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Research Paper

Anammox bacteria are an indispensable part of the nitrogen removal process in the Changjiang estuarine seasonal hypoxia zone*

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Abstract Anthropogenic nitrogen pollution threatens estuarine ecosystems globally, yet the coupling between the seasonal hypoxic zone (SHZ) and anaerobic N-loss microbiomes in major estuaries like the Changjiang estuary (CJE) remains poorly understood. Through a multidisciplinary approach combining geochemistry and high-throughput molecular sequencing, we investigated the spatiotemporal dynamics of N-loss microorganisms in the CJE. We reveal distinct niche differentiation: *nirS*-type denitrifiers dominate in summer near the estuary, while anammox bacteria exhibit peak abundance and diversity in spring in offshore waters. These significant community shifts are primarily driven by the C/N ratio and dissolved oxygen (DO) levels. Crucially, the superior competitiveness of anammox bacteria in anoxic conditions suggests their seasonal variation functions as a specific biological indicator for the SHZ. These findings clarify the distinct ecological roles of N-loss pathways and their feedback mechanisms regarding estuarine hypoxia.

Keyword: N loss; Changjiang estuary (CJE); anammox bacteria; denitrifier; seasonal hypoxic zone (SHZ)

1 INTRODUCTION

Biologically available nitrogen is an essential element for all livings in aquatic ecosystems and is required for the biosynthesis of key cellular components, such as proteins and nucleic acids (Devol, 2015). This bioavailable nitrogen is rare in many environments, and the availability of this growth-limiting nutrient is controlled primarily by microbial reactions that alter the oxidation state of nitrogen (Hutchins and Capone, 2022). However, owing to human activities, the high input of industrial pollutions and nitrogen-based fertilizers in agriculture have accelerated the rate and extent of eutrophication through directly discharges and

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loadings of limiting nutrients, such as nitrate and ammonia, into aquatic ecosystems (Akinnawo, 2023; Jeppesen et al., 2025). Eutrophication is a leading cause of impairment of many freshwater and coastal marine ecosystems in the world (Smith, 2003).

Nitrogen-transforming microorganisms can transform biologically available nitrogen compounds to dinitrogen gas releasing to atmosphere, alleviating eutrophication (Kuypers et al., 2018). The microbial degradation of organic nitrogen, ammonification, releases a molecule of ammonia, which is subsequently oxidized to nitrate through nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) and eventually converted back to a molecule of dinitrogen gas through denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) or anaerobic ammonium oxidation (anammox; $NO_2^- + NH_4^+ \rightarrow N_2$) (Kuypers et al., 2018). Four enzymes participate in complete denitrification processes (mainly refers to alienation denitri fi cation): nitrate reductase (Nar, $NO_3^- \rightarrow NO_2^-$), nitrite reductase (Nir, $NO_2^- \rightarrow NO$), nitric oxide reductase (Nor, $NO \rightarrow N_2O$) and nitrous oxide reductase (Nos, $N_2O \rightarrow N_2$)(Ward et al., 2009). Alternatively, anammox is catalyzed by three enzymes: nitrite reductase (Nir, $NO_2^- \rightarrow NO$), hydrazine synthase (HZS, $NO_2^- + NH_4^+ \rightarrow N_2H_4$), hydrazine dehydrogenase (HDH, $N_2H_4 \rightarrow N_2$) (Kartal et al., 2011). The *nirS* gene encoding nitrite reductase is key functional gene for denitrification and anammox processes and is an effective and widely used gen-marker for fast and accurate detection and quantification of both of processes in environments (Zhuang et al., 2020; Zhang et al., 2023, 2024; Ming et al., 2024).

Estuarine sediments act as critical biogeochemical filters that mitigate the export of land-derived reactive nitrogen through microbially mediated nitrogen loss processes, primarily denitrification and anammox. Among these, denitrification has long been considered the dominant N_2 production pathway in estuaries, recent syntheses demonstrate that anammox can contribute substantially to nitrogen removal, particularly in eutrophic systems with strong salinity and redox gradients (Yao et al., 2023; Lai et al., 2024; Ye et al., 2024). Emerging evidence highlights that the relative importance of denitrification and anammox is governed by microbial niche separation driven by dissolved oxygen (DO), organic matter stoichiometry, and substrate availability. Seasonal hypoxia, in particular, restructures sediment redox zonation and nitrogen fluxes, promoting suboxic niches favorable to obligately anaerobic anammox bacteria while constraining heterotrophic denitrifiers (Wang et al., 2019; Naufal and Wu, 2024; Sun et al., 2024). Such shifts in microbial dominance have important implications for nitrogen removal efficiency and the coupling of nitrogen transformation pathways in eutrophic estuaries. Despite growing recognition of anammox in coastal nitrogen cycling, the extent to which seasonal hypoxia mediates microbial niche partitioning and alters dominant nitrogen loss pathways along estuary-shelf continua remain poorly constrained, especially in large river-dominated estuaries.

Denitrification and anammox were dominant pathways for N-loss process in riverine and estuarine ecosystems, however, the variation of geochemical factors may change their contributions to N-loss process (Wang et al., 2019; Ming et al., 2021; Fortune et al., 2022; Hu et al., 2022). Seasonality is a primary driver of the ecological dynamics of microbial communities, and community composition and function also are affected with seasonal biotic and abiotic factors (Gilbert et al., 2012; Liu et al., 2021; Fang et al., 2023; Guo et al., 2023; Wang et al., 2024). The seasonal dynamics of estuarine ecosystems often create transient and unique conditions (e.g., temperature, DO, and chlorophyll content), which may affect the ecological patterns of microbial communities at multiple scales (Giovannoni and Vergin, 2012; Fang et al., 2023; Wang et al., 2024; Yu et al., 2024; Hervé et al., 2025). Previous studies revealed that the seasonality of temperature, nutrient content and oxygen concentration may shift microbial diversity, function and species coexistence in aquatic ecosystems (Fang et al., 2023; Wang et al., 2024). The water-level of seasonality can regulate the interactions and metabolisms of microbial functional guilds, e.g. nitrogen-transforming microbial community (Guo et al., 2023). Therefore, deciphering the structure and function of nitrogen-transforming microbial communities in estuarine under seasonal variation is necessary for the management of eutrophication and maintenance of environment sustainable.

Changjiang (CJ) is the third-largest river in the world and accounts for large load of sediment and freshwater discharges in the estuarine area. Due to the CJ Basin have large number of cropping and industrialized aera, the CJ estuary (CJE) is one of hypereutrophic estuaries around the world (Xu et al., 2013; Chen et al., 2016), and contributes about 66% of the nitrogen load among all main rivers

flowing into China's coastal seas (Chen et al., 2016; Tong et al., 2017). The nitrogen-transforming microorganisms in estuary sediment play important roles in reducing severe eutrophication (Hou et al., 2013; Shi et al., 2020). Previous studies focusing on nitrogen removal processes (including denitrification and anaerobic ammonium oxidation) in the sediments of the CJE (Wang et al., 2019; Ming et al., 2021; Song et al., 2021; Fortune et al., 2022), but few studies have examined the diversity and function of microbial community under seasonality. The effects of seasonal change driving the diversity and distribution of nitrogen-transforming microorganisms is still unclear. In this study, we collected the surface sediment samples from estuary of CJ in two different seasons. The primary objectives of this study are to characterize the spatiotemporal dynamics of nitrogen-loss microorganisms in the CJE and to elucidate the environmental drivers shaping their community structure. Specifically, we hypothesized that seasonal fluctuations in key environmental parameters, including DO, C/N ratio, and salinity, impose selective pressures, driving distinct shifts in the dominant nitrogen-loss microbial guilds. Ultimately, our findings will provide a theoretical basis for optimizing nitrogen removal strategies to mitigate eutrophication in the CJE.

2 MATERIAL AND METHOD

2.1 Study area, sample collection, and environmental factor analysis

23 and 14 surface sediments were collected from CJE and adjacent area among two cruises during April–May and August–September in 2017 by the R/V *Kexue No.3*, respectively (Fig.1). All sediment samples were packed into sterile plastic bags and stored in -80 ℃ for molecular analysis. The bottom water overlaying the sediment was collected and measured geophysical and geochemical properties for the surface sediments. One portion (~100 mL) of the overlay water was stored at -20 ℃ for dissolved inorganic nitrogen analysis.

The depth of each site was detected and several environmental parameters (temperature, salinity, and DO concentration) in the bottom water were recorded in situ with an RBR XR-620 Multi-Channel CTD (Elcee, Malaysia). Other parameters were measured in the laboratory. Ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) concentrations in the bottom water were measured with QuAAtro nutrient

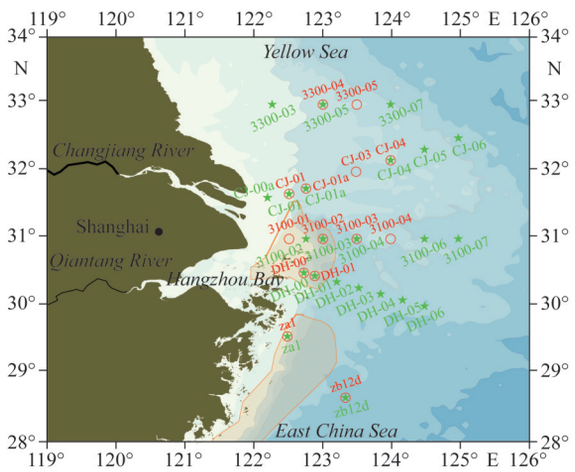


Fig.1 Sampling sites in CJE and adjacent area in the YS and ECS during April–May (surface sediments marked by green solid pentacles) and August–September (surface sediments marked by red hollow circles) in 2017

The orange shadows are the mud area at the CJE and along the coasts of Zhejiang and Fujian provinces.

auto analyzer (Seal Analytical Ltd., UK). TOC and TN of sediments were measured after acid fumigation using an elemental combustion analyzer interfaced (Zhao et al., 2021). In brief, ~30 mg of freeze-dried sediment in silver capsules was acidified with 12-mol/L HCl vapor bath in a desiccator for 12 h to remove carbonates and then dried in an oven at 60 °C and crimp-sealed in tin capsules prior to analysis.

2.2 DNA extraction and quantification of anammox and denitrifiers

Genomic DNA from each sampling site was extracted independently using DNeasy® PowerSoil® Pro kit (QIAGEN, Germany) according to the manufacturer's instruction, and the DNA extraction yield and quality are shown in Supplementary Table S1. For amx-16S rRNA, *Scalindua-nirS*, and denitrifying *nirS* genes, different primer set procedure and protocol were used (details in Supplementary Table S2) to detect the quantity of the anammox and denitrifiers. All samples were processed with an ABI PRISM® 7500 Sequence Detection System (Version 1.3.1; Applied Biosystems, Foster City, CA, USA), and reactions were performed in triplicate. The standard curves were showed in Supplementary Fig.S1. Target genes were quantified by the specific primers mentioned above. The 20-μL reactions consisted of 10-μL FastStart Universal SYBR Green Master (ROX) (Roche

Diagnostics, Mannheim, Germany), 0.6-μL each primer, 0.2-μL bovine serum album (BSA) (0.2 μg/μL), 6.6-μL ddH_2O and 2.0-μL sediment DNA. The specificity of PCR amplification was verified by a melting curve analysis and agarose gel electrophoresis. The threshold cycle (C_t) values obtained from all samples were against to standard curves to determine the initial copies of the three genes.

2.3 High-thoughout sequencing of denitrifying and anammox bacteria

The community characteristics of denitrifying and anammox bacteria were analyzed by Illumina MiSeq platform based on *nirS* and amx-16S rRNA genes. These genes were amplified using the specific primer mentioned above. Each sample was distinguished by barcode oligonucleotides ligated to 5′ end of primers. After PCR production purified, pyrosequencing was performed on the Illumina Miseq (PE300) platform at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China. Paired end reads obtained by pyrosequencing were initially assigned to every sample based on its unique barcode. The paired-end reads were then merged by using some of the read overlap generated from the opposite end of the same DNA fragment. The merged reads were subsequently subjected to quality-based filtering with QIIME2 (Caporaso et al., 2010). Raw sequencing reads were processed using the QIIME 2 pipeline, incorporating quality filtering (score≥Q20), chimera removal via UCHIME. After trimmed, the remaining sequences were clustered into operational taxonomic units (OTUs) using Usearch (version 7.0, http://drive5.com/uparse/) at 97% similarity levels, and the representative sequences were chosen out based the most common sequences. α and β diversity were measured by QIIME2 (Caporaso et al., 2010) based on the processed pyrosequencing data. After randomly reducing the number of reads to the lowest number of reads in any individual sample, the richness indices (Chao1 estimates), diversity indices (Shannon index) and Good's coverage were obtained with QIIME2 (Caporaso et al., 2010). Furthermore, unweighted pair group method with arithmetic mean (UPGMA) clustering based on a weighted UniFrac distance matrix was conducted using QIIME2 to examine the differences in the bacterial communities between the sediment samples. The phylogenetic analyses were performed by MEGA (version 11) software (Tamura et al., 2021).

2.4 Statistical analysis

The relationships between community composition and sediment characteristics were examined using BIOENV, detrended correspondence analysis (DCA) and redundancy analysis (RDA) with the R package vegan. The DCA analysis indicated that a linear-model-based RDA was more suitable than unimodal CCA for our data. Gene abundance data were log-transformed prior to correlation and multivariate analyses to satisfy normality assumptions. The relationship among the activities, abundances of microbial N loss community (including ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), denitrifiers and anammox bacteria, and sediment characteristics were examined by Pearson correlation analysis using R (version 4.4.3). BIOENV provides the subset of environmental variables the best explains the community variation among the sampling stations using Spearman Rank Correlation, and RDA determines the percentage of the community composition variation explained by this subset of environmental variables. The significance of the environmental variables was tested by a Monte Carlo permutation test (999 unrestricted permutations, $P<0.05$).

3 RESULT

3.1 Geophysicochemical measurement

The geophysicochemical characteristics of the sediment samples were showed in Fig.2 and Supplementary Table S3. In April–May, NH_4^+ concentrations were 0.50–2.74 μmol/L, and much higher than that of NO_2^- (0.07–0.42 μmol/L) but lower than NO_3^- (0.37–20.66 μmol/L) except Site 3100-06. The maximum value of DIN was at Site CJ-00a (~20.65 μmol/L), nearest located at CJE, and the minimum occurred at Site DH-06 (~3.36 μmol/L). DO concentration in the bottom water was between 5.66 and 8.37 mg/L, Site CJ-04 shared the highest, with Site DH-00 had the lowest. Salinity value was 20.23–34.6, the lowest occurred in Site CJ-00a, which was the nearest at CJE. The TOC and TN were among 0.233%–1.063% and 0.013%–0.132%, respectively. Concentration tendency of detected nitrogen factors in August-September, the maximum DIN concentration at Site CJ-01a and minimum at Site CJ-04; and the highest NH_4^+/NO_x^- ratio was observed in Site CJ-04, while Site 3100-02 owned the lowest (Supplementary Table S3). Notably, the bottom water DO concentration in August-

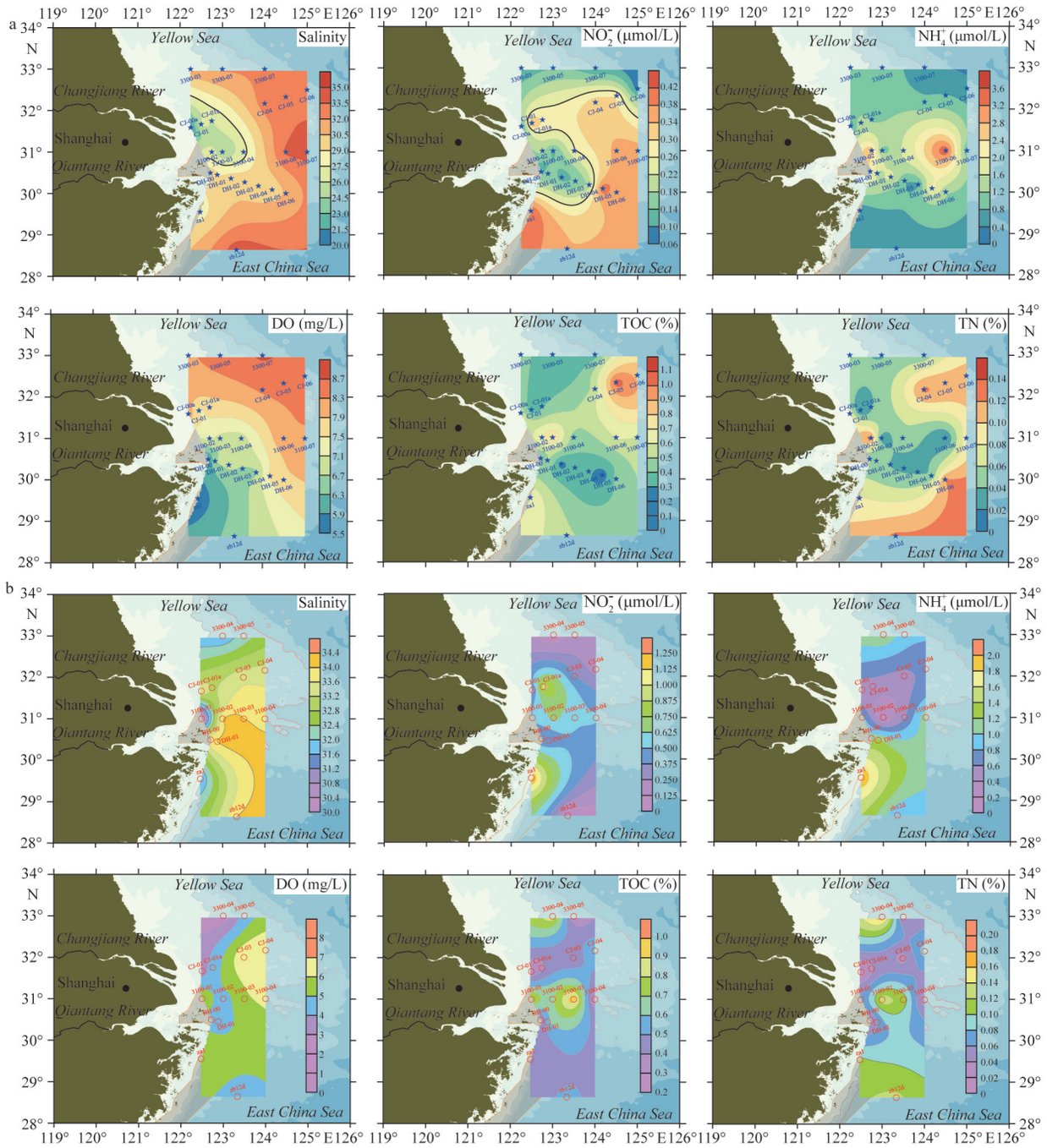


Fig.2 Spatial gradient of concerned environmental factors in April–May (a), August–September (b) at sampling sites in CJE and its adjacent area

September was between 4.48 and 6.92 mg/L, which were lower than April–May. Among the detected sites, salinity in August–September was little higher than April–May, and lowest at Site CJ-00a. The TOC and TN were among 0.261%–0.981% and 0.013%–0.174%, respectively.

3.2 Quantification analysis of *nirS*, amx-16S rRNA and *ScnirS* genes

The amx-16S rRNA and *nirS* gene were used to estimate the anammox and denitrifying bacterial abundance, respectively. The abundances of *nirS*-type denitrifiers ranged from $(4.04\pm0.88)\times10^5$ to $(6.57\pm1.99)\times$

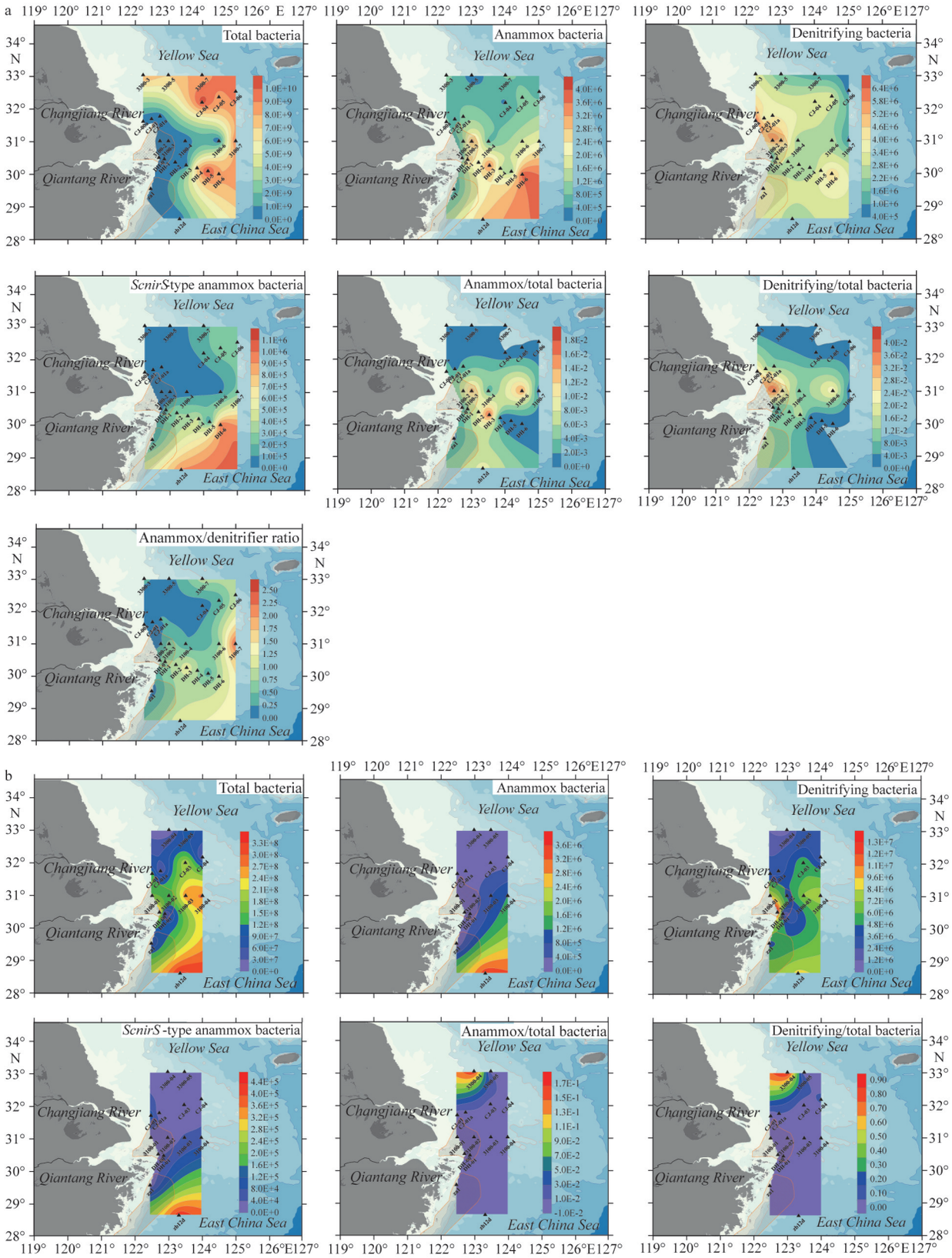
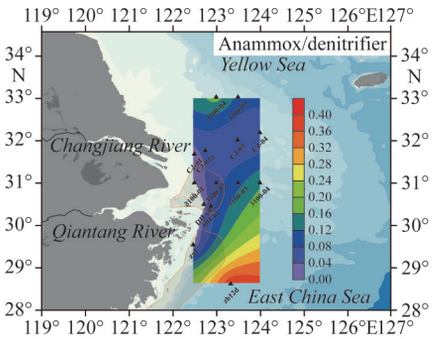


Fig.3 Horizontal distribution of total-, *nirS*-type denitrifying-, anammox-, *Ca.* Scalindua genus-bacteria abundance in April–May (a) and August–September (b)

To be continued

Fig.3 Continued



10^6 copies/g (April–May; fresh weight, Fig.3a), and (6.08±0.51)×10^5 to (1.54±0.01)×10^7 copies/g (August–September; fresh weight, Fig.3b). There was the highest abundance at the Site CJ-00a and DH-00, and the lowest abundance at the Site DH-00 and 3300-04 in April–May and August-September. The abundance of *nirS* gene in August–September (~5.48×10^8 copies/g) was higher than that in April–May (~2.98×10^7 copies/g). The abundance of *nirS* gene increased gradually with increasing pH (r=-0.549, P<0.05), NO_x^- (r=0.663, P<0.01), NH_4^+ (r=0.479, P<0.05), DIN (r=0.733, P<0.01), with negatively correlation with salinity value (r=-0.534, P<0.01, n=23). Contrast to the abundance of *nirS* gene, that of amx-16S rRNA gene ranged from (1.71±0.21)×10^5 to (3.86±3.34)×10^6 copies/g (April–May; Fig.3a) and (4.73±3.98)×10^4 to (3.49±1.01)×10^6 copies/g (August–September; Fig.3b). Higher abundances of the amx-16S rRNA gene were measured in April–May (~1.43×10^6 copies/g) than that in August–September (~5.30×10^5 copies/g) and the proportion of anammox bacteria in total bacteria was also slightly lower in August–September. There was a negative correlation between anammox abundance and C/N ratio (r=-0.545, P<0.01). The amx-16S rRNA gene abundance positively correlated with pH (r=0.428, P<0.05). In all sediment samples, *nirS* gene abundances were 1–2 orders of magnitude higher than amx-16S rRNA. Considering the major genus of anammox bacteria was *Ca*. Scalindua, the specific functional gene, *ScnirS*, for *Ca*. Scalindua was detected. The abundance of *Ca*. Scalindua positively correlated with amx-16S gene that covered all anammox bacteria in April–May (coefficient=0.75, P<0.01), and August-September (P<0.01, r=0.92). Alternatively, the abundance of amx-16S rRNA and *ScnirS* gene positively correlated with salinity value (P<0.05, r=0.52). This positive correlation also explained *Ca*. Scalindua was the main anammox bacterial clade. The overall spatial distribution of anammox bacteria in studied area was lower in lower salinity coastal area and higher in higher-salinity offshore area (Fig.3).

3.3 Alpha diversity analysis of denitrifying and anammox bacteria community

The total numbers of high-quality sequences from *nirS* and amx-bac16S rRNA gene obtained from each sample were 21 546–44 423 and 22 160–46 666 in April–May 2017, respectively. Good's coverage values of these samples were all above 99%, indicating that the obtained sequences could adequately reflect the diversity of denitrifying and anammox bacteria in the sediments. The corresponding richness (Chao1 estimator) and diversity indexes (Shannon index) and coverage calculated for each sample were showed in Fig.4 and the detailed information listed in Supplementary Table S4. Using the 97% similarity cutoff, the *nirS* gene sequences were clustered into 1 579 OTUs. Within every sample, OTU numbers ranged from 383 to 734, with the highest richness at Site 3100-06 and the lowest at Site DH-06. Meanwhile, amx-16S rRNA gene sequences with 97% similarity cutoff, were clustered into 159 OTUs. Within every sample, OTUs ranged from 25 to 39, indicating the highest richness at Site DH-03 and the lowest at Site CJ-01a. Obviously, the richness of anammox bacteria was far less than denitrifying bacteria (Fig.4). The α-diversity indices, Chao1 and Shannon index of *nirS*-type denitrifying bacteria were much higher than anammox bacteria. However, the Simpson index of the *nirS*-type denitrifying bacterial community was lower than that of the anammox bacterial community. As a result, the *nirS*-type denitrifying bacterial species richness and balances richness were both higher than anammox bacteria. And anammox bacteria had a greater number of rare species.

3.4 Community composition and phylogenetic analysis of denitrifying and anammox bacteria

To further investigate the community composition and the relative abundance of anammox and *nirS*-type denitrifying bacterial OTU, the top 30 of dominant OTUs were selected for phylogenetic analysis (Fig.5). Based on the phylogenetic analysis of amx-16S rRNA gene, *Ca*. Scalindua, *Ca*. Kuenenia, and *Ca*. Anammoxodans were three discovered genera, the proportion of relative abundance reached 95% of total bacterial abundance

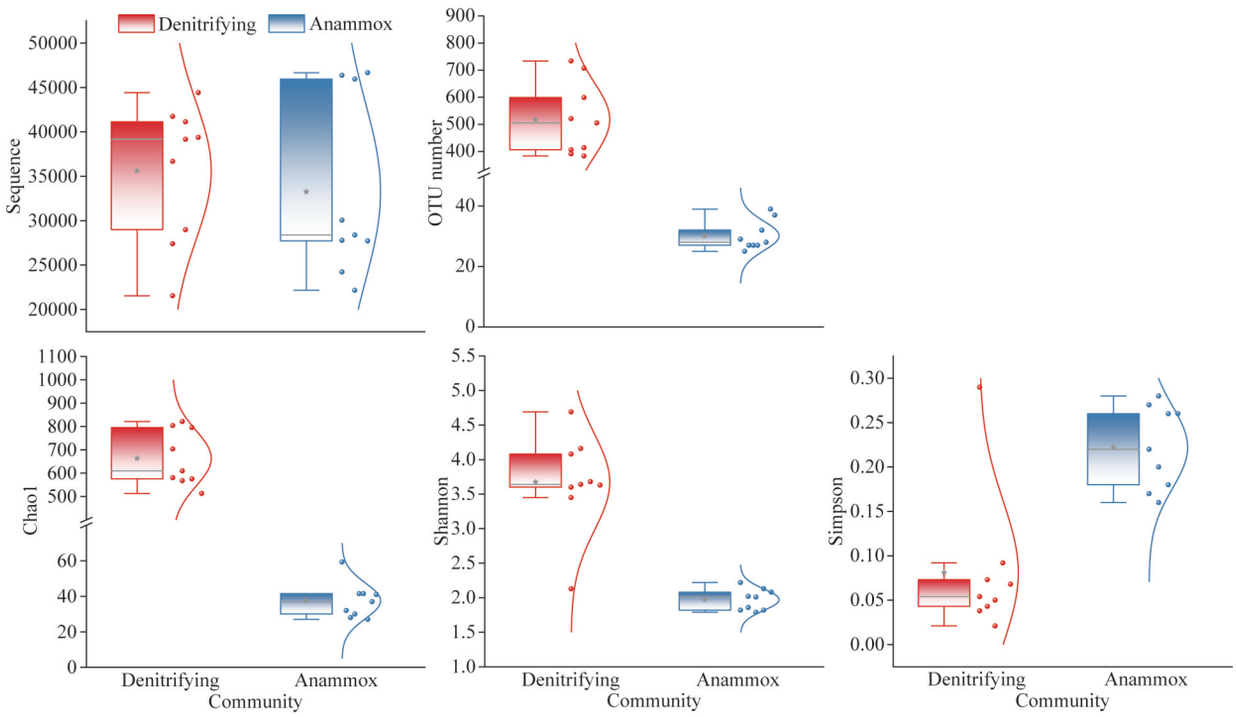


Fig.4 The *α*-diversity indices of denitrifying and anammox bacteria communities

in every sample, except Site DH-00. One clade of the unclassified Planctomycetes might be a new *Ca*. Scalindua branch with relative abundance of 14.2%, and was a dominant group in Site DJ-0a and Site DH-00. *Ca*. Scalindua (OTU130, OTU59, and OTU118) were the dominant OTUs, collectively accounting for >50% of the community in most samples (e.g., site CJ-04, 3100-02, DH-00) (Fig.5a). The others, comprising rare OTUs, representing a substantial proportion (15%–25%) in certain samples (e.g., site DH-00), highlighting the presence of a diverse rare biosphere. These top10 OTUs were all belonged to *Ca*. Scalindua, but OTU147 belonged to *Ca*. Aestuarianus (Fig.5c).

For *nirS*-type denitrifying bacteria, a comprehensive analysis of OTUs samples from CJE surface sediments shows that, at genus level, six clades were detected (Fig.5d). The selected OTUs all belong to Proteobacteria, as the dominant groups of *nirS*-type denitrifying bacteria in the sediments from CJE. Pseudomonas, including OTU1 251, 1 313, 1 336, 203, and 453 were present in all the detected samples. Notably, OTU495, 1 270, 1 313 and 375 showed marked variability, with OTU495 peaking in site 3100-04 (~40%), OUT1 270, 1 313 in site DH-00 (~70%) and OTU375 in site DH-06 (~20%). Such disparities suggest microenvironmental selection, possibly driven by differences in nitrate availability or organic carbon inputs. The others were consistently higher (20%–30%) compared to the anammox community, implying greater taxonomic diversity among *nirS*-type denitrifiers. These results demonstrated that both anammox and *nirS*-type denitrifying communities are structured by a combination of dominant taxa and a persistent rare biosphere. These top30 OTUs of *nirS*-type denitrifying bacteria were almost affiliate with known Betaproteobacteria (*Alcaligenes faecalis* and *Dechloromonas* sp.) and Gammaproteobacteria (*Pseudomonas aeruginosa* and *Pseudomonas grimontii*), which were usually observed in seawater column, coastal and estuary sediments. In total, our results showed that the CJE *nirS*-type nitrite-reducing bacteria were more abundant than anammox bacteria in CJE subseafloor sediments. The differential distribution of dominant OTUs across samples underscored the influence of localized environmental conditions on microbial assemblage composition, with implications for nitrogen cycling efficiency in estuarine ecosystems.

3.5 Relationship of denitrifying and anammox bacterial community abundance with environmental variables

The analysis revealed significant relationships between key environmental variables and microbial abundance (Fig.6). In April–May, NH_4^+, DO, salinity, and C/N ratio exhibited statistically significant correlations with bacterial abundance. NH_4^+, TN, and

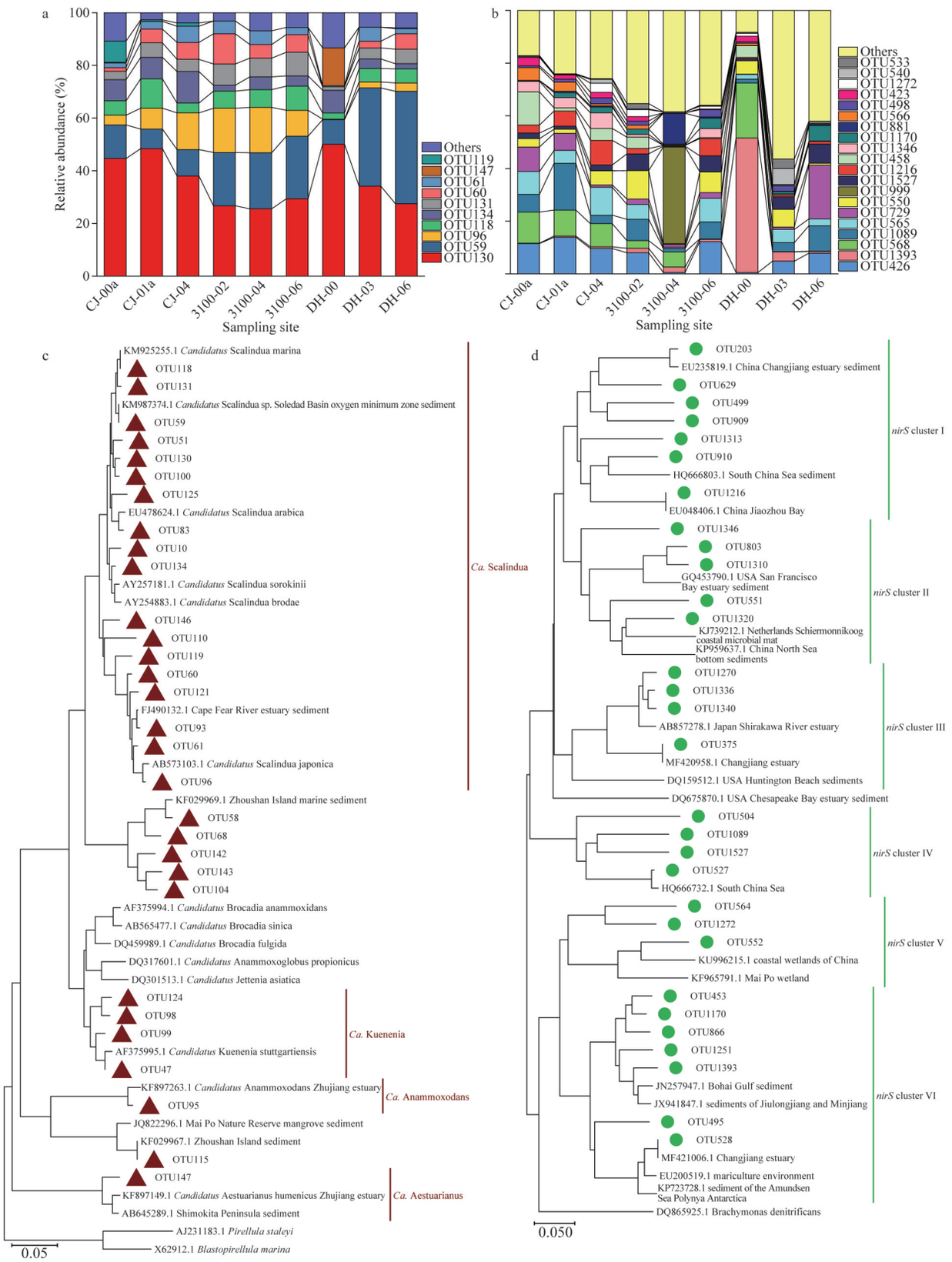


Fig.5 The relative abundance of anammox (a) and denitrifying (b) bacterial communities and, phylogenetic relationships of amx-16S rRNA (c) and *nirS* (d) genes in nine CJE stations where are significantly affected by the diluted water subseafloor sediments

Bootstrap values represent 1 000 replicates, and only values above 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Red triangle and green circles referred to the top OTUs obtained from anammox and *nirS*-type denitrifying bacteria in this study, respectively.

C/N ratio exhibited statistically significant correlations with *Scnir*-type and denitrifying bacterial abundance (Fig.6a). In August–September, DO exhibited statistically significant positive correlation with bacterial abundance and the ratio of anammox to total bacteria, while significant negative correlation with the ratio of denitrifying to total bacteria (Fig.6b). And C/N ratio and TN are significant positive correlation with *nirS*-type denitrifying and the ratio of denitrifying to total bacteria, respectively. The direction and strength of correlations suggest that bacterial abundances are influenced by nutrient availability (NH_4^+, NO_2^-), redox conditions (DO), and organic matter composition (TOC, TN, C/N ratio). Overall, these results demonstrate that DO, C/N ratio, and TN serve as some sensitive indicators of environmental conditions, reflecting shifts in nutrient balance, organic matter input, and physicochemical parameters.

The relationship between community composition of N-loss microbial community and the sediment properties were analyzed by BIO-ENV analysis (Supplementary Table S5). NH_4^+/NO_x^- was the best environment variable to explain the variance in community structure based on the relative abundance of *nirS*-type denitrifying bacteria (ρ_{nirS}= 0.239 9), and a combination of salinity, NH_4^+/NO_x^-, TN explained best in anammox bacterial community structure (ρ_{amx}=0.489 6). NH_4^+/NO_x^- (ρ=0.489 6) and a combination of salinity, NH_4^+/NO_x^- (ρ=0.472 3) also explained anammox bacteria better in April–May 2017. Meanwhile, a combination of NH_4^+/NO_x^-, TOC, C/N ratio (ρ_{nirS}=0.220 8) and salinity, NH_4^+/NO_x^-, TN (ρ_{amx}=0.489 6) were chosen for RDA analysis. According to the Monte Carlo test, there was no environmental factor combination significantly influenced the N-loss bacterial community. The two main axes accounted for 70.87% and 23.56% of the total variation in N-loss microbial abundance structure in April–May and August–September 2017 (Fig.7).

4 DISCUSSION

4.1 Significant seasonal and spatial variation of the N-loss bacterial abundance

This study investigated the abundance and diversity of total and N-loss bacteria (including anammox and *nirS*-type denitrifying bacteria) in an estuary-marine ecosystem, which provided a continuous environmental gradient to show the shift in microbial community. There was no significant discrepancy of the N-loss bacterial abundance between July–August 2011 (Fu et al., 2019) and August–September 2017 (summer, this study) in surface sediments (one way ANOVA, P>0.05), which were higher than that in April–May 2017 (spring, this study). This suggested that microbially driven processes were more active in summer than in spring, highlighting key factors influencing nitrogen cycling. From the perspective of the distribution trend of anammox and denitrifying bacterial abundance consistent with total bacterial abundance, where summer showed higher than spring (Fig.3). This meant the potential N-loss process based on abundance, including anammox and denitrification, became active in summer. Although there are significant seasonal variations, the changes in regional distribution among different seasons are not substantial. The abundance of anammox, *ScnirS*-type anammox and *nirS*-type denitrifying bacteria in CJE showed the consistent distribution trend between spring and summer. As

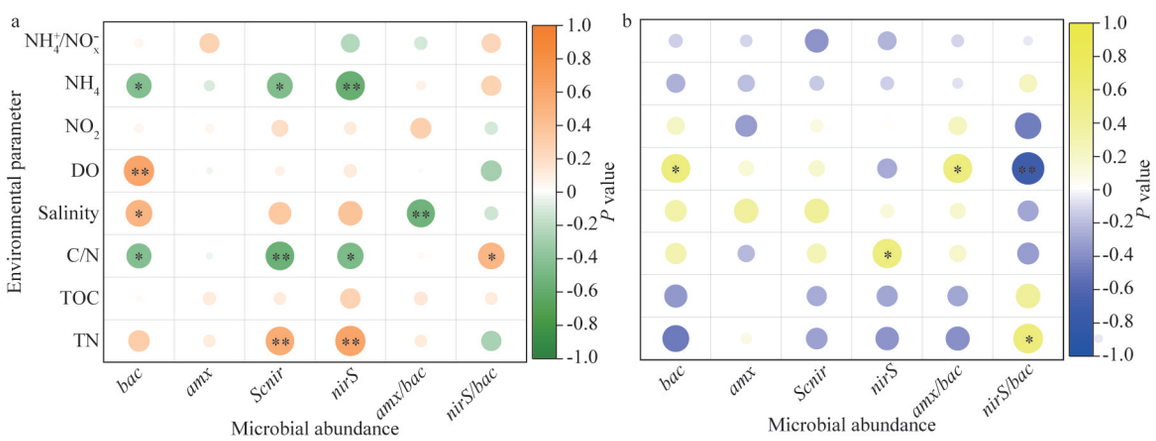


Fig.6 Pearson analysis between the environmental parameters and N loss microbial abundance in April–May (a) and August–September (b)

*: P≤0.05; **: P≤0.01.

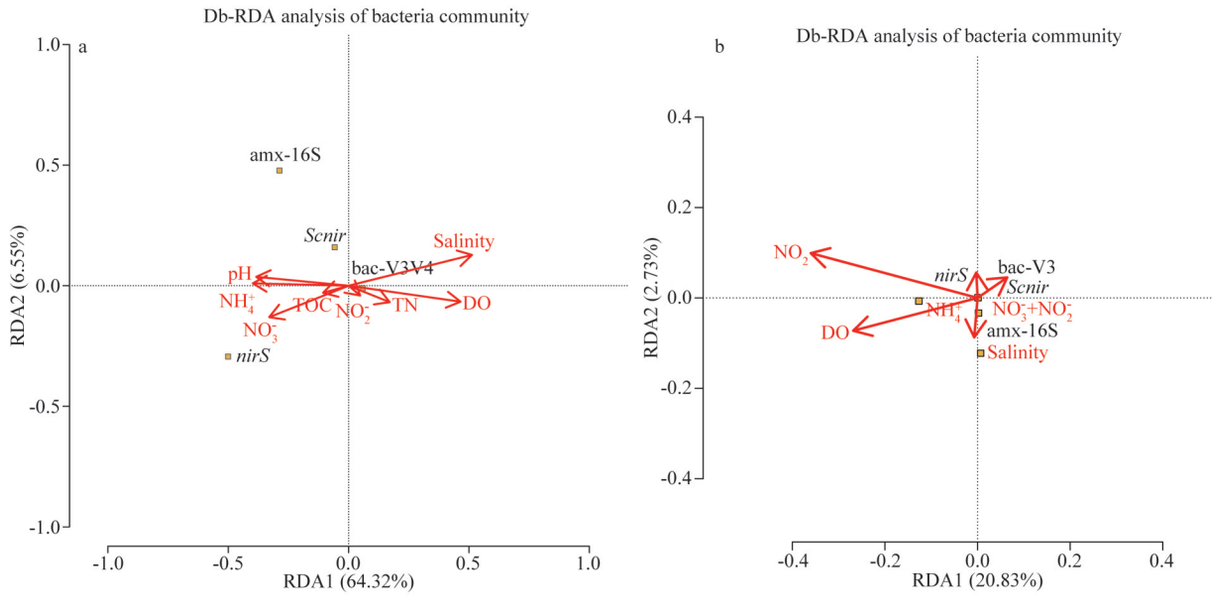


Fig.7 The RDA analysis between the environmental parameters and N-loss microbial abundance in April–May (a) and August–September (b)

the seasons change, the areas with strong N-loss processes did not exhibit migration. Anammox bacterial abundance was lower at nearshore area and higher in open ECS, that is because the environmental filtering, including salinity, substrate. Besides, the doubling time of anammox bacteria was 14–21 d (Wang et al., 2014). The nearshore areas are greatly affected by the inflow of fresh water from CJ and human activities, while the ECS shelf areas are less likely to be affected. *NirS*-type denitrifying bacterial abundance was higher near CJE, lower in the ECS shelf area. In contrast, the abundance ratio of *nirS*-type denitrifying or anammox bacteria to total bacteria showed consistent tendency, and was low in spring and high in summer (Fig.4). These trends were most likely result from nutrients concentration low in spring, and high in summer (Wang et al., 2014). *NirS*-type denitrifying bacteria had a much higher abundance than that of anammox bacteria, no matter spring or summer, which was consistent with that in the Arabian Sea OMZ (Ward et al., 2009) and South China Sea (Li et al., 2013a), indicating *nirS*-type denitrifiers might play a more important role on contributing to the overall nitrogen removal in estuarine surface sediments. Considering the abundance ratio of *nirS*-type denitrifying or anammox bacteria to total bacteria, there was a significant difference between spring and summer. It is possible that the dominant functional bacterial groups undergo slight changes with seasonal variations in CJE. Meanwhile, the N-loss process was most active near CJE during spring, while it was relatively more active in the north region of CJE during summer. The N-loss process exhibits seasonal shifts in the microbe-driven geochemical cycles, which may be influenced by the changes in seasonal hypoxic zones.

Anammox and *nirS*-type denitrifying bacterial abundance detected by amx-16S rRNA, *nirS* and *Scalindua-nirS* genes, showed similar distribution patterns in CJE (Fig.3). Anammox and *nirS*-type denitrifying bacteria had a synergistic relationship to the substrate. *NirS*-type denitrifying bacteria can reduce nitrate to nitrite, providing necessary reaction substrate for anammox bacteria. Meanwhile, both *nirS*-type denitrifying and anammox bacteria take nitrite as their substrate. The *nirS* and *ScnirS* genes maintained the same trend, were both positively correlated with TN (P<0.01), and negatively correlated with ammonium concentration and the C/N ratio (Fig.6a). A significant positive correlation between NH_4^+ and NH_4^+/NO_x^- would indicate that ammonium availability drives the ratio, potentially reflecting reduced conditions favoring ammonium accumulation over nitrate. Conversely, a negative correlation with NO_2 might suggest rapid nitrification or denitrification processes consuming nitrite. DO and salinity could also play critical roles; low DO often promotes ammonification and inhibits nitrification, while salinity may affect microbial activity and nitrogen speciation. The C/N ratio and TOC might further modulate these processes by influencing microbial metabolism, with higher organic carbon potentially enhancing denitrification,

thereby altering NH_4^+/NO_x^- dynamics. In summer, total bacterial abundance and the abundance ratio of anammox to total bacteria were positively correlated with DO (P<0.05), meanwhile, the abundance ratio of denitrifying to total bacteria was positively correlated with the C/N ratio, negatively correlated with DO (P<0.01). The relationship in summer may reveal similar trends but could differ in the magnitude or significance of correlations, possibly reflecting spatial or temporal variability in environmental conditions. It is plausible that anammox bacteria prefer the high-salinity offshore environment, which coincidentally had higher bottom DO during our sampling than the inner estuary, but their activity takes place in the anoxic sediment micro-niches. This allows microbes to respond rapidly to changing environmental conditions, but also means that denitrification is utilized as an auxiliary respiration under aerobic conditions when imbalances occur in electron donor and acceptor supply (Marchant et al., 2017). Meanwhile, ammonia oxidation is an aerobic process, which consumes oxygen and provides an anaerobic environment for anammox process. Ammonia oxidation, denitrification and anammox process are mutually coupled and synergistic (Risgaard-Petersen et al., 2004; Rysgaard et al., 2004; Meyer et al., 2005; Lam et al., 2007). In addition to DO and organic matter stoichiometry, pH may indirectly influence N-loss microbial distributions by regulating ammonia speciation and substrate availability. Increasing pH shifts the NH_4^+/NH_3 equilibrium toward free NH_3, which is more membrane-permeable and can enhance reduced nitrogen accessibility in sediments (Krishnan et al., 2008; Garvin et al., 2009; Zhang et al., 2018). Although anammox bacteria primarily utilize NH_4^+, pH-driven changes in ammonia chemistry can modulate ammonium turnover, nitrification-derived nitrite supply, and thus substrate coupling among nitrifiers, denitrifiers, and anammox bacteria (Kartal et al., 2011). Therefore, the observed correlation between pH and N-loss microbial abundance likely reflects indirect biogeochemical regulation of nitrogen availability rather than a direct physiological pH effect.

SHZ in the Changjiang estuary is dynamic, with seasonal and spatial variations (Zhou et al., 2025). Anammox bacteria abundance levels varied with the seasons. Anammox bacterial abundance may serve as an indicator of the seasonal SHZ in estuary. In spring, as temperature gradually increased and the SHZ formed, the sedimentary environment gradually became suitable for the extremely sensitive anammox bacteria. However, after entering the autumn, the temperature decreases, and the range of low oxygen area shrinks rapidly, thus reducing the abundance of anammox bacteria. The abundance of the dominant genus *Ca*. Scalindua was (1.39×10^4)–(9.94×10^5) copies/g (April–May) and (4.85×10^2)–(4.31×10^5) copies/g (August–September), respectively. The proportion of anammox bacteria in total bacteria was very low. But the ratio of anammox bacteria to total microbial biomass reported in this study was higher than Fu et al. (2015) in Zhujiang (Pearl) River estuary and Dang et al. (2010) in Jiaozhou Bay. This might be related to anammox bacterial own growth cycle, seasonal SHZ in CJE sedimentary environment. The intensified stratification during the summer and the impact of upwelling contribute to the formation of the seasonal hypoxic zone in CJE (Wei et al., 2015), which provides a suitable environment for denitrifying and anammox bacteria. To some extent, the variation trend of N loss microorganisms and the development of seasonal SHZ overlapped and complemented each other.

According to the average labile POM stoichiometry in the ocean, $C_{6.6}H_{10.9}O_{2.6}N$, Babbin et al. (2014) had proved anammox contribute 28% of the N loss in the ocean. Largely affected by the freshwater plume, anammox and denitrification exhibited distinct spatial patterns (regionalization). In sediments of the subterranean estuaries in Gloucester Point, Virginia (Wu et al., 2021), anammox and denitrification played equal roles in N loss, while the abundance of anammox bacteria was an order of magnitude less than denitrification, which meant the genes abundance ratio of amx-16S rRNA to *nirS* was around one in ten. As C/N ratio ranged 3.6–10.7 in this study, fixed N was sufficient compared the average OM level with the C/N ratio at 3.6–6.6 in Babbin et al.'s study (2014). The model developed by Babbin et al. (2014) can be helpful in evaluating the effects of SHZ expansion on future climate, potential shifts in average C/N ratios with changing atmospheric CO_2 concentrations, and negative feedbacks to primary production via N loss. The organic carbon content may affect NO_2^- production through reduction or other unknown reactions (Hu et al., 2012) and promote NH_4^+ accumulation (Li et al., 2013a), which jointly affect anammox bacterial community diversity and abundance. The N-loss contribution of anammox should be greater than the average level in the ocean, such as the Site DH-06, CJ-04, and 3100-07 in this study. Besides, the genes abundance ratio range of amx-16S rRNA to *nirS* was 0.03–0.54 in April–May, 0.002–0.15 in July–August, and 0.01–0.40

in August–September, respectively (Supplementary Table S6). Also, this ratio was lower near CJE and higher away from CJE, further indicating that near the CJE, denitrification was the dominant N loss way especially in summer, and anammox lead the N loss away from CJE and was more active in April–May. This was consistent with Song's study (Song et al., 2021). And owing to the N content is modified by the largest coefficient, and the H and O contents tend to compensate for each other, the C/N ratio is the most important parameter in setting the N loss balance between anammox and denitrification. When the C/N ratio is more than 6.6, the anammox's N loss contribution is less than 28%. In studied area, C/N ratio of Site 3100-07 and DH-02 was 6.43 and 6.50, and the predicted anammox proportioned ~45% of N loss according the model in Babbin's paper (2014). Previous modeling work by Babbin et al. (2014) demonstrated that the relative contribution of anammox to fixed nitrogen loss declines sharply when organic matter availability increases, with anammox accounting for <28% of total N loss at C/N ratios >6.6. In our study area, the C/N ratios at Sites 3100-07 and DH-02 were slightly below this threshold (6.43 and 6.50, respectively), which would predict a relatively high anammox contribution (~45%) based on Babbin's model. However, our microbial community data show that denitrifying bacteria were substantially more abundant than anammox bacteria across the CJE, indicating a potential dominance of heterotrophic denitrification over anammox in terms of genetic potential for N_2 production. This apparent discrepancy suggests that, despite marginally favorable C/N conditions, additional factors—such as elevated organic carbon lability, stronger electron donor availability, or intensified oxygen depletion—may have preferentially stimulated denitrifiers and suppressed anammox activity. Together, these results indicate that C/N ratio alone is insufficient to predict N loss pathways in this system, and that local biogeochemical and microbial constraints modulate the balance between denitrification and anammox. The ratio of denitrifying to anammox bacteria in our study indicated that N_2 production was mainly contributed by denitrification process in the CJE, which agreed with the observations from the CJ marsh and estuary sediments (Zhou et al., 2014; Hou et al., 2015), and another eutrophic coastal sediment (Thamdrup and Dalsgaard, 2002).

Overall, the N-loss bacteria and environment factors highlights the complexity of nitrogen dynamics, where multiple interacting factors govern NH_4^+/NO_x^-. These findings align with previous studies emphasizing the interplay between redox conditions, organic matter, and microbial processes in nitrogen cycling (Dang et al., 2010; Li et al., 2013b; Zhao et al., 2023). Future studies could expand on these findings by incorporating isotopic tracers or metagenomic data to elucidate mechanistic pathways. Such insights are crucial for managing ecosystems affected by eutrophication or anthropogenic nitrogen inputs.

4.2 Anammox bacterial community is significantly influenced by the diluted water

For N-loss bacteria, denitrifying bacteria had far higher diversity and larger abundance than anammox. It was possible that the intensity of other processes coupled to anammox was changing, for denitrification process was increasing to compete for substrate. The relative abundance analysis of anammox and *nirS*-type denitrifying bacteria across different samples reveals distinct community structures and potential ecological dynamics in marine environments. The low diversity is typical for anammox guilds compared to the highly diverse denitrifiers (Schmid et al., 2007). This suggests their adaptability to varying environmental conditions, possibly linked to substrate availability or oxygen gradients. Notably, the presence of multiple OTUs at lower abundances highlights the coexistence of niche-specific taxa, which may contribute to functional redundancy in nitrogen cycling. The predominant OTUs of *nirS*-type denitrifying bacteria differed among samples (Fig.5b). Betaproteobacteria and Gammaproteobacteria were prevalent, indicating their key role in nitrate reduction. The heterogeneity in community composition across samples, such as the prominence of OUT1270, OTU1313 in DH-00 (~70%), OTU495 in 3100-04 (~40%) and OTU375 in DH-06 (~20%), may reflect localized environmental drivers like organic carbon content or salinity fluctuations. These findings align with prior studies emphasizing the spatial variability of microbial nitrogen-cycling communities in marine systems. The dominance of specific OTUs suggests selective pressures favoring certain metabolic traits, while the rare biosphere may serve as a genetic reservoir for ecosystem resilience.

High salinity inhibited the growth of denitrifying bacteria and provided a good ecological niche for anammox bacteria *Ca*. Scalindua (Hou et al., 2018; Pan et al., 2023). Considering *Ca*. Scalindua is strictly marine/halophilic, its increasing abundance offshore restricted it from the freshwater-dominated

inner estuary (Fig.8). With a certain salinity gradient affected by the diluting water, three sites, DH-00, DH-03, and DH-06, were selected to analyze the influence of salinity on anammox bacterial community structure. Site DH-00 was of great different to the other stations, which located at Hangzhou Bay, greatly affected by diluted water, with lower salinity and greater differences in nutrient status, resulting in high relative abundance of OTU147 (*Ca*. Aestuarianus, Fig.3b). With extending outwards, Site DH-03 and DH-06 were less and least influenced by the fresh water. As a result, there were significant differences in community structure between DH-00 station and other stations. Estuarine genus *Ca*. Aestuarianus was detected at the station DH-00 located nearest the CJE. OTU130 (*Ca*. Scalindua) was particularly abundant in CJ-01a and DH-06 (~45%), suggesting its adaptability to varying redox conditions. In contrast, OTU60, OTU61, OTU119 (*Ca*. Scalindua) and OTU147 (*Ca*. Aestuarianus) were present at lower abundances but exhibited sample-specific distributions, potentially indicating niche partitioning. The relative abundance of *Ca*. Aestuarianus at Site DH-00 was 14.2%, belonging to the dominant clade, was consistent with Fu et al. (2015) in Zhujiang River estuary, suggesting that it was a new genus growing in the estuarine habitat. Some non-dominant genera *Ca*. Kuenenia occurred in Site CJ-00a where was near CJE, also *Ca*. Brocadia was detected at Site 31 located near Zhoushan Islands in previously Cruise 2011 (Fu et al., 2019). Non-dominant genera *Ca*. Brocadia and *Ca*. Kuenenia were also detected in estuary sediments. *Ca*. Brocadia and *Ca*. Kuenenia might originate from land and had a little certain tolerance to the high-salt environment.

Ca. Scalindua, with widely distributed had a broad ecological niche in marginal and estuarine sedimentary environment. *Ca*. Scalindua was the predominant group of anammox bacteria (relative proportion was over 90%). High denitrifying and anammox bacterial similarities were represented between the results in this study and Cape Fear estuary (Dale et al., 2009), Peruvian OMZ (Woebken et al., 2008) and the marginal sea of China, including Jiaozhou bay (Dang et al., 2010), the CJ tidal flat wetland (Hou et al., 2013, 2015), the Zhujiang estuary (Fu et al., 2015). The high OTU diversity also confirmed the high diversity detected in estuarine and marine environments (Schmid et al., 2007; Dang et al., 2010; Li et al., 2011a, b). The higher diversity of anammox bacteria in estuarine ecosystems than that in the oceans may be due to the interaction between land and sea. As the dominant one, *Ca*. Scalindua was presumed to play a vital role in nitrogen removal process. It is reported that *Ca*. Scalindua was widely distributed in salt-water lakes and oceans, as *Ca*. Brocadia and Kuenenia widely occurred in river ecosystems (Devol, 2015). The

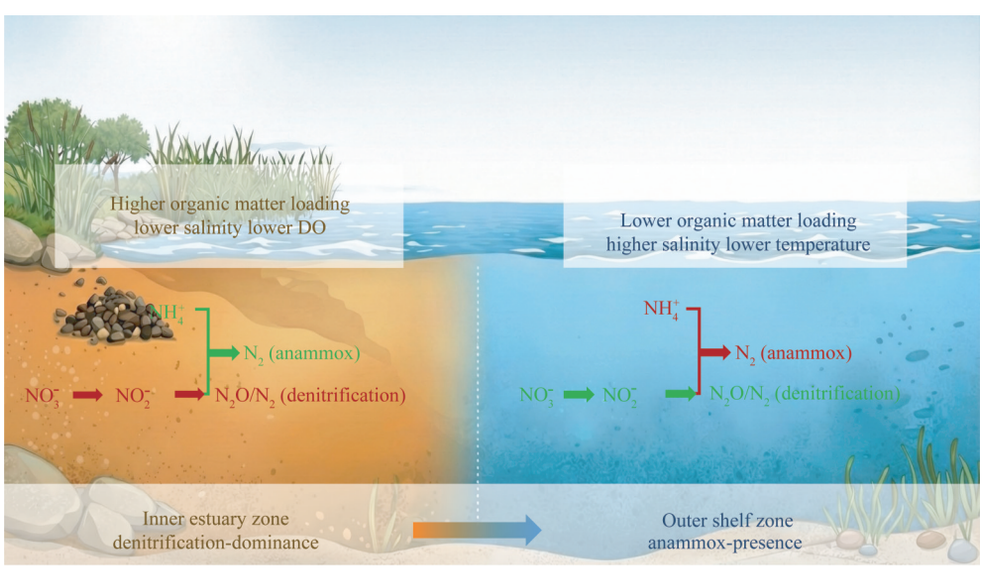


Fig.8 Seasonal shift of nitrogen loss pathways from denitrification in the inner estuary to anammox in the outer shelf

This conceptual diagram illustrates the seasonal and spatial transition of dominant nitrogen loss pathways along the estuary-shelf continuum. In the inner estuary, elevated organic matter input, high C/N ratios, and reduced DO favor heterotrophic denitrification as the primary N_2 production pathway. Moving offshore toward the outer shelf, lower organic carbon availability, higher salinity, and more stable low-oxygen conditions promote the persistence and relative importance of anammox, which couples ammonium and nitrite oxidation to N_2 production. This shift highlights the complementary roles of denitrification and anammox in regulating nitrogen removal under seasonally varying hypoxic conditions.

existence of different dominant genera may be the significant difference in salinity between oceans and rivers (Sun et al., 2014). Previously studies concluded that salinity was an important environmental factor affecting the dominant genus of anammox bacteria and some terrestrial anammox bacteria clades can tolerate in marine environment. The diversity of anammox bacteria was low in marine environment (Kuypers et al., 2018; Tamura et al., 2021; Ren et al., 2022; Zhao et al., 2023). The dominant OTU was different owing the gradient salinity value (Supplementary Fig.S2). Salinity increased, the dominant OTU(s) changed from OTU 134 in Site DH-00 to OTU 59 in Site DH-06, and OTU134 and OTU59 were both the dominant in Site DH-03. The dominant OTU147 (*Ca*. Aestuarianus) was not detected in high salinity value Site DH-03 and -06. The tolerance degree of *Ca*. Aestuarianus was weak in high salt environment, while *Ca*. Scalindua was strong. Moreover, in *Ca*. Scalindua genus, OTU 134 had a relatively low tolerance to salinity while OTU 59 had a high tolerance. Therefore, it is believed that, in CJE surface sediments, the predominant genus *Ca*. Scalindua may have more detailed branches at the next classification level, which are suitable for the environment with different salinity values. *Ca*. Scalindua occupies a relatively wide niche distribution in the estuarine ecosystems like CJE. This study also found a relatively low proportion of non-*Ca*. Scalindua anammox bacteria, *Ca*. Brocadia, *Ca*. Kuenenca, *Ca*. Aestuarianus, and some other unidentified. Further research integrating physicochemical data (e.g., NO_3^-, NH_4^+ concentrations) is warranted to elucidate the mechanistic links between community structure and biogeochemical processes. Such insights are critical for predicting microbial responses to anthropogenic disturbances, including eutrophication and climate change.

The significant spatiotemporal co-occurrence of *nirS*-coding denitrifiers and anammox bacteria suggests a synergistic metabolic coupling centered on nitrite availability rather than competitive exclusion (Risgaard-Petersen et al., 2004; Meyer et al., 2005; Lam et al., 2007). Given that *nirS*-type denitrifiers were orders of magnitude more abundant than anammox bacteria and nitrate concentrations far exceeded nitrite in the CJE, denitrifiers likely function as the primary source of nitrite via nitrate reduction. This "metabolic handoff" is further supported by the consistent positive correlation of both functional genes with TN and their negative correlation with the C/N ratio (Hu et al., 2012; Li et al., 2013a). Under the observed conditions of limited labile organic carbon, incomplete heterotrophic denitrification may facilitate nitrite accumulation, which is subsequently scavenged by anammox bacteria (Thamdrup and Dalsgaard, 2002). This implies a "partial denitrification-anammox" pathway where denitrifiers provide the essential electron acceptor (NO_2^-) for anammox, enhancing overall nitrogen removal efficiency through metabolic sharing.

5 CONCLUSION

This study elucidates how seasonal environmental gradients restructure nitrogen loss pathways in the surface sediments of the CJE, demonstrating that denitrification and anammox are functionally partitioned rather than redundant processes along the estuary-shelf continuum. Denitrification dominates in the inner estuary under high organic carbon availability and elevated C/N ratios, whereas anammox becomes relatively more important in offshore areas and during spring, particularly at stations with C/N ratios around ~6.6 and persistent low-oxygen conditions. These seasonal microbial shifts highlight the sensitivity of estuarine nitrogen cycling to organic matter stoichiometry, oxygen availability, and salinity, implying that changes in carbon quality can disproportionately alter N_2 production pathways in eutrophic estuaries. Notably, anammox bacteria exhibit a narrower ecological niche and stronger coupling to DO, C/N ratio, and the SHZ than denitrifiers, identifying anammox abundance as a sensitive biological indicator of hypoxia development. This trait distinguishes anammox from more ubiquitous denitrifiers and underscores its utility for tracking redox-driven biogeochemical transitions in estuarine sediments. From a management perspective, integrating seasonal monitoring of anammox functional genes with conventional physicochemical measurements could improve early detection and assessment of hypoxia risk in the CJE and other eutrophic estuaries. Future work combining long-term time series, process-based rate measurements, and multi-omics approaches will be essential to generalize anammox-based microbial indicators and strengthen their application in adaptive estuarine management under ongoing eutrophication and climate change.

6 DATA AVAILABILITY STATEMENT

Illumina MiSeq sequence data from this study

were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP130581 (for anammox) and SRP144762 (for *nirS*-denitrifiers).

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Electronic supplementary material

Supplementary material (Supplementary Tables S1–S6 and Figs.S1–S2) is available in the online version of this article at https://doi.org/10.1007/s00343-026-5296-0.