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Shifts in the anammox bacterial community structure and abundance in sediments from the Changjiang Estuary and its adjacent area

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is an important process in marine nitrogen cycle. In this study, diverse anammox bacteria were identified in the sediments of the Changjiang (Yangtze) Estuary and its adjacent area. Specifically, the community characters of anammox bacteria in the studied area were studied by quantitative polymerase chain reaction (qPCR), as well as 16S rRNA gene- and functional gene (*hzo*)-based Roche 454 sequencing. The abundance of denitrifying bacteria detected by the *nirS* gene was greater than that of anammox bacteria. 16S rRNA and hzo gene fragments affiliating with known anammox bacterial lineages were recovered, and the two major phylotypes belonged to the *Candidatus Scalindua* (*Ca.* Scalindua) genus, with >90% sequence similarity. A phylogenetic analysis detected the *Scalindua* and *Brocadia* genera together with some anammox-like bacterial clusters, which suggested a higher diversity in the studied ecosystem than in open ocean environment, where only *Scalindua* genus was detected. A redundancy analysis (RDA) showed that total organic carbon (TOC) and total nitrogen (TN) content in sediments significantly influenced anammox bacterial abundance of. Spearman correlation analyses confirmed that the spatial variation in anammox bacterial abundance was highly correlated with TOC ($P < 0.01$) and TN ($P < 0.01$) contents in sediments.

Introduction

Most anthropogenic N is delivered to estuarine and coastal areas through river flow, groundwater discharge, and atmospheric deposition, which consequently represents a serious threat to these aquatic ecosystems. Understanding N removal and related microbial processes is important for developing N management strategies in order to protect such ecosystems [5]. In the global nitrogen cycle, bacterial denitrification was recognized for decades as the only quantitatively important process that converted fixed nitrogen to atmospheric nitrogen gas, N_2, thereby influencing many aspects of ecosystem functions and global biogeochemistry. However, a process novel to the marine nitrogen cycle, the anaerobic oxidation of ammonium and its coupling to nitrate reduction, has been shown to contribute substantially to N_2 production in marine sediments. Anaerobic ammonium oxidization (anammox) is a microbe-mediated process that was predicted in 1977 [3] but was first described in 1995 in the bioreactors of wastewater treatment plants in the Netherlands [37]. This process represents effective microbial nitrogen transformation under anoxic conditions [20]. The processes for removing nitrogen from oceans have since been recognized as anammox, the autotrophic oxidation of ammonium to N_2 by nitrite, and denitrification, a stepwise heterotrophic reduction of nitrate to N_2. Anammox removes bioavailable nitrogen and accounts for up to 28% of the total N_2 production in the world's marine environments [2]. Therefore, understanding the balance between anammox and denitrification is very important for understanding N cycling in suboxic zones.

Six anammox genera have been described, with 16S rRNA gene sequence identities between any two species ranging between 87 and 99% [22,24,50]. However, the high divergence between any two anammox bacterial genera (<87.1% similarity) makes the detection

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of anammox bacteria difficult when they are present at low concentrations [22]. One main limitation of using the 16S rRNA gene as a molecular marker is that it is not always related to the physiology of the bacteria [23]. The genes of anammox bacteria involved in anammox biochemical metabolism therefore provide a better alternative for the study of anammox bacterial communities [9,22,51]. Hydrazine oxidoreductase (*hzo*), which encodes a key anammox enzyme, has been suggested as an efficient tool for investigating anammox bacteria [25,29,43,45]. Li et al. [29] and Hirsch et al. [15] designed several PCR primers to detect the *hzo* gene in various marine sediment samples. They demonstrated that the anammox bacterial *hzo* gene was widely distributed in marine ecosystems and could be used to identify the diversity of anammox bacteria at high resolution. Amx-16S rRNA and *hzo* genes have thus been confirmed as functional biomarkers for investigating the community characteristics of anammox bacteria in natural ecosystems.

The Changjiang (Yangtze) Estuary is a large-river delta-front estuary (LDE) that receives large inputs of terrestrial materials from the Changjiang River. This river strongly influences the geochemistry, environment and ecosystem of the Changjiang Estuary and its adjacent area. Once discharged to the East China Sea (ECS), these terrestrial materials are subject to long-term hydrodynamic sorting and reworking, which results in changing formations of the surface sediment [49,58]. The frequent physical reworking of mobile muds has led their microbial communities to always be more active, diverse, and abundant than those of other sediment deposits [1]. Hou et al. [16] showed that high anammox bacterial diversity existed in the Changjiang Estuary and that community composition and diversity varied among different regions of the estuary. In addition, by performing ^{15}N tracing experiments, they found that denitrifying bacteria may be a primary source of nitrite for anammox bacteria in the estuarine marshes. Although denitrification makes the largest contribution to the total N loss [32], anammox plays a significant role in benthic nitrogen cycling in ESC sediments [48]. Investigations have revealed the coexistence of anammox, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) through slurry incubations using the ^{15}N isotope pairing technique. There have been several reports of anammox and denitrification, as well as DNRA, in the Changjiang Estuary and its adjacent area [16,32,48]; however, the contributions of different functional microbial groups to these processes remain unclear. In the present study, the community structure of anammox bacteria was determined in sediment samples from the Changjiang Estuary and its adjacent area by Roche 454 sequencing. 16S rRNA and *hzo* genes were both used as targets to measure the features of anammox bacterial communities, and the responses of native anammox bacteria to different local environmental properties were evaluated. In addition, the relationship between anammox and denitrification was inferred from the abundance data from the perspective of molecular biology. This work may provide novel insights into the microbial nitrogen cycle in the aforementioned area.

Materials and methods

Samples and collection

Twenty samples of surface sediment and bottom water were collected from the Changjiang Estuary and its adjacent area in the East China Sea during July and August 2011 by the R/V *Run-Jiang* (Fig. 1). Surface sediments were obtained from all sampling stations using a box corer, which scraped surface sediment samples with a thickness of 3 cm. The sediment samples were placed into sterile plastic bags and stored at −20 °C for molecular analysis. One portion of the bottom water was stored at −20 °C for dissolved inorganic nitrogen analysis, and the rest was kept at room temperature for silicate analysis after the addition of chloroform.

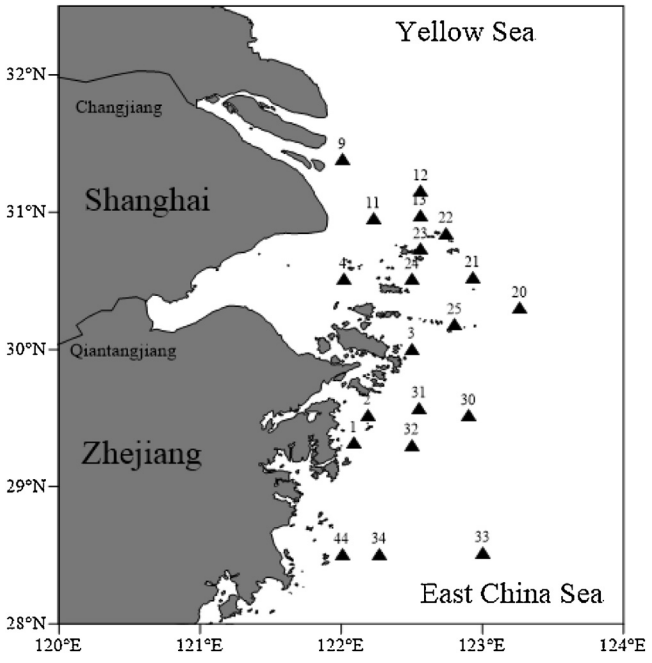

Fig. 1. Sampling sites in the Changjiang Estuary and its adjacent area in the ECS during July and August 2011.

Determination of environmental parameters

The depth at each site was determined. Several environmental parameters (temperature, salinity, and dissolved oxygen concentration) of the bottom water samples were recorded in situ with an RBR XR-620 Multi-Channel CTD (Elcee, Malaysia). Other parameters were measured on return to the laboratory. A QuAAtro nutrient auto analyzer (Seal Analytical Ltd., UK) was used as previously reported in order to measure the ammonium, nitrite, and nitrate concentrations in the bottom water from all sampling sites [34,35]. The total organic carbon (TOC) and total nitrogen (TN) contents in the surface sediments were measured using a FLASH 2000 elemental analyzer (Thermo Fisher Scientific Inc., USA), according to a previously published method [19,33]. Briefly, freeze-dried sediment samples (~30 mg) were placed in silver capsules and acidified in an HCl vapor bath for 8 h to remove inorganic carbon (IC) (mainly in the form of carbonate). Prior to the instrumental analysis of TOC and TN, the IC-free samples were dried and then carefully and tightly crimped in the tin capsules.

DNA extraction and polymerase chain reaction (PCR)

The sediments were thawed and mixed thoroughly, and approximately 0.25 g of sediment was then placed in a Lysing Matrix E Tube. Total genomic DNA of the sediment samples was extracted using the Power Soil DNA Isolation Kit (MO Bio, USA), according to the manufacturer's instructions. The integrity of the DNA was confirmed with 1% agarose gel electrophoresis, and the DNA concentration was determined with a Picodrop microliter spectrophotometer (Picodrop, Saffron Walden, Essex, UK). The 16S rRNA gene fragments (477 bp) of anammox bacteria were amplified by using specific PCR primers Amx368F (5′-TTC GCA ATG CCC GAA AGG) and Amx820R (5′-AAA ACC CCT CTA CTT AGT GCC C) [16]. The *hzo* gene fragments (463 bp) were amplified by primers *hzo*5F (5′-AGT ATG GGT ATG TCH AAT G) and *hzo*5R (5′-CAT CWG TCC

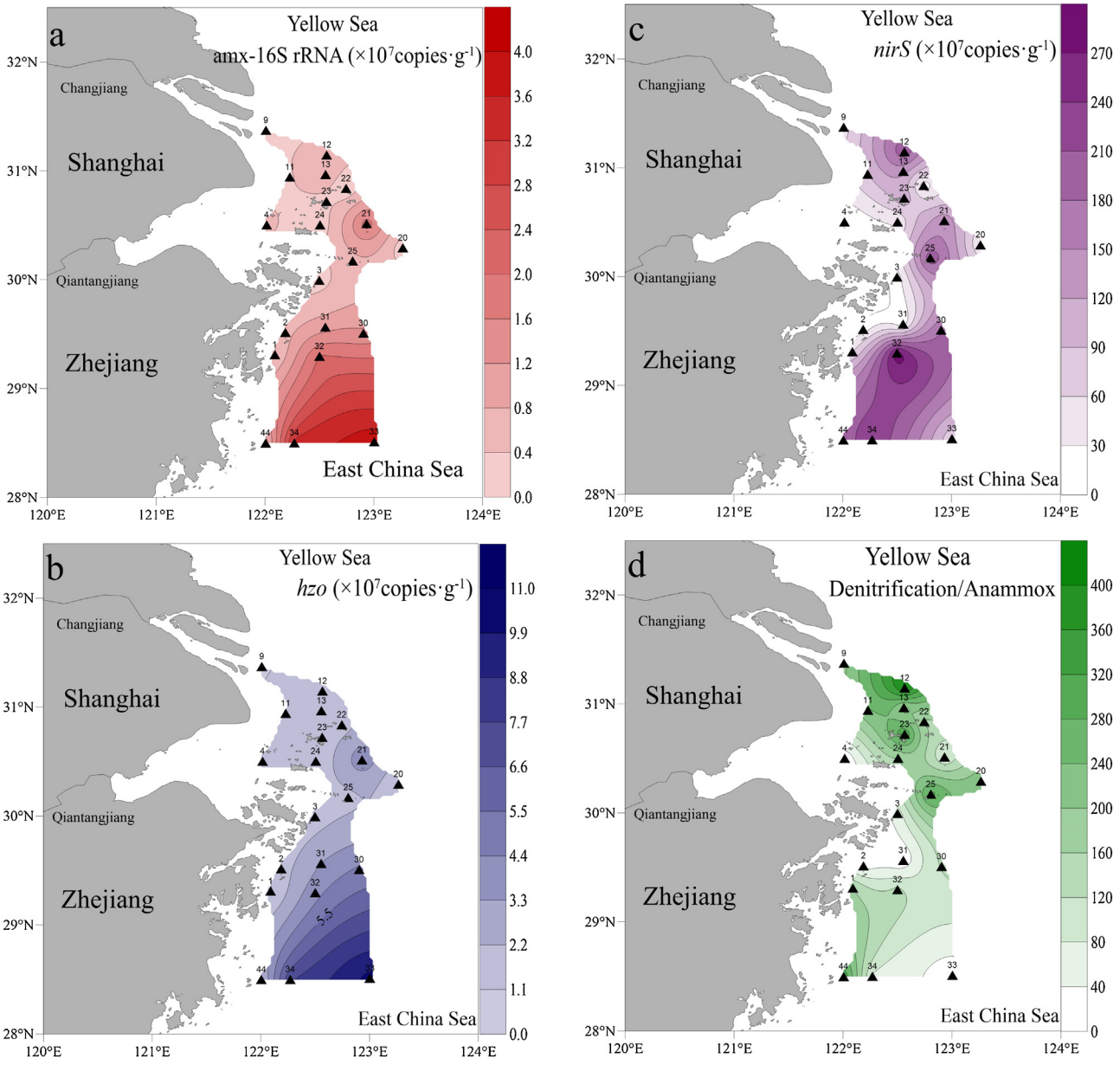

Fig. 2. Abundances of amx-16S rRNA (a), *hzo* (b) and *nirS* (c) genes, and the ratio of denitrification to anammox (d) at different sampling locations in the Changjiang Estuary and its adjacent area.

Table 1
Diversity characteristics of anammox bacterial 16S rRNA and *hzo* genes.

Anammox bacterial gene	Site	Available sequences	Optimized sequences	OTUs[a]	Chao1[b]	Shannon[c]	Simpson[d]	Coverage[e] (%)
16S rRNA	13	30,847	30,464	54	76.2	0.72	0.17	99.95
	20	18,323	18,101	63	83.0	2.50	0.70	99.89
	31	13,275	13,036	54	135.0	2.57	0.75	99.81
	33	30,962	30,406	73	104.9	1.29	0.30	99.93
hzo	13	19,323	18,633	491	601.1	7.09	0.99	99.44
	20	19,607	19,102	531	628.7	7.49	0.99	99.53
	31	19,150	18,488	472	572.1	6.76	0.98	99.47
	33	15,895	15,120	559	715.2	6.59	0.98	99.05

[a] OTUs were classified to the genus level and defined at 3% nucleotide divergence.
[b] Non-parametric statistical prediction of OTU total richness based on the distribution of singletons and doubletons.
[c] Shannon diversity index. A higher number indicates higher diversity.
[d] Simpson diversity index. A higher number indicates higher diversity.
[e] Percentage coverage: percentage of observed number of OTUs divided by the Chao1 estimate.

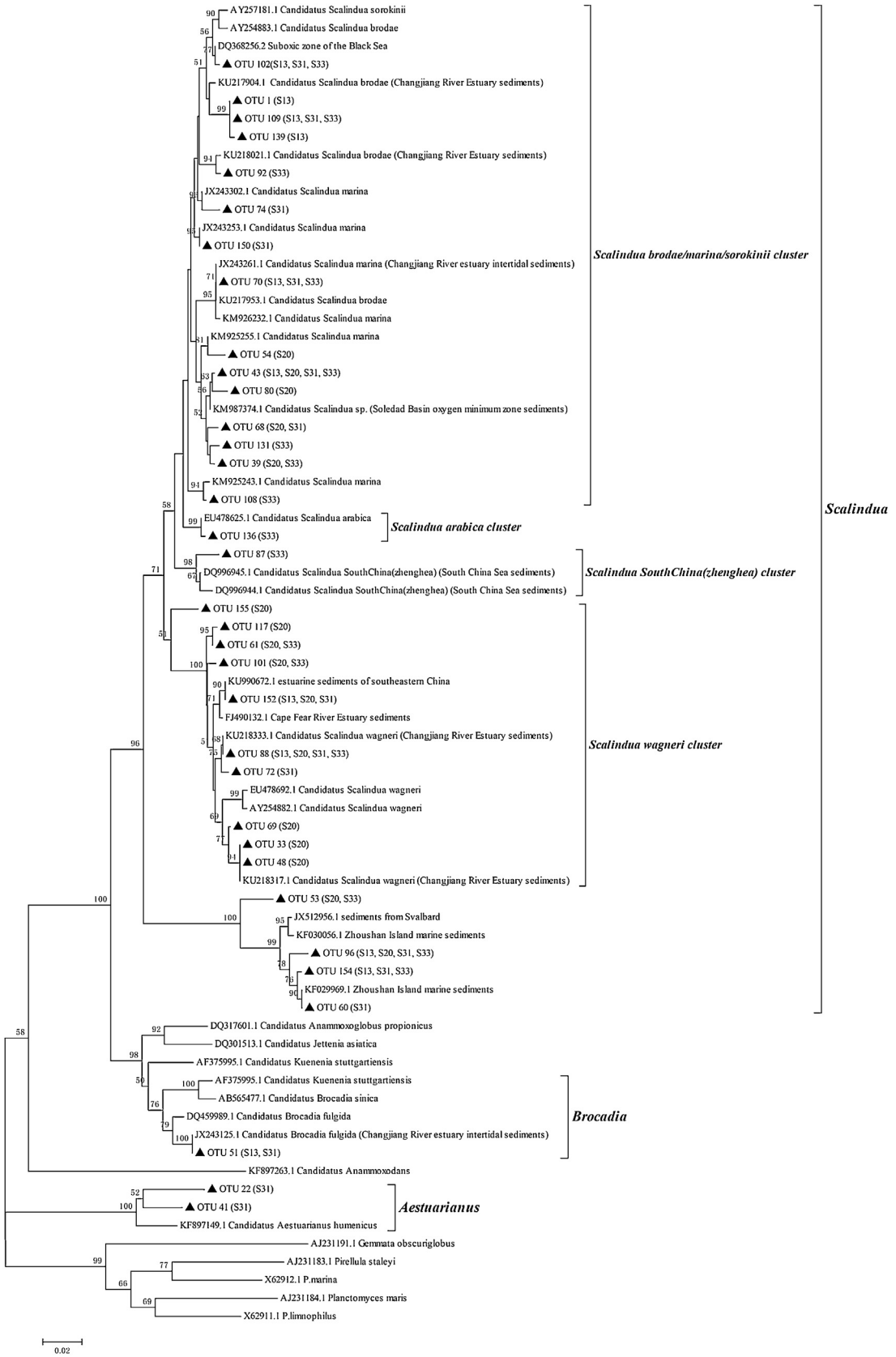

Fig. 3. Neighbor-joining phylogenetic tree of anammox bacterial 16S rRNA gene sequences (with relative abundance ≥1% in at least one sediment sample) and the reference sequences from GenBank. Bootstrap values greater than 50% for 1,000 resamplings are shown close to the nodes. OTUs were defined based on a nucleotide acid divergence of 3%. Bar: the scale bar represents 2% of sequence divergence.

ATA CCA AA) [31]. The *nirS* gene fragments (410 bp) were amplified by primers cd3aF (5′-GTS AAC GTS AAG GAR ACS GG) and R3cd (5′-GAS TTC GGR TGS GTC TTG A) [54]. The PCR conditions using Amx368F/Amx820R were as follows: 94 °C for 4 min; 32 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. The products were then stored at 4 °C. The PCR conditions with the *hzo*5F/*hzo*5R primers consisted of 94 °C for 4 min; 32 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min; and the products were then stored at 4 °C. The PCR conditions with the *nirS* gene primers were 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min; and 72 °C for 10 min. The products were then stored at 4 °C. The presence and size of the amplification products were determined by running 1.0% agarose gels in TAE buffer at 110 V and 200 mA for 25 min.

Quantification of three target genes in surface sediments

Appropriately sized fragments were separated on 1.0% agarose gels, purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Dalian, China), following the manufacturer's instructions, and ligated into pMD18-T vectors (TaKaRa, Dalian, China) in order to construct plasmids that were then transformed into competent *Escherichia coli* Trans 5α cells. The plasmids carrying the target gene fragments constructed in this study were extracted from *E. coli* hosts with a FastPlasmid Mini Kit (CWBIO, Beijing China), and plasmid DNA concentrations were measured by Picodrop microliter spectrophotometry (Picodrop, Saffron Walden, Essex, UK). The standard curves were obtained by using serial 10-fold dilutions of the plasmids described above.

DNA quantification was based on the fluorescent dye SYBR Green, which binds double-stranded DNA during amplification. All samples were processed with an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and reactions were performed in triplicate. 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 of each primer, 0.2 μL bovine serum album (BSA), 2.0 μL of template DNA, and 6.6 μL ddH_2O. The specificity of PCR amplification was verified by a melting curve analysis and agarose gel electrophoresis. qPCR for the *hzo* gene was performed with the following conditions: 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of 45 s at 95 °C, 1 min at 51 °C, and 1.5 min at 72 °C. The PCR conditions for amplification of the *nirS* gene were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 30 s at 95 °C, 30 s at 53 °C, and 45 s at 72 °C. For the amx-16S rRNA gene, qPCR was performed according to a previous study [11]. In all experiments, negative controls, which contained no template DNA, were subjected to the same qPCR procedure in order to detect and exclude any possible contamination or carry-over.

Sequencing by Roche 454

The reaction mixtures were pooled in equimolar ratios, and the paired-end reads of the primer pairs Amx368F/Amx820R and *hzo*5F/*hzo*5R were generated on a Roche 454 instrument (Personal Biotechnology Co., Ltd., Shanghai, China). Paired-end reads were assigned to samples based on their unique barcode, and the barcode and primer sequence were truncated. The paired-end reads were then merged by using some of the read overlap from the read generated from the opposite end of the same DNA fragment. Sequencing data were collected from raw sequences, and the spliced sequences were interpreted and deposited in a database by QIIME (quantitative insights into microbial ecology), which provided a wide range of microbial community analyses and visualizations, such as identification of operational taxonomic units (OTUs), sequence alignment, inference of phylogenetic trees and phylogenetic- and taxon-based analysis of diversity within and between samples [4]. Quality filtering of the raw tags was performed under specific filtering conditions with QIIME (Version 1.9.0, http://qiime.org/index.html) in order to obtain high-quality clean tags. The tags were compared with the reference database (Gold database; http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (UCHIME Algorithm; http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimeric sequences, which were then removed. Effective tags were then finally obtained. OTUs were clustered using a 97% similarity cutoff using UPARSE [8], and the most common sequences in each OTU were selected as representatives. An "OTU table" showing the number of reads from each sample assigned to each OTU was created by using the usearch_global command. α and β diversity were calculated by QIIME based on processed pyrosequencing data, and the phylogenetic analyses were then conducted by MEGA (Version 5.1) software [53].

Statistical analysis

The data obtained from quantification were analyzed with ABI PRISM® 7500 SDS software (Version 1.3.1; Applied Biosystems). Gene abundances were calculated based on standard curves and then converted to gene copy numbers assuming 100% DNA extraction efficiency. Correlations between environmental parameters and community abundances were identified with RDA analyses using CANOCO for Windows (Version 4.5) and Spearman's moment correlation using SPSS Version 17.0, with differences at the $P < 0.05$ level considered statistically significant.

Nucleotide sequence accession numbers

Roche 454 sequence data from this study were submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP112655:PRJNA394772.

Results

Physiochemical parameters at the sampling sites

The temperature, salinity, DO, TOC and concentrations of dissolved inorganic nitrogen compounds (NO_2^-, NO_3^- and NH_4^+) in the bottom water samples during the R/V *Run-Jiang* cruise are shown in Table S1. The water depth of the sampling sites varied between 10 and 72 m. Temperature, salinity, and DO values ranged from 17.9 to 27.2 °C, 15.65 to 34.49‰, and 1.11 to 4.04 mg L^{-1}, respectively. The DO concentration in the bottom water of the Changjiang Estuary and its adjacent area varied from 1.11 to 4.04 mg L^{-1}, which was the inverse trend to that of the abundance of anammox bacteria quantified according to the amx-16S rRNA gene [11]. The TOC content in sediments varied from 0.21 to 0.85%. The concentrations of nitrate (0.00–187.00 mol L^{-1}) in the bottom water were higher than those of nitrite (0.00–1.48 mol L^{-1}) (one-way ANOVA, $P < 0.05$). In addition, the concentrations of ammonium in the bottom water ranged from 0.92 to 1.20 mol L^{-1}.

Abundance and diversity distribution pattern of anammox bacteria at different sites

Anammox bacterial community abundance

In this study, melting curve analyses confirmed that fluorescent signals were derived from the specific PCR products during qPCR quantification. A significant linear relationship of amx-16S rRNA gene amplification ($R_{amx\text{-}16S}^2 = 0.9973$), with 100.5% amplification efficiency, was obtained between the $\log_{10}$ values of the standard plasmid DNA concentration (5.13×10–5.13×10^6 copies L^{-1}) and

the associated threshold cycles (Ct). The standard curves of functional gene *hzo* were also obtained ($R_{hzo}{}^2 = 0.9973$) with a 97.2% amplification efficiency by recording the $\log_{10}$ values of plasmid DNA concentration (4.71×10–4.71×10^6 copies L^{-1}) and the Ct values (Fig. S1a, b).

The abundance of the amx-16S rRNA gene varied between 6.73×10^5 and 3.81×10^7 copies g^{-1} (fresh weight) [11], with the highest number of copies being recorded at site 33, and the lowest at site 22. The abundance of the anammox bacterial functional gene *hzo* was between 9.86×10^6 and 1.02×10^8 copies g^{-1} (fresh weight). Site 33 had the highest copy number, whereas site 1 had the lowest copy number. The abundance of *nirS*, the denitrifying bacterial functional gene, varied from 3.35×10^7 to 2.89×10^9 copies g^{-1} (fresh weight) among the sites, with the highest number occurring at site 32, and lowest number occurring at site 4. The sediments at site 33 had significantly greater anammox bacterial abundance than those at the other sites (Fig. 2a, b). In addition, the sediments at site 32 had a higher abundance of denitrifying bacteria than those at the other sites. These results indicated pronounced spatial variation in the abundances of anammox and denitrifying bacteria among the sediments of the Changjiang Estuary and its adjacent area. The abundance of anammox bacteria was lower in the estuary than in the offshore area of southern Zhejiang Province (Fig. 2a, b). The abundance of denitrifying bacteria was higher in the offshore area of Changjiang Estuary and southern Zhejiang Province than in the lower immediately offshore area (Fig. 2c). The abundance of denitrifying bacteria was much higher than that of anammox bacteria (Student's t test, $P < 0.01$). The ratio of denitrification to anammox was higher in the northern estuarine area than in the southern offshore area (Fig. 2d). Moreover, the abundances of anammox and denitrifying bacteria were positively correlated ($P < 0.05$, $r = 0.447$).

Anammox bacterial diversity and richness in marine sediments

Anammox bacterial communities in the sediments of the Changjiang Estuary and coastal zone were successfully detected with a PCR method. Samples from selected sites 13, 20, 31, and 33 were sequenced by high-throughput sequencing on a Roche 454 platform. The retrieved sequences of amx-16S rRNA and *hzo* genes were screened, discarded and analyzed by QIIME, confirming that all the qualified sequences represented anammox-like sequences. In the rarefaction curves of the amx-16S rRNA and *hzo* genes (Fig. S2), the lines of sites 13, 20, and 33 eventually became approximately smooth, indicating that the amplified amx-16S rRNA and *hzo* genes had been sequenced deeply enough. However, the curve for studying site 31 by the amx-16S rRNA gene continued to rise, showing that the sequencing depth was not satisfactory for this site's sediment sample.

In the present study, the number of high-quality anammox bacterial sequences from each sediment sample quantified by the amx-16S rRNA gene copy number ranged between 13,275 and 30,962, and was optimized to between 13,036 and 30,464; whereas the *hzo* gene yielded between 13,275 and 30,962 sequences, which were optimized to 13,036 and 30,464 sequences for analyzing the anammox bacterial community richness, diversity, and evenness (Table 1).

Good's coverage estimator revealed that 99–100% of the anammox bacterial OTUs were obtained for all marginal sea sediment samples, which indicated that the OTUs of each anammox bacterial library had been well captured. Afterwards, sequences acquired by amplifying 16S rRNA and *hzo* genes were grouped into 54–73 and 427–559 operational taxonomic units (OTUs) at 3% nucleotide divergence (Table 1), respectively. The efficiently subsampled anammox bacterial sequences covered by the amx-16S rRNA and *hzo* genes were grouped into 54–73 and 472–559 OTUs, respectively. The Chao1 richness estimators of the anammox bacterial

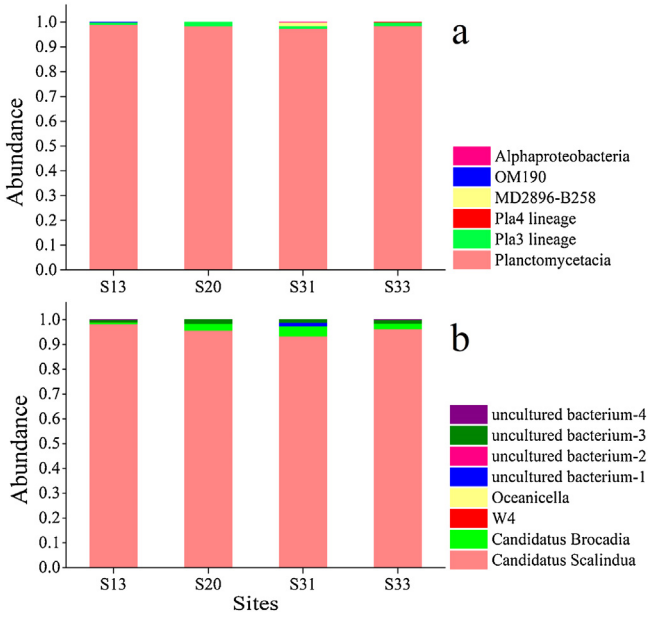

Fig. 4. Anammox bacterial community structure quantified based on the amx-16S rRNA gene at the class (a) and genus (b) levels.

communities defined by the amx-16S rRNA and *hzo* genes were 76.2–135.0 and 572.1–715.2, respectively. Sediment anammox bacterial communities in the Changjiang Estuary and its adjacent area defined by the amx-16S rRNA and *hzo* genes exhibited Shannon diversity indices of 0.72–2.57 and 6.59–7.49, respectively. Regardless of whether the community members were identified by the amx-16S rRNA or *hzo* gene, the same clear spatial variations in sediment anammox bacterial OTU number, Chao1 richness, and Shannon diversity were observed in the study area. In addition, the Simpson evenness indices of anammox bacterial communities quantified by amx-16S rRNA and *hzo* genes were 0.17–0.75 and 0.98–0.99, respectively, which also illustrated the spatial variation in the communities throughout the study area.

Sediment anammox bacterial community evenness quantified by the 16S rRNA gene increased at site 31, but decreased when quantified by the *hzo* gene. On the basis of Shannon-Wiener and Chao1 diversity indices of the amx-16S rRNA gene at the 3% distance level, site 31 showed the most OTU diversity, whereas the minimum OTU diversity appeared at site 13. The 3% *hzo* gene distance level indicated that sites 20 and 31 showed the most and least OTU diversity, respectively (Table 1).

The phylogenetic analysis indicated that three known genera of anammox bacteria, namely, *Ca.* Scalindua, *Ca.* Brocadia, and *Ca.* Aestuarianus [10], and certain unknown anammox-like groups were represented in the sediments from the Changjiang Estuary and the adjacent coastal area (Fig. 3).

At the class level, *Planctomycetacia* was dominant (>90%) among the sediment bacteria at the representative sites (S13, S20, S31, and S33) (Fig. 4a). The remaining sequences were affiliated with MD2896-B258, Pla3 lineage, Pla4 lineage, and *Alphaproteobacteria*. At the genus level, *Ca.* Scalindua and *Ca.* Brocadia, members of *Planctomycetales*, were observed to coexist in these areas by amx-16S rRNA gene quantification. *Ca.* Scalindua, representing almost 90% of the total site sequences, was the dominant anammox bacterial genus in the studied area. The other anammox bacterial genera were affiliated to four types of uncultured *Planctomycetales* bacteria, an *Oceanicella* uncultured bacterium, a W4 uncultured bacterium and *Ca.* Brocadia, which were present in very low quantities (Fig. 4b).

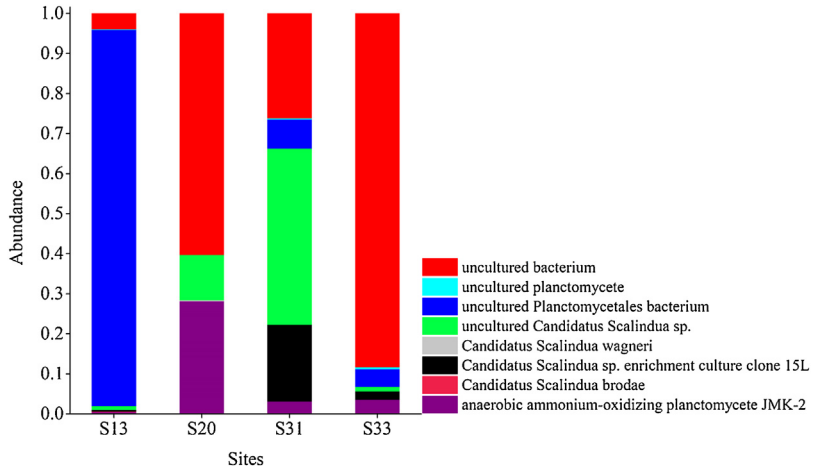

Fig. 5. *Scalindua* anammox bacterial structure quantified based on the amx-16S rRNA gene at the species level.

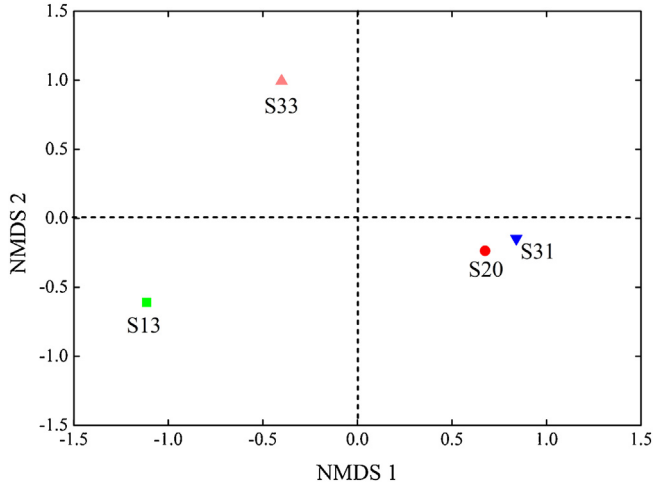

Fig. 6. NMDS analysis of the anammox bacterial assemblages as revealed by the 16S rRNA gene sequences. The first two principal coordinate axes (MDS1 and MDS2) are shown.

As the *Ca.* Scalindua genus was clearly dominant in the construction of the anammox bacterial community, analyses of the population structure at the species level in the *Ca.* Scalindua genus were of great interest. Uncultured anammox bacteria already contained a high proportion of the *Ca.* Scalindua genus. Known species, such as the anaerobic ammonium-oxidizing planctomycete JMK-2, *Ca.* Scalindua brodae, *Ca.* Scalindua sp. enrichment culture clone 15L, and *Ca.* Scalindua wagneri, were detected at the studied sites. The anaerobic ammonium-oxidizing planctomycete JMK-2 was present in a higher proportion at site 20 than at the other sites, whereas *Ca.* Scalindua sp. enrichment culture clone 15L was more abundant at site 31 than at the other sites (Fig. 5).

A non-metric multidimensional scaling (NMDS) analysis revealed geographically specific distributions of anammox bacterial communities along the Changjiang Estuary and coastal zone (Fig. 6). The plot of the first two axes showed that anammox bacterial assemblages fell into three groups. Anammox bacterial communities at sites 20 and 33 were grouped together and possessed similar community structures, while other sites were in different clusters and the three groups differed greatly.

Even though an attempt was made to cluster anammox bacteria using the functional gene *hzo*, the high-throughput sequencing results showed that all sequences belonged to uncultured genera and were difficult to classify, while traditional phylogenetic analyses were required to cluster the *hzo* genes. The results also showed that the most prevalent and dominant anammox bacterial genus at the studied sites identified by the *hzo* gene was *Ca.* Scalindua (Fig. 7).

Distribution of denitrifying bacterial abundance across different sites

The standard curves of the functional gene *nirS* were obtained ($R_{nirS}{}^2 = 0.9999$) with 83.3% amplification efficiency by recording the $\log_{10}$ values of plasmid DNA concentration (5.68×10^2–5.68×10^7 copies L^{-1}) and the Ct values (Fig. S1c). The copy number of *nirS*, the denitrifying bacterial functional gene, varied from 3.35×10^7 to 2.89×10^9 copies g^{-1} (fresh weight) among all sites, with the highest number recorded at site 32, and the lowest recorded at site 4. The sediments at site 33 had significantly greater anammox bacterial abundance than those at the other sites (Fig. 2a, b). In addition, the sediments at site 32 had significantly higher denitrifying bacterial abundance than those at the other sites. These results indicated pronounced spatial variation in the abundances of anammox and denitrifying bacterial abundance among the sediments of the Changjiang Estuary and its adjacent area. The abundance of denitrifying bacteria was higher in the offshore area of Changjiang Estuary and southern Zhejiang Province than in the lower immediately offshore area (Fig. 2c). The abundance of denitrifying bacteria was much higher than that of anammox bacteria (Student's t test, $P < 0.01$). The ratio of denitrification to anammox was higher in the northern estuarine area than in the southern offshore area (Fig. 2d). Moreover, the abundances of anammox and denitrifying bacteria were positively correlated ($P < 0.05$, $r = 0.447$).

Relationships of anammox bacterial abundance and community structure to environmental variables

Spearman's correlation analysis indicated that the abundance of anammox bacteria in sediments based on the 16S rRNA gene was positively correlated with TOC ($P < 0.01$, $r = 0.718$) and TN ($P < 0.01$, $r = 0.811$) in sediments, and negatively correlated with the NO_3^- ($P < 0.05$, $r = -0.464$), NO_x^- ($P < 0.05$, $r = -0.472$), and PO_4^{3-} ($P < 0.05$, $r = -0.506$) concentrations in bottom water (Table 2). The amx-16S rRNA gene qPCR results showed that the abundance of anammox bacteria exhibited a geographically heterogeneous distribution pattern along the TOC and TN gradients in sediments from the Changjiang Estuary and its adjacent coastal zone ($P < 0.01$). The anammox bacteria were generally more abundant at the high-TOC

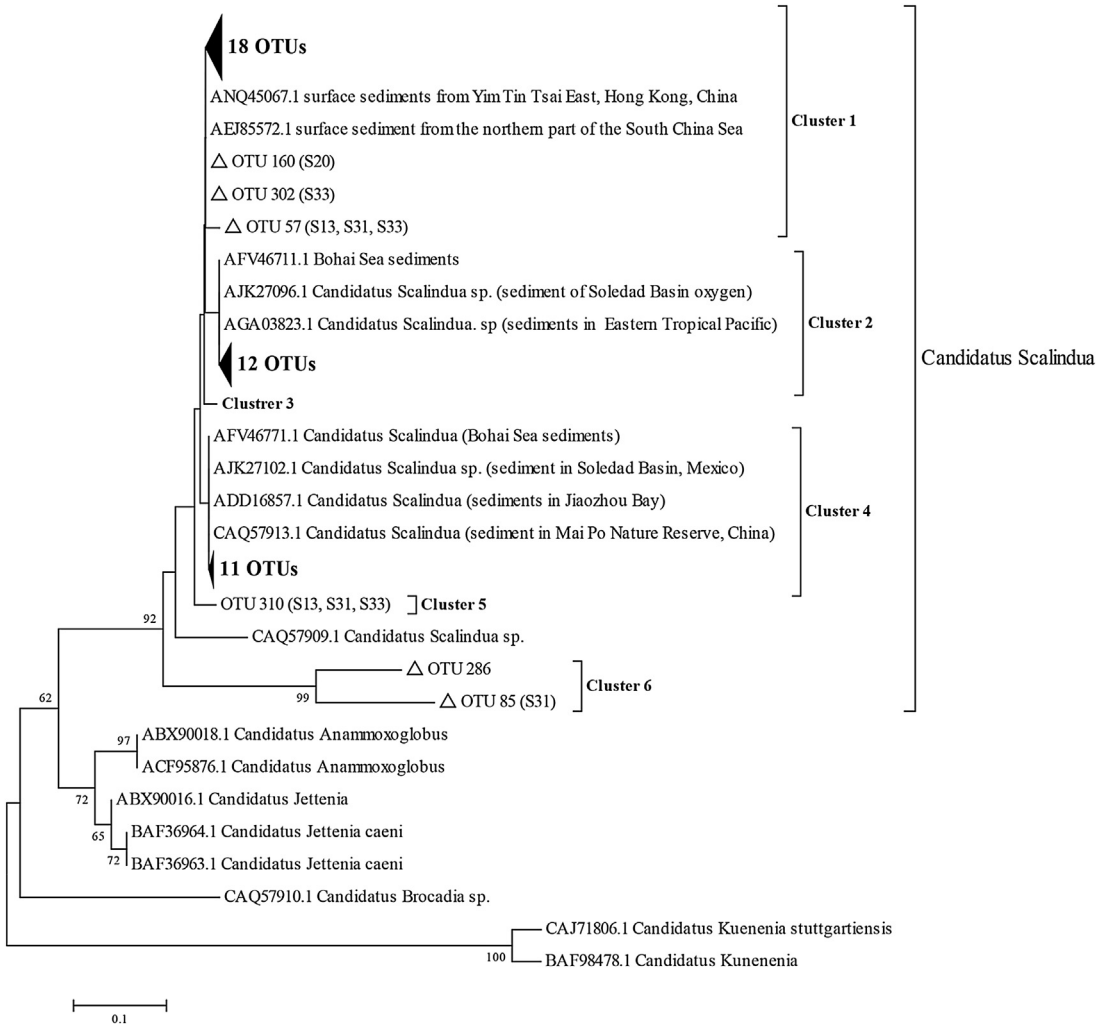

Fig. 7. Neighbor-joining phylogenetic tree of anammox bacterial *hzo* protein sequences from the respective sequences of OTUs (with relative abundance ≥1% in at least one sediment sample) and the reference sequences from GenBank. OTUs were defined based on a nucleotide divergence of 3%. Bootstrap values greater than 50% for 1000 resamplings are shown close to the nodes. Bar: the scale bar represents 10% of sequence divergence. Neighbor-joining phylogenetic tree of anammox bacterial *hzo* gene sequences were provided as Fig. S4 in the attachments.

Table 2
Spearman correlation analysis of environmental factors with the abundance of anammox bacteria.[a]

Anammox bacterial genes	Salinity	DO	NH_4^-	NO_2^-	NO_3^-	NO_x^-[b]	PO_4^{3-}	TOC (%)	TN (%)	C/N
amx-16S rRNA	0.355	−0.176	0.112	−0.317	−0.464*	−0.472*	−0.506*	0.718**	0.811**	−0.288
hzo	0.603**	−0.067	−0.001	−0.451*	−0.660**	−0.659**	−0.669**	0.699**	0.747**	−0.254

* Correlation significant at the 0.05 level (one-tailed).
** Correlation significant at the 0.01 level (one-tailed).
[a] Salinity, DO, NH_4^-, NO_2^-, NO_3^-, NO_x^-, and PO_4^{3-} are properties of bottom water; TOC, TN and C/N are properties of sediments.
[b] $NO_X^- = NO_2^- + NO_3^-$.

sites than at the low-TOC sites [11]. Moreover, the proportions of anammox bacteria quantified based on the *hzo* gene abundance were also positively correlated with TOC ($P < 0.01$, $r = 0.699$) and TN ($P < 0.01$, $r = 0.747$) in sediments, and negatively correlated with the NO_2^- ($P < 0.05$, $r = -0.451$), NO_3^- ($P < 0.01$, $r = -0.660$), NO_x^- ($P < 0.01$, $r = -0.659$), and PO_4^{3-} ($P < 0.01$, $r = -0.669$) concentrations in bottom water (Table 2).

RDA analyses were conducted to reveal the potential relationships of anammox bacterial abundance and community composition with environmental factors (Figs. 8 and 9). Sediment environmental factors, including depth, salinity, dissolved inorganic nitrogen (DIN), TOC, and NO_2^-, NO_3^-, NH_4^+, and PO_4^{3-} concentrations, illustrated various contributions to the anammox bacterial abundance and community–environment relationships. The sediment environmental factors covered by the first two RDA axes explained 70.5 and 2.9% of the total variance in the sediment anammox bacterial abundance, 72.8 and 23.1% of the variance in community composition determined based on the 16S rRNA gene, and 60.7 and 30.3% of the variance in community composition determined based on the *hzo* gene. These results confirmed that TOC ($F = 15.21$, $P < 0.01$, 499 permutations) had a significant relationship with the abundance of the anammox communities, and these terms together provided 46% of the total RDA explanatory power; similarly, the relationship of the NO_2^- concentration to the anammox bacterial composition provided 57% of the total RDA explanatory power. Additionally, the abundance and composition

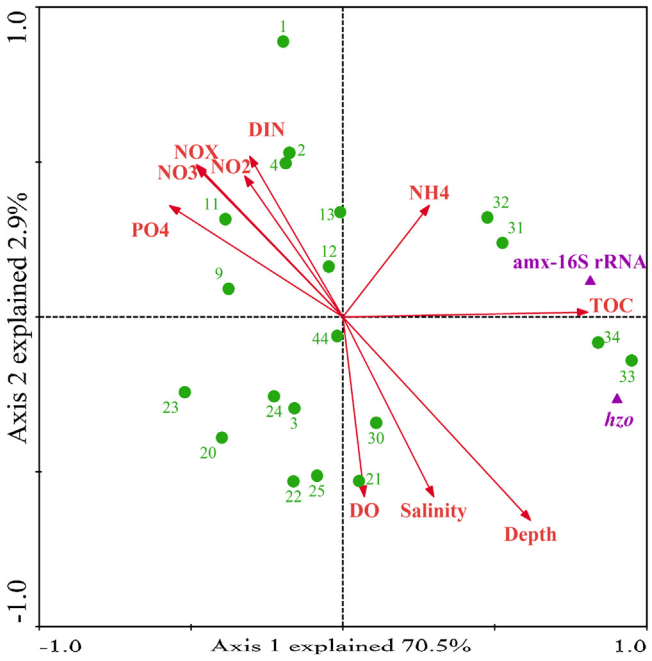

Fig. 8. RDA ordination plot for the first two principal dimensions of the relationships between sediment anammox bacterial abundance and sediment parameters. (Green filled circles indicate the 20 detected sediment samples in the Changjiang River Estuary, and purple filled triangles indicate the anammox bacterial abundance covered by the 16S rRNA and *hzo* genes, respectively).

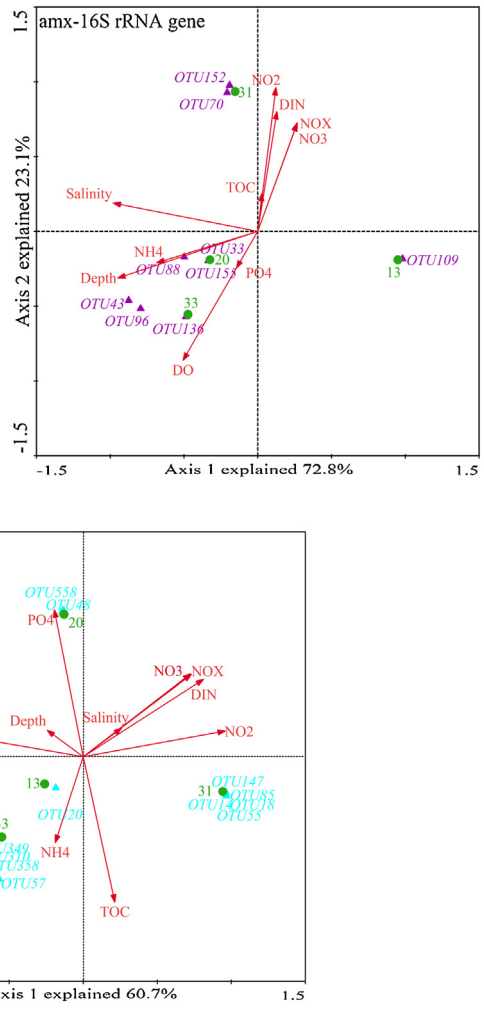

Fig. 9. RDA ordination plot for the first two principal dimensions of the relationships between sediment anammox bacterial OTU composition and sediment parameters (green filled circles indicate the four detected sediment samples in the Changjiang River Estuary, and purple and blue filled triangles indicate the anammox bacteria covered by the 16S rRNA and *hzo* genes, respectively).

of anammox bacterial communities were correlated with depth, salinity, and NH_4^+, DIN and PO_4^{3-} concentrations.

Discussion

Anammox bacterial abundance in the sediments of the Changjiang Estuary and adjacent area

Several previous studies have revealed the spatial distribution of anammox bacterial abundance in various sediment ecosystems, such as lake sediment [62], river sediment [17], estuary sediment and marine sediment [5,16]. However, only a few recent studies have documented the community structure of anammox bacteria in sediments by applying high-throughput sequencing technology [62]. In this study, geographical distributions and variations in anammox bacterial communities and abundances were examined along the Changjiang Estuary and adjacent coastal zone. Amx-16S rRNA gene abundance in the sediments was between 6.73×10^5 and 3.81×10^7 copies g^{-1} (fresh weight) throughout the area, which was higher than the abundances observed in the Cape Fear River estuary (1.3×10^5–8.4×10^6 copies 16S rRNA gene g^{-1}) [5], Jiaozhou Bay (3.5×10^5–5.9×10^6 copies *hzo* gene g^{-1}) [6], and the Pearl Estuary (4.22×10^5–2.55×10^6 copies 16S rRNA gene g^{-1}) [10]. These estimated numbers meant that anammox bacterial cell numbers varied greatly by region. Anammox activity was possibly higher in the Changjiang Estuary and coastal zones than in the other investigated areas. Anammox bacterial *hzo* gene abundance in the sediments was between 9.86×10^6 and 1.02×10^8 copies g^{-1} (fresh weight) throughout both areas in this study. The average abundance of anammox bacteria as quantified by the *hzo* copy number was much higher (9.86×10^6–1.02×10^8 copies g^{-1} fresh sediment) than that quantified by the amx-16S rRNA gene copy number (6.73×10^5–3.81×10^7 copies g^{-1} fresh sediment) (Student's t test, $P < 0.01$). In addition, the abundance of the *hzo* gene, which codes the main protein involved in the process of anaerobic ammonia oxidation, in the anammox bacteria was approximately twice that of the 16S rRNA gene at all sites ($P < 0.05$) (Fig. S3). These results agreed with those of Shimamura et al. [47], who studied the anammox bacterial functional gene *hzo* and identified two genes coding for *hzo*, *hzoA* and *hzoB* within the metagenomic DNA from the enrichment culture of a reactor in which only one anammox bacterium was dominant.

The abundance of denitrifying microorganisms quantified by the *nirS* gene was 3.35×10^7–2.89×10^9 copies g^{-1} (fresh weight), which was higher than the abundance of anammox bacteria at each site (Student's t test, $P < 0.01$). Considering the disturbed hydrological environment in the Changjiang Estuary and its adjacent area [60,64], it was hypothesized that anammox bacteria might be more weakly adapted to this fluctuating environment due to their slow growth rates [27,57] and that denitrifying bacteria should thus have a competitive advantage. It is possible that the nitrogen removal pathway in the area mainly depends on denitrification; however, the ratio of anammox was significantly enhanced in the southern offshore area (Fig. 2d). Similar to the case in the Arabian Sea OMZs [59], as previously reported by Ward et al., the denitrifying abundance measured by qPCR exceeded the anammox abundance by 3- to 19.2-fold, and the denitrification rate averaged 93.7% of the total N2 production rate. In general, the high abundance of denitrifying microorganisms resulted in superior N_2 production than

that achieved with anammox bacteria. Ward et al. [59] investigated the denitrification and anammox rates and found that denitrification accounted for a large proportion of the total N_2 production rate (93.7%). Denitrification was the dominant process of nitrogen loss in the Arabian Sea [59], whereas anammox acted as a major factor in the loss of N in the Black Sea, the world's largest anoxic basin [26]. In the studied area, the abundance of *nirS*-encoding nitrite-reducing bacteria exceeded that of anammox bacteria by 6.9- to 411.1-fold at the 20 stations, indicating that *nirS*-encoding nitrite-reducing bacteria might play a more important role in nitrite reduction than anammox bacteria. However, the physiological state is more directly related to the process that has an advantage, denitrification or anammox. This deficiency was also present in our study, and RNA analyses are needed to provide a better perspective of the contribution of anammox and denitrifying bacteria to N loss.

Nitrification and denitrification both proceed through NO_2^- as an important free intermediate, which could serve as a substrate for anammox bacteria. However, an undescribed phenomenon that does not appear to be stoichiometrically linked appears to connect anammox and denitrification. According to the C/N ratio of 6.6 in the ocean, anammox is responsible for 28% of the nitrogen loss in oceans [2]. Babbin et al. [2] suggested that the generally conserved average composition of organic matter (OM) is important but that one process or the other dominates the total N loss when measured discretely in space and time. It was previously demonstrated that the OM-driven stoichiometric constraints on the anammox/denitrification ratio were observed in ocean minimum zones but not sediments in estuaries [38]. In addition, the anammox/denitrification ratio is significantly lower in estuaries, with an average ratio of 3:7, compared with that in OMZs [38]. The C/N ratio (mean of 7.14, range from 6.41 to 7.61, as shown in Table S1) in the studied area was higher than that in the OMZs (mean of 6.6). In addition, the abundance of *nirS*-encoding nitrite-reducing bacteria exceeded that of anammox bacteria by 6.9- to 411.1-fold at the 20 stations studied, which indicated that *nirS*-encoding nitrite-reducing bacteria might play a more important role in nitrite reduction than anammox bacteria. In this study, it was found that diverse species of uncultured anammox bacteria contributed to the nitrogen cycle in estuarine and marginal sea ecosystems from a molecular perspective, but their contribution as a N_2-forming pathway might be less significant than that of denitrifying bacteria.

Researchers have argued for years over which of the two nitrogen-removal mechanisms, denitrification or anammox, is more important in the oceans. Babbin et al. [2] linked the rates of nitrogen loss to the supply of organic material and found a balance between the two processes; furthermore, they found that the ratio of anammox to denitrification depended on the stoichiometry of OM. Denitrification removed approximately 70% of the nitrogen, and anammox removed the rest due to the average OM C/N in the ocean being 6.6 [2]. On the other hand, the variation in anammox bacterial abundance between several different areas is probably related to study differences in the efficiency of extracting DNA from sediments and the differences of amplification efficiency between primers. Considering the controversy over whether the main N loss term in the fixed nitrogen inventory is the heterotrophic denitrification or autotrophic anammox process, further studies using incubation experiments and RNA analyses of the processes may be required in order to gain a better understanding of the abundance and activity of anammox and denitrifying bacteria.

In the present study, a significantly lower anammox bacterial abundance in the Changjiang River Estuary compared to the other sites, where the TOC was lower (0.21–0.44) than the other sites, was quantified by the amx-16S rRNA and *hzo* genes (Fig. 2) (sites 9 and 11; 2.46×10^6–3.76×10^6 copies g^{-1} and 3.35×10^7–3.81×10^7 copies g^{-1}) (sites 33 and 34; 1.01×10^7–1.50×10^7 copies g^{-1} and 7.57×10^7–1.02×10^8 copies g^{-1}) (one-way ANOVA, $P < 0.05$). The significant increase in anammox bacterial abundance with increasing TOC ($P < 0.01$, $r_{amx\text{-}16S} = 0.718$; $r_{hzo} = 0.699$) in the present study might be attributed to the fact that more OM in sediments can generate more NH_4^+ through ammonification and more NO_2^- via denitrification, leading to a positive correlation between anammox abundance and TOC. A correlation of anammox with the organic content of the sediment may also be caused by NO_2^- availability, with greater reductive NO_2^- production in sediments richer in TOC [27]. Higher N_2 contributions from anammox have been found to be positively correlated with both the organic carbon content of the sediments and the concentration of NO_3^- in the overlying water at the river estuary [39]. The organic carbon content of the sediment decreased towards the coast, but its reactivity remained largely constant, which suggested that the amount of organic carbon was more important to anammox bacteria than NO_3^- [39].

Since NH_4^+ and NO_2^- are the substrates for anammox bacteria, the release of NH_4^+ and NO_2^- might favor these bacteria and thus lead to higher anammox bacterial abundance [57]. However, the correlation between the concentrations of NH_4^+ or NO_2^- and anammox abundance was insignificant ($P > 0.05$). This result indicated that part of the correlation between the concentrations of NH_4^+ or NO_2^- and anammox abundance was attributable to its correlation with TOC. NH_4^+ is not a limiting factor for the growth of anammox bacteria as it is always present in natural environments due to the degradation of OM. Low concentrations of NO_2^- cannot be accurately measured because it is unstable in natural environments. The concentration of NO_2^- in the bottom water of the Changjiang Estuary and its adjacent area was very low (less than 1.5 mM) (Table S1). It has been well established that anammox is reliant on a supply of NO_2^- to fuel the oxidation of NH_4^+ and that this NO_2^- is produced within the suboxic sediment by the reduction of NO_3^- [36]. Since NO_2^- is a substrate participating in the anammox process, the very low NO_2^- concentration is probably the limiting factor for anammox bacteria in the estuarine subtidal sediments. Although an in situ supply of NO_2^- was found to be modulated by total organic carbon (TOC) and/or the concentration of NO_3^- in the overlying water column of estuaries [39], anammox has been shown to proceed at equal rates via either the direct reduction of NO_2^- or indirect reduction following the initial reduction of NO_3^- [55]. However, in the present study, the correlation analysis revealed that anammox abundance and the NO_3^- ($P < 0.05$, $r_{amx\text{-}16S} = -0.464$; $P < 0.01$, $r_{hzo} = -0.660$) and/or NO_x^- ($P < 0.05$, $r_{amx\text{-}16S} = -0.472$; $P < 0.01$, $r_{hzo} = -0.659$) concentration were significantly negatively correlated, which is not in accordance with past findings [39,41]. A possible reason for this difference was that anammox bacterial abundances were determined by the abundances of amx-16S rRNA and *hzo* genes rather than mRNA levels, which might represent the fraction of active anammox bacteria. Anammox might be coupled with other processes that are components in the marine nitrogen cycle. On the other hand, the measured physiochemical factors all came from bottom water rather than sediment; hence, it was understandable that anammox abundance showed no correlation with the concentrations of NO_2^- or NH_4^+. Further studies of the connection between anammox bacterial abundance when anammox is active and environmental factors would therefore be needed.

In previous studies, significant correlation was also detected between anammox bacterial numbers and salinity. Dale et al. [5] found that variation in anammox bacteria abundance was highly related to the changes in salinity in the Cape Fear River estuary, where the salinity ranged between 0 and 9.9. In 2016, Zhang et al. found that anammox bacterial abundance was significantly associated with salinity ($P < 0.05$) in the Changjiang Estuary [65]. The salinity thus appeared to be a strong influence on anammox abun-

dance in both the Cape Fear River estuary and the Changjiang Estuary. The correlation between salinity and anammox bacterial 16S rRNA gene abundance was insignificant ($P_{amx\text{-}16S} > 0.05$), but a significant correlation was found in the present study between salinity and anammox bacterial 16S rRNA gene abundance ($P_{hzo} < 0.01$, $r = 0.603$). The *hzo* gene sequences only covered the dominant oceanic genus, *Ca.* Scalindua, whose distribution trend was consistent with the salinity gradient; thus, it is possible that salinity was the most significant environmental factor affecting the abundances of *Ca.* Scalindua lineages. The highest abundance of anammox bacteria (3.81×10^7 copies 16S rRNA gene g^{-1}, 1.02×10^8 copies *hzo* gene g^{-1}) occurred at site 33, where the salinity was 34.47, and lower anammox bacterial abundance was observed in areas with lower or higher salinity (15.65–34.46 or 34.47–34.48). The very small irregular range of salinity and other synergistic physical and chemical factors in the measured area might have caused this behavior.

Anammox bacterial community characteristics in the sediments of the Changjiang Estuary and adjacent area

The Changjiang River exhibits a high discharge rate in China and transports huge amounts of freshwater into the adjacent sea, where the sedimentological status is influenced by many environmental aspects. These aspects include dynamic hydrology factors, such as currents, tides, waves, and upwelling, as well as the durations of these activities, sediment source-material supplies from rivers, and the compositions and abundances of nutrients and environmental contaminants [64]. Large quantities of terrestrial materials have also been carried into the estuarine and coastal regions. During these interactions, anammox species originating from marine and terrestrial environments may be mixed together by tidal currents and river runoff in the estuarine ecosystem, thereby enhancing anammox bacterial biodiversity [16]. The seasonal hypoxic zone off the Changjiang River Estuary represents one of the most important scientific phenomena and ecological issues on the continental sea shelf of China. In addition, a very serious phenomenon, seasonal hypoxia, occurred in August [60]. The studied area exhibited low DO concentrations, and OMZs are appropriate suitable locations for the growth of highly diverse anammox bacteria [11,22]. Most amx-16S rRNA gene-based studies have found that the diversity of anammox bacteria in marine environments is low, with *Ca.* Scalindua occupying a dominant position [12,26,46]. Most 16S rRNA gene PCR primers do not cover all known anammox bacterial lineages at the genus level, and using the *hzo* gene might improve the coverage [29]. High-throughput sequencing was selected for use in this study for detection of the amx-16S rRNA and *hzo* genes because it can acquire large numbers of effective sequences and yield comprehensive information about the diversity and construction of anammox bacterial communities in sediments of low-abundance areas. The diversities were detected in the present study using both gene markers, and the results were similar to previous findings obtained for sediments from the Bohai Sea and Jiaozhou Bay [6,7]. The *Ca.* Scalindua genus was highly prevalent and was shown to be predominant in all sites by both primers. This genus accounted for more than 90% of the obtained sequences, with high diversity, in surface sediments of the Changjiang Estuary and its adjacent area, where non-*Scalindua* anammox bacteria were also detected (Fig. 4). The amx-16S rRNA gene sequences retrieved from the area were affiliated closely to sequences obtained from other estuarine and marine environments, including Cape Fear River estuarine sediments [5], the Peruvian OMZs [61], and the sediments of the marginal seas of China, such as those of Jiaozhou Bay [6], the Changjiang Estuary [16], the Zhoushan island marine environment [63], and the Pearl Estuary [10]. These sequences shared 95–100% sequence identity, indicating that *Ca.* Scalindua pacifica bacteria were widely distributed in the Pacific Ocean and may prefer estuarial and offshore sediment environments. The *hzo*-based approach detected diverse anammox bacterial lineages from the Changjiang Estuary and its adjacent area, whereas the amx-16S rRNA gene primers confirmed the previously found high microdiversity of the *Ca.* Scalindua lineage in estuarine and marine environments [6,30,44].

Diverse anammox bacterial phylotypes were found in the sediments of the Changjiang Estuary and its adjacent coastal sediments based on sequences of both the 16S rRNA and *hzo* genes (Figs. 3 and 7). At the genus level, the 16S rRNA gene PCR primers detected the anammox bacteria *Ca.* Scalindua, *Ca.* Brocadia and other unknown anammox-like lineages (Fig. 3), whereas almost all the OTUs detected by the *hzo* primers corresponded to *Ca.* Scalindua (Fig. 7). The topologies of the phylogenetic analyses for these two sets of biomarkers were consistent. Comparative studies of 16S rRNA and *hzo* genes of anammox bacteria are helpful in comprehensively understanding the distributions and diversities of anammox bacteria in sediments. The prevalence of the *Ca.* Scalindua lineage found by both gene biomarkers validated members of *Ca.* Scalindua as belonging to a major anammox bacterial genus in marine environments and suggested that relative to other anammox lineages, they play a significant role in nitrogen production in the studied area. The higher anammox bacterial diversity in the estuarine ecosystem was likely because of the land-sea interaction, which is in agreement with previous studies [5,10,16]. In general, analyses based on the *hzo* gene resulted in a higher estimate of the diversity of anammox bacteria according to the number of OTUs rather than the genus level compared with the estimate obtained based on the 16S rRNA gene, which was consistent with previous conclusions obtained in studies of sediments from Jiaozhou Bay [6] and the north South China Sea [31].

Phylogenetic analyses based on these two biomarkers of anammox bacteria demonstrated that *Ca.* Scalindua was the dominant genus in the sediments of the Changjiang Estuary and its adjacent area. Although the different coverage of the anammox bacterial lineages by these distinct primers made direct parallel comparison of the 16S rRNA gene- and *hzo* gene-based results impossible, both helped decode at different levels and thus provided complementary views of the anammox bacterial ecological characteristics in the estuarine sediments. However, the 16S rRNA gene was only useful for bacterial taxonomy and not functional capability, whereas the detection of functional anammox bacteria by the *hzo* gene was more promising, reflecting the potential roles of anammox bacteria in natural ecosystems [15]. *Ca.* Scalindua may therefore comprise the most functional anammox bacteria in the sediments of the Changjiang Estuary and its adjacent area. A previous phylogenetic analysis of the *hzo* gene identified several novel anammox bacterial clades in Jiaozhou Bay [6] and, in the current study, *hzo* sequences yielded more OTUs but less genera of anammox bacteria than the 16S rRNA sequences. The study identified different environmental responses of the *Ca.* Scalindua assemblages and the whole anammox bacterial communities in the sediments. The *Ca.* Brocadia clade was also recovered with 16S rRNA gene primers from site 31 near the Zhoushan Islands, which might be the possible source of the clade. Some genera of anammox bacteria tolerated various environments but others could not; as a result, some allochthonous anammox bacteria genera survived in marine environments. These genera were related to the "*Ca.* Brocadia" and "*Ca.* Kuenenia" lineages that were found at low-salinity sites in the Cape Fear River estuary [5]. In contrast, the detected DNA might have originated from dead or dormant anammox bacteria. Further study with a reliable quantitative method is therefore needed to obtain more information concerning anammox bacteria in estuarine ecosystems and to determine anammox activity in nitrogen removal.

Anammox bacterial diversity and community structure differ among various ecosystems by showing niche-specific distributions [5,13]. The geochemical conditions of estuarine sediments may show significant impacts on the diversity and abundance of anammox bacterial communities. Using an ordination analysis and a non-parametric analysis of the distance matrix of many marine samples from all over the world, Sonthiphand et al. [50] confirmed that salinity was the dominant factor governing the global distribution of anammox bacteria. The *Ca.* Scalindua genus was assumed to have a higher tolerance to salinity than *Ca.* Kuenenia and *Ca.* Brocadia [21], and it has been present and dominant in most studied marine and estuary ecosystems [6,15,44,61]. *Ca.* Brocadia may be ubiquitous in river ecosystems, whereas the presence of *Ca.* Scalindua may rely on the significantly higher salinity in the sediments of marine environments [52]. As the salinity in the studied area was higher than 29 PSU, *Ca.* Scalindua was the most dominant genera, in accordance with previous findings in the Cape Fear River estuary [5] and the Changjiang Estuary [65]. Salinity might therefore be a key environmental variable in controlling the biogeographical distribution of anammox bacteria [16]. However, the diversity and abundance of anammox bacteria in sediments did not clearly correlate with salinity, possibly because the growth of anammox bacteria was not directly related to salinity in the Changjiang Estuary and its adjacent area, which were deeply influenced by fresh water influent from the Changjiang River. The growth of anammox bacteria was negatively correlated with the concentration of NO_3^-, which is the oxidized product of the metabolized substrate NO_2^- in natural environments that may also be reduced to form NO_2^-. To some extent, the anammox substrates may have impacted the diversity and abundance of anammox bacteria more strongly than other factors. However, ammonium concentration and the diversity and abundance of anammox bacteria did not significantly correlate in this study. The bottom-water samples contained relatively high contents of DIN that originated from OM and eventually became ammonium. The concentration of nitrite was actually rather lower than that of ammonium. The ammonium content was thus much higher than that of nitrite and may not be a rate-limiting factor for anammox bacterial growth in the sediments of the Changjiang Estuary and its adjacent area. These results were consistent with observations of sediments from Australian subtropical tidal rivers fringed by mangrove vegetation, the Thames estuary in the United Kingdom and Jiaozhou Bay in China, where ammonium concentration was reported not to be a limiting factor that regulated the abundance of anammox bacteria [6,36,56]. In the present study, the abundance and diversity of anammox bacteria were strongly related to nitrate concentration rather than the metabolic substrate nitrite. Although anammox bacteria do not directly reduce nitrate in nature, nitrate concentration was previously identified as an important factor influencing the distributions of anammox communities in estuarial sediments of the Mai Po Nature Reserve and Jiaojiang Estuary [18,30]. It has been well established that anammox bacteria are reliant on a supply of NO_2^- to fuel the oxidation of NH_4^+ and that NO_2^- is produced within suboxic sediment by the reduction of NO_3^- [36]. In turn, the heterotrophic reduction of NO_3^- to NO_2^- cannot proceed without a source of electron donors. The greatest potential for anammox bacteria was predominantly found in the upper reaches of the estuaries, where the organic carbon content of the sediment and concentration of NO_3^- in the bottom water were both greatest and, together, could maintain a critical supply of NO_2^- [39]. Moreover, the number of anammox bacteria in different ecosystems, such as the South China Sea and a full-scale wastewater treatment plant, was positively correlated with nitrate concentration [13]. The direct contributions of nitrate reduction by denitrification and ammonium oxidization by nitrification may cause nitrite accumulation at different levels for the anammox process, which may depend on the surrounding environmental conditions [28,36,42]. The relative contribution of nitrite concentration to the anammox process needs to be further investigated in estuary ecosystems given the competition for nitrite that potentially exists between anammox and denitrification, although nitrite production and consumption processes are very complex and poorly understood in natural ecosystems [6].

In an incubation-based study providing evidence of a novel symbiotic consortium between anammox bacteria and the nitrate-sequestering sulfur-oxidizing *Thioploca* spp. in anoxic sediments of the Soledad basin [40], *Thioploca*-anammox symbiosis intensified the benthic fixed nitrogen losses, whereas anammox bacteria relied on *Thioploca* species for a supply of metabolic substrate in the anoxic sediments. Prokopenko et al. [40] suggested that the molar ratio of fixed N loss to TOC oxidation could diagnose the degree of coupling between the N and C cycles. Owing to the diffusion limitations of the nitrate supply below the sediment-water interface, this ratio is decreased in sediments underlying suboxic/anoxic bottom waters worldwide [14,40]. The TOC content might have influenced the production of nitrite via reduction or other unknown reactions [18] and promoted ammonium accumulation [31], which then cooperatively influenced the community distributions of anammox bacteria. TOC could be considered a common and widespread influence on the anammox community and was identified as a key factor influencing the diversity and distribution of anammox bacteria in sediments of the Changjiang Estuary (Fig. 9). It is also reasonable that uncorrelated variables related the concentrations of NO_3^-, NO_2^-, and NH_4^+ in bottom water to the distribution of anammox bacteria in sediments. Furthermore, TOC, organic nitrogen and other materials participating in, directly or indirectly, the anammox process may relieve the strict dependence of the anammox bacteria on the direct availability of inorganic N nutrients, such as NO_2^-, NO_3^- and NH_4^+, which may be the limiting aspect in certain marine environments. However, given the limited ecological datasets currently available, additional physicochemical profiles are needed in order to acquire a more comprehensive understanding of their true roles in anammox bacterial communities in such dynamic ecosystems. Future studies of the relationships between the activity of anammox processes, verified by either mRNA viability or bacterial incubation, and community composition and abundance will be of great value for understanding estuarine and coastal ecosystems.

Several local sedimentological parameters were found to have key roles influencing the community structure, distribution and abundance of sediment anammox bacteria in the Changjiang Estuary and its adjacent area, where diverse *Ca.* Scalindua genera, other anammox-like bacteria and many uncultured species were located. Anammox bacterial community properties may also influence the ecological environment. The importance of inorganic N nutrients, TOC and many other environmental factors to the anammox bacteria suggested that microbiotas participating in the nitrogen cycle were active in the sediment N-transforming process and maintained a versatile lifestyle well adapted to the varying environmental conditions of the Changjiang Estuary.

Conclusions

The geochemical conditions of the estuarine sediments significantly impacted the anammox bacterial community, but the key influencing factors shaping the community structures may vary because they depend on local environmental conditions. The study aids in understanding the versatile lifestyle of the marine anammox bacteria in the Changjiang Estuary revealed by high-throughput sequencing technology. This study is the first to use qPCR and high-throughput sequencing technology to provide evidence for the existence, distributions and diversities of anammox bacteria in sediments of the Changjiang Estuary and its adjacent area,

where environmental conditions are relatively complicated. Comparisons of phylogenetic analyses using the 16S rRNA genes and *hzo* sequences of anammox bacteria confirmed that the known genera of *Ca.* Scalindua and *Ca.* Brocadia were present in sediments of the studied area, and that *Ca.* Scalindua-related sequences were the dominant functional anammox bacterial phylogenetic type. The qPCR results revealed that denitrifying bacteria were more abundant than anammox bacteria. In the studied area, although anammox bacteria were widespread, they were found at low abundance, and denitrification might be the dominant route of nitrogen loss. The anammox bacterial abundances were significantly correlated with the contents of NO_3^-, NO_x^-, PO_4^- and TOC, and the diversities of anammox bacterial communities covered by the *hzo* gene were negatively correlated with TOC contents, which highlighted the role of anammox bacteria as a significant microorganism in offshore ecosystems. Sedimentological conditions thus played important roles in shaping the sediment anammox microbiotas in marine environments. In summary, these findings have promoted the understanding of the distributions and influencing factors of anammox bacterial abundances and communities in sediments from the Changjiang Estuary and its adjacent area in the East China Sea.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.12.008.

References

[1] Aller, J.Y., Aller, R.C. (2004) Physical disturbance creates bacterial dominance of benthic biological communities in tropical deltaic environments of the Gulf of Papua. Cont. Shelf Res. 24 (19), 2395–2416, http://dx.doi.org/10.1016/j.csr.2004.07.015.

[2] Babbin, A.R., Keil, R.G., Devol, A.H., Ward, B.B. (2014) Organic matter stoichiometry, flux, and oxygen control nitrogen loss in the ocean. Science 344 (6182), 406–408, http://dx.doi.org/10.1126/science.1248364.

[3] Broda, E. (1977) Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. 17 (6), 491–493, http://dx.doi.org/10.1002/jobm.19770170611.

[4] Caporaso, J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F., Costello, E., Fierer, N., Peña, A., Goodrich, J., Gordon, J., Huttley, G., Kelley, S., Knights, D., Koenig, J., Ley, R., Lozupone, C., McDonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., Knight, B. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336 https://10.1038/NMETH.F.303.

[5] Dale, O.R., Tobias, C.R., Song, B. (2009) Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River estuary. Environ. Microbiol. 11 (5), 1194–1207, http://dx.doi.org/10.1111/j.1462-2920.2008.01850.x.

[6] Dang, H.Y., Chen, R.P., Wang, L., Guo, L.Z., Chen, P.P., Tang, Z.W., Tian, F., Li, S.Z., Klotze, M.G. (2010) Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. Appl. Environ. Microbiol. 76 (21), 7036–7047, http://dx.doi.org/10.1128/aem.01264-10.

[7] Dang, H.Y., Zhou, H.X., Zhang, Z.N., Yu, Z.S., Hua, E., Liu, X.S., Jiao, N.Z. (2013) Molecular detection of *Ca.* Scalindua pacifica and environmental responses of sediment anammox bacterial community in the Bohai Sea, China. PLoS One 8 (4), e61330 https://doi.org/10.1371/journal.pone.0061330.

[8] Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10 (10), 996–998, http://dx.doi.org/10.1038/nmeth.2604.

[9] Francis, C.A., Beman, J.M., Kuypers, M.M.M. (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J. 1 (1), 19–27, http://dx.doi.org/10.1038/ismej.2007.8.

[10] Fu, B.B., Liu, J.W., Yang, H.M., Hsu, T.C., He, B.Y., Dai, M.H., Kao, S.J., Zhao, M.X., Zhang, X.H. (2015) Shift of anammox bacterial community structure along the Pearl Estuary and the impact of environmental factors. J. Geophys. Res. Oceans 120 (4), 2869–2883, http://dx.doi.org/10.1002/2014jc010554.

[11] Fu, L.L., Zhen, Y., He, H., Zhang, Y., Mi, T.Z. (2016) Distribution characteristics of anaerobic ammonia oxidation bacteria in sediments from the adjacent seas of Yangtze estuary. Environ. Sci. 37 (10), 3914–3922, http://dx.doi.org/10.13227/j.hjkx.2016.10.033.

[12] Hamersley, M.R., Lavik, G., Woebken, D., Rattray, J.E., Lam, P., Hopmans, E.C., Damsté, S.J., Krüger, S., Graco, M., Gutiérrez, D., Kuypers, M.M.M. (2007) Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. Limnol. Oceanogr. 52 (3), 923–933, http://dx.doi.org/10.4319/lo.2007.52.3.0923.

[13] Han, P., Gu, J.D. (2013) More refined diversity of anammox bacteria recovered and distribution in different ecosystems. Appl. Microbiol. Biotechnol. 97 (8), 3653–3663, http://dx.doi.org/10.1007/s00253-013-4756-6.

[14] Hartnett, H.E., Devol, A.H. (2003) Role of a strong oxygen-deficient zone in the preservation and degradation of organic matter: a carbon budget for the continental margins of northwest Mexico and Washington State. Geochim. Cosmochim. Acta 67 (2), 247–264, http://dx.doi.org/10.1016/s0016-7037(02)01076-1.

[15] Hirsch, M.D., Long, Z.T., Song, B. (2011) Anammox bacterial diversity in various aquatic ecosystems based on the detection of hydrazine oxidase genes (hzoA/hzoB). Microb. Ecol. 61 (2), 264–276, http://dx.doi.org/10.1007/s00248-010-9743-1.

[16] Hou, L.J., Zheng, Y.L., Liu, M., Gong, J., Zhang, X.L., Yin, G.Y., You, L. (2013) Anaerobic ammonium oxidation (anammox) bacterial diversity, abundance, and activity in marsh sediments of the Yangtze Estuary. J. Geophys. Res. 118 (3), 1237–1246, http://dx.doi.org/10.1002/jgrg.20108.

[17] Hu, B.L., Shen, L.D., Zheng, P., Hu, A., Chen, T.T., Cai, C., Liu, S., Lou, L. (2012) Distribution and diversity of anaerobic ammonium-oxidizing bacteria in the sediments of the Qiantang River. Environ. Microbiol. Rep. 4 (5), 540–547, http://dx.doi.org/10.1111/j.1758-2229.2012.00360.x.

[18] Hu, B.L., Shen, L.D., Du, P., Zheng, P., Xu, X.Y., Zeng, J.N. (2012) The influence of intense chemical pollution on the community composition, diversity and abundance of anammox bacteria in the Jiaojiang Estuary (China). PLoS One 7 (3), e33826, http://dx.doi.org/10.1371/journal.pone.0033826.

[19] Hu, J., Peng, P., Jia, G., Mai, B., Zhang, G. (2006) Distribution and sources of organic carbon, nitrogen and their isotopes in sediments of the subtropical Pearl river estuary and adjacent shelf, Southern China. Mar. Chem. 98 (2), 274–285, http://dx.doi.org/10.1016/j.marchem.2005.03.008.

[20] Jetten, M.S.M., Strous, M., Van, d.P.K.T., Schalk, J., Van Dongen, U.G.J.M., Van, d.G.A.A., Logemann, S., Muyzer, G., Van Loosdrecht, M.C.M., Kuenen, J.G. (1998) The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22 (5), 421–437, http://dx.doi.org/10.1016/S0168-6445(98)00023-0.

[21] Jetten, M.S.M., Sliekers, O., Kuypers, M., Dalsgaard, T., van Niftrik, L., Cirpus, I., van de Pas-Schoonen, K., Lavik, G., Thamdrup, B., Le Paslier, D., Op den Camp, H.J.M., Hulth, S., Nielsen, L.P., Abma, W., Third, K., Engström, P., Kuenen, J.G., Jørgensen, B.B., Canfield, D.E., Sinninghe Damsté, J.S., Revsbech, N.P., Fuerst, J., Weissenbach, J., Wagner, M., Schmidt, I., Schmid, M., Strous, M. (2003) Anaerobic ammonium oxidation by marine and freshwater planctomycete-like bacteria. Appl. Microbiol. Biotechnol. 63 (2), 107–114 https://doi.org/10.1007/s00253-003-1422-4.

[22] Jetten, M.S., van Niftrik, L., Strous, M., Kartal, B., Keltjens, J.T., Op den Camp, H. (2009) Biochemistry and molecular biology of anammox bacteria. Crit. Rev. Biochem. Mol. Biol. 44 (2–3), 65–84, http://dx.doi.org/10.1080/10409230902722783.

[23] Kartal, B., Geerts, W., Jetten, M.S. (2011) Cultivation, detection, and ecophysiology of anaerobic ammonium-oxidizing bacteria. Methods Enzymol. 486, 89–108, http://dx.doi.org/10.1016/b978-0-12-381294-0.00004-3.

[24] Khramenkov, S.V., Kozlov, M.N., Krevbona, M.V., Drofeev, A.G., Kazakova, E.A., Grachev, V.A., Kuznetsov, B.B., Polyakov, D.Y., Nikolaev, Y.A. (2013) A novel bacterium carrying out anaerobic ammonium oxidation in a reactor for biological treatment of the filtrate of wastewater fermented residue. Microbiology 82 (5), 628–636, http://dx.doi.org/10.1134/S002626171305007X.

[25] Klotz, M.G., Stein, L.Y. (2008) Nitrifier genomics and evolution of the nitrogen cycle. FEMS Microbiol. Lett. 278 (2), 146–156, http://dx.doi.org/10.1111/j.1574-6968.2007.00970.x.

[26] Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jørgensen, B.B., Kuenen, J.G., Damsté, J.S., Strous, M., Jetten, M. (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422 (6932), 608–611, http://dx.doi.org/10.1038/nature01472.

[27] Kuypers, M.M.M., Lavik, G., Bo, T. 2006 Anaerobic ammonium oxidation in the marine environment. In: Past and Present Water Column Anoxia, Springer, Netherlands, pp. , 311–335, http://dx.doi.org/10.1007/1-4020-4297-3_13.

[28] Lam, P., Lavik, G., Jensen, M.M., van de Vossenberg, J., Schmid, M., Woebken, D., Gutiérrez, D., Amann, R., Jetten, M.S.M., Kuypers, M.M.M. (2009) Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proc. Natl. Acad. Sci. U. S. A. 106 (12), 4752–4757, http://dx.doi.org/10.1073/pnas.0812444106.

[29] Li, H., Chen, S., Mu, B.Z., Gu, J.D. (2010) Molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in high-temperature petroleum

reservoirs. Microb. Ecol. 60 (4), 771–783, http://dx.doi.org/10.1007/s00248-010-9733-3.
[30] Li, M., Cao, H., Hong, Y.G., Gu, J.D. (2011) Seasonal dynamics of anammox bacteria in estuarial sediment of the Mai Po nature reserve revealed by analyzing the 16S rRNA and hydrazine oxidoreductase (*hzo*) genes. Microbes Environ. 26 (1), 15–22, http://dx.doi.org/10.1264/jsme2.me10131.
[31] Li, M., Cao, H., Hong, Y.G., Gu, J.D. (2013) Using the variation of anammox bacteria community structures as a bio-indicator for anthropogenic/terrestrial nitrogen inputs in the pearl river delta (PRD). Appl. Microbiol. Biotechnol. 97 (22), 9875–9883, http://dx.doi.org/10.1007/s00253-013-4990-y.
[32] Lin, X.B., Liu, M., Hou, L.J., Gao, D.Z., Li, X.F., Lu, K.J., Gao, J. (2017) Nitrogen losses in sediments of the East China Sea: spatiotemporal variations, controlling factors, and environmental implications. J. Geophys. Res.: Biogeosci. 122, 2699–2715, http://dx.doi.org/10.1002/2017JG004036.
[33] Liu, D.Y., Li, X., Emeis, K.C., Wang, Y., Richard, P. (2015) Distribution and sources of organic matter in surface sediments of Bohai Sea near the yellow river estuary, China. Estuar. Coast. Shelf Sci. 165, 128–136, http://dx.doi.org/10.1016/j.ecss.2015.09.007.
[34] Liu, S.M., Hong, G.H., Ye, X.W., Zhang, J., Jiang, X.L. (2009) Nutrient budgets for large Chinese estuaries and embayment. Biogeosciences 6 (10), 2245–2263, http://dx.doi.org/10.5194/bg-6-2245-2009.
[35] Liu, S.M., Li, L.W., Zhang, G.L., Liu, Z., Yu, Z., Ren, J.L. (2012) Impacts of human activities on nutrient transports in the Huanghe (Yellow River) Estuary. J. Hydrol. 430–431, 103–110, http://dx.doi.org/10.1016/j.jhydrol.2012.02.005.
[36] Meyer, R.L., Risgaard-Petersen, N., Allen, D.E. (2005) Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. Appl. Environ. Microbiol. 71 (10), 6142–6149 10.1128/AEM.71.10.6142-6149.200551.
[37] Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16 (3), 177–184, http://dx.doi.org/10.1111/j.1574-6941.1995.tb00281.x.
[38] Naeher, S., Huguet, A., Roose-Amsaleg Céline, L., Laverman, A.M., Fosse, C., Lehmann, M.F., Derenne, S., Zopfi, J. (2015) Molecular and geochemical constraints on anaerobic ammonium oxidation (anammox) in a riparian zone of the seine estuary (France). Biogeochemistry 123 (1-2), 237–250 https://doi.org/10.1007/s10533-014-0066-z.
[39] Nicholls, J.C., Trimmer, M. (2009) Widespread occurrence of the anammox reaction in estuarine sediments. Aquat. Microb. Ecol. 55 (2), 105–113, http://dx.doi.org/10.3354/ame01285.
[40] Prokopenko, M.G., Hirst, M.B., De, B.L., Lawrence, D.J.P., Berelson, W.M., Granger, J., Chang, B.X., Dawson, S., Crane, E.J., III, Chong, L., Thamdrup, B., Townsend-Small, A., Sigman, D.M. (2013) Nitrogen losses in anoxic marine sediments driven by Thioploca-anammox bacterial consortia. Nature 500 (7461), 194–198, http://dx.doi.org/10.1038/nature12365.
[41] Rich, J.J., Dale, O.R., Song, B., Ward, B. (2008) Anaerobic ammonium oxidation (anammox) in Chesapeake Bay sediments. Microb. Ecol. 55 (2), 311–320, http://dx.doi.org/10.1007/s00248-007-9277-3.
[42] Rysgaard, S., Dalsgaard, T. (2004) Denitrification and anammox activity in Arctic marine sediments. Limnol. Oceanogr. 49 (5), 1493–1502, http://dx.doi.org/10.4319/lo.2004.49.5.1493.
[43] Schalk, J., de Vries, S., Kuenen, J.G., Jetten, M.S.M. (2000) Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry 39 (18), 5405–5412, http://dx.doi.org/10.1021/bi992721k.
[44] Schmid, M.C., Risgaard-Petersen, N., van de Vossenberg, J., Kuypers, M.M.M., Lavik, G., Petersen, J., Hulth, S., Thamdrup, B., Canfield, D., Dalsgaard, T., Rysgaard, S., Sejr, M.K., Strous, M., Camp, H.J., Jetten, M. (2007) Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. Environ. Microbiol. 9 (6), 1476–1484, http://dx.doi.org/10.1111/j.1462-2920.2007.01266.x.
[45] Schmid, M.C., Hooper, A.B., Klotz, M.G., Woebken, D., Lam, P., Kuypers, M.M.M., Pommerening-Roeser, A., Camp, H.J., Jetten, M.S.M. (2008) Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. Environ. Microbiol. 10 (11), 3140–3149, http://dx.doi.org/10.1111/j.1462-2920.2008.01732.x.
[46] Shehzad, A., Liu, J., Yu, M., Qismat, S., Liu, J.L., Zhang, X.H. (2016) Diversity, community composition and abundance of anammox bacteria in sediments of the north marginal seas of China. Microbes Environ. 31 (2), 111–120, http://dx.doi.org/10.1264/jsme2.me15140.
[47] Shimamura, M., Nishiyama, T., Shigetomo, H., Toyomoto, T., Kawahara, Y., Furukawa, K., Fujii, T. (2007) Isolation of a multiheme protein with features of a hydrazine-oxidizing enzyme from an anaerobic ammonium-oxidizing enrichment culture. Appl. Environ. Microbiol. 73 (4), 1065–1072, http://dx.doi.org/10.1128/aem.01978-06.
[48] Song, G.D., Liu, S.M., Marchant, H., Kuypers, M.M.M. (2013) Anaerobic ammonium oxidation, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment. Biogeosciences 10 (11), 6851–6864, http://dx.doi.org/10.5194/bgd-10-4671-2013.
[49] Song, G.D., Liu, S.M., Zhu, Z., Zhai, W., Zhu, C., Zhang, J. (2015) Sediment oxygen consumption and benthic organic carbon mineralization on the continental shelves of the East China Sea and the Yellow Sea. Deep Sea Res. II: Topical Stud. Oceanogr. 124, 53–63, http://dx.doi.org/10.1016/j.dsr2.2015.04.012.
[50] Sonthiphand, P., Hall, M.W., Neufeld, J.D. (2014) Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria. Front. Microbiol. 5 (399), 1–14, http://dx.doi.org/10.3389/fmicb.2014.00399.
[51] Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., Dutilh, B.E., Camp, H.J.M., Drift, C., Cirpus, I., Pas-Schoonen, K.T., Harhangi, H.R., Niftrik, L., Schmid, M., Keltjens, J., Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M.A., Mewes, H., Weissenbach, J., Jetten, M., Wagner, M., Paslier, D.L. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440 (7085), 790–794, http://dx.doi.org/10.1038/nature04647.
[52] Sun, W., Xu, M.Y., Wu, W.M., Guo, J., Xia, C.Y., Sun, G.P., Wang, A.J. (2014) Molecular diversity and distribution of anammox community in sediments of the Dongjiang River, a drinking water source of Hong Kong. J. Appl. Microbiol. 116 (2), 464–476, http://dx.doi.org/10.1111/jam.12367.
[53] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739, http://dx.doi.org/10.1093/molbev/msr121.
[54] Throbäck, I.N., Enwall, K., Jarvis, A., Hallin, (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49 (3), 401–417, http://dx.doi.org/10.1016/j.femsec.2004.04.011.
[55] Trimmer, M., Nicholls, J.C., Deflandre, B. (2003) Anaerobic ammonium oxidation measured in sediments along the Thames Estuary, United Kingdom. Appl. Environ. Microbiol. 69 (11), 6447–6454, http://dx.doi.org/10.1128/aem.69.11.6447-6454.2003.
[56] Trimmer, M., Nicholls, J.C., Morley, N., Davies, C.A., Aldridge, J. (2005) Biphasic behavior of anammox regulated by nitrite and nitrate in an estuarine sediment. Appl. Environ. Microbiol. 71 (4), 1923–1930, http://dx.doi.org/10.1128/aem.71.4.1923-1930.2005.
[57] Wang, Y.F., Gu, J.D. (2014) Effects of allylthiourea, salinity, and pH on ammonia/ammonium-oxidizing prokaryotes in mangrove sediment incubated in laboratory microcosms. Appl. Microbiol. Biotechnol. 98 (7), 3257–3274, http://dx.doi.org/10.1007/s00253-013-5399-3.
[58] Wang, Y.Y., Zhang, G.D., Zhu, J.C., Zhang, F.G. (1991) The early diagenesis of the sediments in the estuary of Yangtze River and its adjacent shelf. Acta Sedimentol. Sin. 9 (1), 54–61, http://dx.doi.org/10.14027/j.cnki.cjxb.1991.01.007.
[59] Ward, B.B., Devol, A.H., Rich, J.J., Chang, B.X., Bulow, S.E., Naik, H., Pratihary, A., Jayakumar, A. (2009) Denitrification as the dominant nitrogen loss process in the Arabian Sea. Nature 461 (7260), 78–81, http://dx.doi.org/10.1038/nature08276.
[60] Wei, Q.S., Wang, B.D., Chen, J.F., Xia, C.S., Qu, D.P., Xie, L.P. (2015) Recognition on the forming-vanishing process and underlying mechanisms of the hypoxia off the Yangtze River estuary. Sci. China: Earth Sci. 58 (04), 628–648, http://dx.doi.org/10.1007/s11430-014-5007-0.
[61] Woebken, D., Lam, P., Kuypers, M.M.M., Naqvi, S.W.A., Kartal, B., Strous, M., Jetten, M.S., Fuchs, B.M., Amann, R. (2008) A microdiversity study of anammox bacteria reveals a novel *Candidatus* Scalindua phylotype in marine oxygen minimum zones. Environ. Microbiol. 12 (8), 3106–3119, http://dx.doi.org/10.1111/j.1462-2920.2008.01640.x.
[62] Yang, Y., Dai, Y., Li, N.N., Li, B.X., Xie, S.Q., Liu, Y. (2017) Temporal and spatial dynamics of sediment anaerobic ammonium oxidation (anammox) bacteria in freshwater lakes. Microb. Ecol. 73 (2), 285–295, http://dx.doi.org/10.1007/s00248-016-0872-z.
[63] Zhang, D.S., Liu, Z.S., Zhang, H.F., Wang, X.G., Wang, C.S. (2015) Diversity of anaerobic ammonium oxidizing bacteria in marine sediments from the Zhoushan Islands. Acta Ecol. Sin. 35 (19), 6250–6258, http://dx.doi.org/10.5846/stxb201402210303.
[64] Zhang, J., Wu, Y., Jennerjahn, T.C., Ittekkot, V., He, Q. (2007) Distribution of organic matter in the Changjiang (Yangtze River) Estuary and their stable carbon and nitrogen isotopic ratios: implications for source discrimination and sedimentary dynamics. Mar. Chem. 106 (1), 111–126, http://dx.doi.org/10.1016/j.marchem.2007.02.003.
[65] Zheng, Y.L., Jiang, X., Hou, L.J., Liu, M., Lin, X., Gao, J., Li, X., Yin, G., Yu, C., Wang, R. (2016) Shifts in the community structure and activity of anaerobic ammonium oxidation bacteria along an estuarine salinity gradient. J. Geophys. Res. Biogeosci. 121 (6), 1632–1645, http://dx.doi.org/10.1002/2015jg0033.