore, anammox bacterial abundance was no obvious spatial heterogeneity at the sampling sites (Figure 3A).

The high-throughput sequencing of the 16S rRNA gene of anammox bacteria could provide adequate data, as compared to data from cloning libraries, to identify community changes and even recognize unusual bacteria with low relative abundance (Zhao et al., 2013; Shen et al., 2016). Based on the OTUs and the Shannon index, the AMX 16S genes in the surface sediment represented a very varied community of anammox bacteria (Supplementary Table 4). A wide variety of anammox bacteria was found in the surface sediment of Yangtze Estuary and the adjacent sea, in similarity to other estuarine sediments (Dale et al., 2009; Hong et al., 2014; Lisa et al., 2014). Salinity is a significant environmental factor that affects the diversity of anammox bacteria, according to earlier research (Schmid et al., 2007; Hamersley et al., 2009; Hu et al., 2012). However, in this

present study, the Chao1 had significantly correlated with NH_4^+ concentration in bottom water and sediment (Supplementary Table 6). These results implied that the substrates contents might be the main limiting factors for anammox bacteria.

In this study, the dominant OTUs were closely related to *Ca. Anammoximicrobium moscowii* and *Candidatus Anammoximicrobium* was the dominant genera at genus level (Supplementary Figures 9, 10B). In contrast to previous studies (Schmid et al., 2007; Fernandes et al., 2016; Fu et al., 2019; Wu et al., 2021), *Ca. Scalindua* are the dominant anammox bacteria in marine environments. Some studies reported that *Ca. Scalindua* has a relatively high tolerance for salinity and it can adapt to high hypersaline environment (Oshiki et al., 2017; Speth et al., 2017). Recently, the other genera have been found and *Ca. Scalindua* was not the only genus in the marine environments (Wu et al., 2019). *Ca. Anammoximicrobium moscowii* also can carry out anaerobic ammonium oxidation, which was firstly isolated in the Moscow region (Khramenkov et al., 2013), and the key role of this genus in N loss process cannot be ignored. Some studies reported that the different anammox bacteria lineages can become the dominant populations based on the slight change in the environment (Wang et al., 2021). In addition, the community composition study of anammox bacteria can be directly impacted by primer sets and sequencing depth. In the present study, considering the primer efficiency, Amx808F and Amx1040R was the appropriate choice for capturing anammox bacteria with good coverage, however, the specificity was not strong for *Ca. Scalindua* detection. From the RDA analysis, the MΦ and bottom water NO_3^- significantly affected anammox bacteria community. In addition, according to Spearman's correlation analysis, *Candidatus Anammoximicrobium* was negatively related by TOC.

Microbial processes for nitrogen loss and their environmental implications

For microbial mediated N transformation, the surface sediment are usually the most vibrant with greater potential N loss rates, particularly the denitrification rates; however, the potential rates of denitrification and anammox were lower than previous studies (Hou et al., 2015; Lin et al., 2017; Wu et al., 2021), denitrification and anammox can exhibit strongly spatiotemporal variabilities, and the rates might be affected by biotic and abiotic factors in sampling sites. The bulk of the samples belong to sandy sediments, with the low organic matter content and low N removal rates. In our study, denitrification was the main contributor of N loss, dominating the N loss process in surface sediment in most sampling sites (Figure 5B). These results were in accordance with those in the previous studies in estuarine and coastal habitats (Fernandes et al., 2012;

Cao et al., 2017; Lin et al., 2017; Huang et al., 2021). Moreover, Huang et al. (2021) found that denitrification rates were substantially correlated with organics and median grain size (MΦ), implying that both MΦ and organic matter were the crucial factors regulating denitrification activities. High surface-to-volume ratios in fine-grained sediments typically result in greater organic matter absorption (Keil et al., 1994). Meanwhile, organic matter (as an electron donor) can directly or indirectly boost N removal processes by providing energy *via* the oxidation of organics (Devol, 2015). Additionally, we verified that denitrification rates were substantially correlated with MΦ and the C/N ratio (Figures 5C, D). Higher specific surface area in sediment with little grains was more suited to microbial colonization, and the required energy for the growth of heterotrophic denitrifiers were provided by organic matter. Moreover, the contribution of anammox was higher than denitrification in few sampling stations with lower C/N ratio and higher median grain size, further implying that sediment texture can control the N loss rates *via* affecting the residence time of organic matter (Huang et al., 2021).

Anammox rates and denitrification rates have a positive correlation in this study (Figure 5A), suggesting that denitrification was probably the main source of NO_2^- for anammox. Coherent to many previous researches in the aquatic environment (Brin et al., 2017; Zhao et al., 2020a), anammox metabolism requires the substrates of NO_2^- , which are main supplied by denitrification rather than nitrification in these anaerobic environments. This coupling between anammox and denitrification may be mediated by an imbalance in NO_3^- and organic carbon needed for denitrification. Furthermore, in the present study, NO_2^- was negatively related to DEN%, but positively correlated to ANA%, implying the coupling of denitrification and anammox. However, there also studies showing that anammox coupled with nitrification rather than denitrification in estuarine sediments in winter (Hou et al., 2015; Lin et al., 2017). The uncertainties in the relationships between anammox and denitrification might be attributed to organic acids, and anammox bacteria can convert NO_3^- to NO_2^- in the presence of organic acids (Brin et al., 2014). Thus, coupled anammox and nitrification or denitrification play a vital role in N removal processes from the surface of marine sediments.

Previous studies have revealed that NH_4^+ and NO_3^- play the crucial and central role in N removal (Lin et al., 2017; Huang et al., 2021), which were as substrates in denitrification and anammox processes. In consistent to our study, NH_4^+ and NO_3^- were the key factors to influence denitrification and anammox rates. In the present study, *Chla* was the key environmental regulator for denitrification rates ($p < 0.01$) (Figure 4, Figure 5E, Supplementary Table 5). The sediment *Chla* is an indicator for the availability of labile organic matter which is obtained from phytoplankton deposited from upper waters, along as phyto-benthos and benthic algal debris in the shallow marginal

system (Franco et al., 2007; Kitidis et al., 2017). The sediment organic matter correlates positively with Chl a in sediment and water column (Li et al., 2013), in similarity to our study (Figure 4, Supplementary Table 5). The growth of phytoplankton may be aided by light intensity and water temperature in March (Shi et al., 2019b), indicating that many phytoplankton cells might be destroyed to release labile DOC (Zhang et al., 2018), which could enhance denitrification by sinking down to anaerobic sediment layers. Meanwhile, anammox rates also positively correlated with Chl a ($p < 0.01$) (Figure 4, Figure 5F, Supplementary Table 5). The main reason for that was the substrates (NH $_4^+$ and NO $_2^-$) of anammox originated from the mineralization of organic matters (Zhang et al., 2018). Thus, both the quality and amount of organic matter in sediment are essential to manage estuarine N-cycling because fresh organics has a higher lability and simpler to be utilized by NO $_x^-$ removal processes (Westrich and Berner, 1984; Henrichs and Doyle, 1986).

There were no significant correlations between N removal rates and related gene abundances in this study. The gene abundance obtained from the DNA levels has limitations. On the one hand, since DNA extracts include a wide variety of cells (e.g., active cells, inactive cells and dead cells) and its degraded or lysed extracellular DNA, gene abundance does not always indicate activity (Gaidos et al., 2011). On the other hand, the number of microbes just indicates potential microbial metabolism and has no direct impact on the activities of N transformation (Dai et al., 2008). It has been reported that the related functional gene transcript abundance is reflective of N transformation process potential, further revealing N transformation potential had a close relationship with active microbes (Li et al., 2022). Thus, to gain an insight into changes in denitrification and anammox potential, the functional transcript abundance combining with N removal rates are needed.

Conclusion

Taken as a whole, the current work described the distribution and diversity of denitrifiers and anammox bacteria in the Yangtze Estuary and the adjacent sea, and the potential rates of denitrification and anammox were measured together. We found the abundance and diversity of *nirS*-denitrifiers were higher than those of *nirK*-denitrifiers, and *nirK*-denitrifiers were more sensitive to the environments. For anammox bacteria, *Ca. Anammoximicrobium moscowii* has been found in surface sediment, and the other genera of anammox bacteria cannot be neglected in marine ecosystems. Compared to anammox, denitrification was the dominant contributor of N removal process and contributed 73.5% on average. Denitrification and anammox rates were regulated by multiple environmental factors, and the median grain size (M Φ), C/N ratio, and Chl a were the key factors.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

ZT: conceptualization, investigation, methodology, data curation, formal analysis, and writing-original draft. YZ: conceptualization, writing-review and editing, supervision, and funding acquisition. TM: writing-review and supervision. ZY: conceptualization, writing-review and editing, and supervision. TC: writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1078653/full#supplementary-material>

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