An overlooked nitrogen loss linked to anaerobic ammonium oxidation in estuarine sediments in China

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Abstract

Purpose Despite its importance, anammox (anaerobic ammonium oxidation) in estuarine sediment systems remains poorly understood, particularly at the continental scale. This study aimed to understand the abundance, diversity, and activity of anammox bacteria and to determine the main factors influencing the anammox process in estuarine sediments in China.

Materials and methods Estuarine sediments were collected from 18 estuaries spanning over 4000 km. Experiments using an ¹⁵N-tracer, quantitative PCR, and clone library construction were used to determine the activity, abundance, and diversity of anammox bacteria. The impact of environmental factors on anammox processes was also determined.

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Results and discussion The abundance of the anammox-specific hydrazine synthase (*hzsB*) gene ranged from $1.8 \times 10^5 \pm 3.4 \times 10^4$ to $3.6 \times 10^8 \pm 7.5 \times 10^7$ copies g⁻¹ dw. *Candidatus Scalindua*, *Brocadia*, *Kuenenia*, *Jettenia*, and two novel unidentified clusters were detected, with *Scalindua* dominating the anammox population. Additionally, the abundances of *Scalindua*, *Kuenenia*, and *Brocadia* were found to be significantly correlated with latitude. The anammox rates ranged from 0.29 ± 0.15 to 13.68 ± 3.98 nmol N g⁻¹ dw h⁻¹ and contributed to 2.39–82.61% of total N₂ production. Pearson correlation analysis revealed that the anammox rate was positively correlated with total nitrogen, total carbon, and temperature, and was negatively correlated with dissolved oxygen (DO). The key factors influencing the *hzsB* gene abundance were ammonium concentration, salinity, and DO. Ammonium concentration, pH, temperature, and latitude were main variables shaping the anammox-associated bacterial community.

Conclusions Our results suggested that anammox bacteria are ubiquitous in coastal estuaries in China and underline the importance of anammox resulting in N loss at a continental scale.

Keywords Activity · Anammox · Estuarine sediments · N loss · Spatial variation

1 Introduction

Estuarine environments are partially enclosed coastal water bodies with rivers or streams and a free connection to the open sea, resulting in potential anthropogenic pollutants, particularly inorganic nitrogen. Anaerobic ammonium oxidation (anammox) couples ammonium oxidation with nitrite reduction under anaerobic conditions. Based on marine geochemical data, Richard (1965) first proposed the hypothesis that a

group of unknown microorganisms might exist in anoxic marine sediments that could carry out anammox. Over a decade later, the existence of anammox bacteria was also predicted via chemical reaction thermodynamic calculations (Broda 1977). Heterotrophic denitrification was considered to be the only known pathway for the loss of fixed nitrogen to the atmosphere for decades until the discovery of anammox bacteria in a wastewater treatment plant (Mulder et al. 1995). Subsequently, anammox bacteria were detected with broad biogeographic distribution in various natural ecosystems, including marine sediments (Thamdrup and Dalsgaard 2002; Trimmer and Nicholls 2009; Brabandere et al. 2014; Shao et al. 2014; Dang et al. 2016), estuarine sediments (Dale et al. 2009; Dang et al. 2010; Li et al. 2011; Wang et al. 2012a, b; Dang et al. 2013), and paddy soils (Zhu et al. 2011; Nie et al. 2015; Yang et al. 2015). The relative contribution of anammox to dinitrogen production can vary widely, with >80% of total N_2 production observed in the eastern tropical South Pacific oxygen minimum zone (OMZ) off the coast of northern Chile (Brabandere et al. 2014).

The anammox process is mediated by bacteria belonging to the Candidate Brocadiales order, which are affiliated with the Planctomycetes phylum (Jetten et al. 2010). Five *Candidatus* genera of anammox bacteria have been described: *Brocadia* (Kartal et al. 2008), *Kuenenia* (Schmid et al. 2000), *Scalindua* (Schmid et al. 2003; Kuypers et al. 2005), *Anammoxoglobus* (Kartal et al. 2007), and *Jettenia* (Quan et al. 2008). Each of these genera has been detected in coastal estuaries (Zhu et al. 2015). The anammox genotype is associated with hydrazine synthase, an intermediate step in the anammox pathway that synthesizes hydrazine from nitric oxide and ammonium (Kartal et al. 2011). We used the *hzsB* gene that encodes one of the hydrazine synthase subunits as a molecular marker (Wang et al. 2012a, b; Zhu et al. 2013; Yang et al. 2015) in order to determine the abundance and diversity of anammox bacteria.

China is the largest rice producer in the world (Zhu et al. 2016), stably consuming ~20 Tg of N-based chemical fertilizers per year (21.6 Tg N in 2005, 23.8 Tg N in 2011; China Agricultural Yearbook 2012). It was estimated that nearly 20% of the total nitrogen loss, mostly in the form of nitrate, was transported into estuarine and coastal ecosystems in the past three decades through riverine discharge and atmospheric deposition (Cui et al. 2013), resulting in water pollution (e.g., coastal eutrophication, hypoxia, harmful algae blooms) (Deegan et al. 2012). Thus, estuaries are thought to be a potential sink of nitrogen, especially nitrate, which is the substrate for many nitrogen cycling processes (e.g., denitrification and anammox). Several studies have reported anammox processes in estuarine sediments in China (Dang et al. 2010; Li et al. 2011; Hu et al. 2012; Dang et al. 2013; Zhu et al. 2013), but understanding of N cycling and the release of fixed N as dinitrogen gas (N_2) is lacking due to the high heterogeneity

of the anammox activity in different estuarine and coastal sediments. Therefore, it is crucial to estimate the contributions of N_2 from anammox in different estuarine environments and discover the key factors governing activity and microbial diversity at a large scale.

On the basis of previous studies (Dang et al. 2010; Li et al. 2011; Hu et al. 2012; Zhu et al. 2013; Hou et al. 2015), we hypothesize that coastal estuaries are hotspots of anammox processes. Therefore, we sampled estuarine sediments from 18 rivers (from north to south China) in order to (i) determine anammox abundance via qPCR of the hydrazine synthase (*hzsB*) gene and investigate the community composition of anammox bacteria; (ii) evaluate the contribution of denitrification and anammox to N_2 production using an ^{15}N -tracer technique; and (iii) elucidate the impact of environmental factors on anammox process in estuarine sediments along the coastal zone in China.

2 Materials and methods

2.1 Sample collection

Surface sediment samples (0–10 cm) of five replicates (ca. 500 g each subsample) were collected from 18 estuaries during July 2013 (total of 90 subsamples), spanning over 4000 km of the coastline and covering a range of climatic and geological zones. The study area and sampling sites are presented in Table S1 (Electronic Supplementary Material). The overlaying water was also collected and kept on ice during transportation. Sediment samples were stored in sterile plastic bags, sealed, and transported to the laboratory on ice. Each replicate sample was partitioned into three subsamples. One subsample was incubated to determine denitrification and anammox activities immediately after arrival, and another subsample was used for analysis of chemical properties. The remainder was stored at $-80^\circ C$ for genomic DNA extraction and further molecular analysis.

2.2 Chemical properties of sediment samples

Sediment pH was determined at a soil/Milli-Q water ratio of 1:2.5 with a pH analyzer (XL60, Fisher, USA). Water temperature, dissolved oxygen (DO), and salinity were measured in situ using a Hydrolab DS5 multiparameter water quality analyzer (Hach, Loveland, CO, USA). The total N (TN) and total C (TC) were analyzed by using dry combustion in a C/N analyzer (vario MAX C/N, Germany). NO_3^- -N and NH_4^+ -N were measured using an ion chromatograph (ICS-3000, USA) after 2 M KCl extraction at a soil/KCl ratio of 1:10 and filtration through a 0.22- μm membrane filter. All analyses were performed in triplicate for each sample.

2.3 Anammox and denitrification activity measurement with ^{15}N -labeled ammonium and nitrate

The rates of anammox and denitrification were measured using the ^{15}N -tracer technique (Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2004) with slight modifications, and their relative contributions to N_2 production were then calculated based on the rates. Briefly, ~3.5 g sediment (wet weight) was transferred to a 12.0-mL glass vial (Exetainer, Labco, High Wycombe, Buckinghamshire, UK) and filled with N_2 -purged water. The resulting sediment slurries were pre-incubated for 24 h to remove intrinsic NO_x^- and oxygen. After the pre-incubation step, both NO_2^- and NO_3^- were under the detection limit of ion chromatography (0.05~0.1 ppm and 0.075~0.1 ppm for NO_2^- and NO_3^- , respectively). Subsequently, the vials were portioned into three treatments, which were spiked through the stopper of each vial with 100 μL of N_2 -purged stock solution of (1) $^{15}\text{NH}_4^+$ (^{15}N -(NH_4) $_2\text{SO}_4$, ^{15}N at. %: 99.14), (2) $^{15}\text{NH}_4^+ + ^{14}\text{NO}_3^-$, and (3) $^{15}\text{NO}_3^-$ (^{15}N - KNO_3 , ^{15}N at. %: 98.15%), resulting in a final concentration of about 100 μM N. The incubations were performed at a temperature of 25 ± 1 °C and blocked at schedule time intervals (0, 3, 6, 12, 24 h) by injecting ZnCl_2 solution (200 μL , 7 M) to stop microbial activity. Concentrations of the produced $^{29}\text{N}_2$ and $^{30}\text{N}_2$ were measured by continuous-flow isotope ratio mass spectrometry (MAT 253 with GasBench II and autosampler (GC-PAL), Bremen, Thermo Electron Corporation, Finnigan, Germany). The rate and potential contribution to N_2 production of either anammox or denitrification were calculated as described before (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003).

2.4 DNA extraction and clone library construction

Approximately 0.5 g soil was used for genomic DNA extraction using a FastDNATM SPIN Kit for Soil (MP Biomedicals, USA) according to the protocols provided by the manufacturer's instructions. A nested PCR approach was used to amplify the anammox 16S ribosomal RNA (rRNA) genes. In the first round of this PCR, the primer set Pla46f-630r was used to amplify the Planctomycetales 16S rRNA genes (Juretschko et al. 1998; Schmid et al. 2005). In the next round, anammox 16S rRNA genes were amplified using Amx368f-Amx820r as the primer set (Schmid et al. 2000; Schmid et al. 2003) and amplicons of Planctomycetales 16S rRNA genes as templates. The PCR reactions and thermal cycles were performed as previously described (Zhu et al. 2011). The amplified products were verified by electrophoresis in a 1.0% agarose gel, and then purified using a Universal DNA Purification Kit (Tiangen, Beijing China). The purified products were cloned into a pMD19-T vector (TaKaRa, Bio Inc., Shiga, Japan) according to the manufacturer's instruction. At least 40 positive clones of each clone library were randomly selected for

sequencing (Major Bio Ltd., Shanghai, China). The quality of sequence was examined using the Chromas Lite (version 2.01, Technelysium Pty, QLD, Australia) program, and the existence of chimeric sequence was further checked using QIIME (version 1.8) (Caporaso et al. 2010). After that, the sequences were aligned with the MEGA (version 6.0) (Tamura et al. 2011) software and manually checked and trimmed.

2.5 Quantitative PCR assay

The abundance of the *hzsB* gene was determined in triplicate using a LightCycler 480 with the primer set HSBeta396F-HSBeta742R (Wang et al. 2012a, b). The 20- μL qPCR reaction contained 10 μL 2 $\times$ TransStart[®] Top Green qPCR SuperMix (AQ131, TransGen Biotech, Beijing, China), 0.25 μM each primer, 0.8 μL bovine serum albumin (BSA, 20 mg mL^{-1}), and 2 μL of fivefold diluted DNA as a template. The standard curve was obtained using tenfold serial dilutions of plasmid DNA with a target gene of *hzsB*. Three non-template controls were carried out for each quantitative assay. The PCR was performed in triplicate with the following thermal profile: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 34 s. Melting curves showed only one peak at 86 °C. Only the reactions with efficiencies between 90 and 110% (Malte et al. 2015) and standard curves with correlation coefficient above 0.99 were employed in this study.

2.6 Phylogenetic analysis

The sequences from each clone library were identified by blasting in NCBI GenBank database. Operational taxonomic units (OTUs) were defined using 97% similarity in the nucleotide sequences by Mothur (version 1.34.0) (Schloss et al. 2009). The related reference sequences and our representative sequences were aligned, and the neighbor-joining phylogenetic tree was constructed by MEGA (version 6.0). A bootstrap analysis based on 1000 replicates was applied to assess the cluster stabilities. The diversity, community composition of anammox, and redundancy analysis (RDA) were performed using R (version 2.14.0) software (<https://www.r-project.org/>). The plots in this study were created using Origin program (version 9.0).

2.7 Statistical analysis

Pearson correlation analyses were used to test the correlations among the anammox bacterial activity, abundance, and different environmental factors, using the SPSS 20.0 (SPSS, Chicago, Illinois, USA).

2.8 Nucleotide sequence accession numbers

The sequences obtained in this study are available in GenBank under accession numbers KU987935–KU990864.

3 Results

3.1 Physicochemical properties of estuarine sediments

The locations and physicochemical properties of the sampling sites can be found in Table S1 (Electronic Supplementary Material). The physicochemical properties exhibited great heterogeneity among samples from northern and southern China. The pH ranged from 8.57 ± 0.59 in LN-LH in the north to 6.49 ± 0.03 in GD-LJ in the south. The TC varied from 0.53 ± 0.33 to 1.84 ± 0.87 g kg⁻¹ dw, while TN ranged from 0.03 ± 0.01 to 0.17 ± 0.08 g kg⁻¹ dw, resulting in the C/N ratio ranging from 7.34 ± 1.7 to 26.84 ± 3.63 . Concentrations of NH₄⁺-N ranged between 0.18 ± 0.1 mg kg⁻¹ dw (ZJ-QTJ) and 12.54 ± 9.59 mg kg⁻¹ dw (TJ-YDXH). NO_x⁻-N concentration varied from 0.03 ± 0.01 mg kg⁻¹ dw (ZJ-JJ) to 5.40 ± 4.72 mg kg⁻¹ dw (LN-LH), while not detected in ZJ-QTJ, GX-NLJ, GX-QJ, and GX-FCJ.

3.2 Anammox rates and contributions to N₂ production

No significant accumulation of ¹⁵N₂-labeled gas (²⁹N₂ and/or ³⁰N₂) was detected in any of the sample slurries amended with only ¹⁵NH₄⁺ (Fig. S1A, Electronic Supplementary Material), indicating that all residual ¹⁴NO_x⁻ had been consumed during pre-incubation. When both ¹⁵NH₄⁺ and ¹⁴NO₃⁻ were added, only ²⁹N₂ accumulated in each soil (Fig. S1B, Electronic Supplementary Material), indicating that anammox must have been occurring. When amended with ¹⁴NH₄⁺ and ¹⁵NO₃⁻, significant production of both ²⁹N₂ and ³⁰N₂ was detected, as a result of both anammox and denitrification (Fig. S1C, Electronic Supplementary Material). Anammox rates ranged from 0.29 ± 0.15 to 13.68 ± 3.98 nmol N g⁻¹ dw h⁻¹, with the lowest and highest rates detected in QTJ in ZheJiang province and ZJ in GuangDong province, respectively (Table 1). Denitrification rates varied substantially from 0.31 ± 0.06 to 56.63 ± 17.29 nmol N g⁻¹ dw h⁻¹. The contribution to N₂ production was calculated based on these rates, with anammox contributing between 2.39% (GX-FCJ) and 82.61% (ZJ-OJ) of total N₂ production (Table 1), with the remaining production attributed to denitrification.

3.3 Abundance and composition of anammox bacteria

The presence of anammox bacteria was confirmed by qPCR in all samples from 18 estuaries (Fig. 1). The abundance of *hzsB* genes in the sediments was from $1.8 \times 10^5 \pm 3.4 \times 10^4$ to

$3.6 \times 10^8 \pm 7.5 \times 10^7$ copies g⁻¹ dw. The ratio of *hzsB* gene copies to total bacterial 16S rRNA gene copies ranged from 0.005% (ZJ-YJ) to 3.72% (GD-ZJ).

A total of 2930 sequences were retrieved from the 18 estuarine sediment samples and clustered into 223 operational taxonomic units (OTUs). The phylogenetic analysis of the 16S rRNA gene showed that 80.17% of sequences were affiliated with the following known anammox bacterial genera, *Candidatus Brocadia*, *Candidatus Scalindua*, *Candidatus Kuenenia*, *Candidatus Jettenia*, and an additional 19.83% belonged to the unclassified Planctomycetes phylum (four clusters, I–IV) (Fig. S2, Electronic Supplementary Material). The most abundant genus was *Candidatus Scalindua*, followed by *Candidatus Brocadia*, *Candidatus Kuenenia*, and *Candidatus Jettenia* (Fig. S2, Electronic Supplementary Material). The distribution of each cluster in 18 estuarine sediments was visualized in Fig. 2. Among them, *Candidatus Scalindua* was found to be prevalent in all sites and occupied up to 43.07% of the relative abundance (1262 sequences). *Candidatus Brocadia* was detected in most of the sites with the exception of GX-FCJ, FJ-HTX, ZJ-OJ, ZJ-JJ, and LN-LH, and accounted for 19.32% (566 sequences). *Candidatus Kuenenia* was found to be present in all sediments except LN-LH, accounting for 16.18% (474 sequences). Sequences belonging to the *Candidatus Jettenia* genus contributed 1.6% (47/2930) overall, but up to 20% of the likely anammox bacteria in TJ-YDXH were assigned to *Candidatus Jettenia*. The remaining sequences (577 sequences) were affiliated to unknown clusters, and up to 60% of these unclassified taxa were found in TJ-YDXH. The ratio of *Candidatus Scalindua* to total anammox bacteria decreased from north to south China based on the latitude, with the exception of GX-FCJ and TJ-YDXH (Figs. 2 and 3). In contrast, the abundance of *Candidatus Brocadia* and *Candidatus Kuenenia* exhibited a general pattern which increased from north to south (Figs. 2 and 3). Additionally, the ratio of the genus of *Candidatus Scalindua* to total anammox bacteria was found to be positively correlated with the latitude, whereas the proportions of genera of *Candidatus Brocadia* and *Candidatus Kuenenia* were both negatively correlated with the increasing latitude (Fig. 3).

3.4 Influence of environmental factors on anammox rate, abundance, and diversity

Pearson correlation analysis was used to illustrate the effects of the environmental factors on the anammox rate and abundance (Fig. 4). Results showed that anammox rates were positively correlated with TC ($P < 0.01$), temperature ($P < 0.01$), NO₃⁻ concentration ($P < 0.05$), *hzsB* abundance ($P < 0.05$), *hzsB*/16S rRNA ($P < 0.05$), and salinity ($P < 0.05$), and negatively correlated with DO ($P < 0.01$). The *hzsB* abundance was positively correlated with salinity ($P < 0.01$) and NO₃⁻ concentration ($P < 0.05$) and negatively with NH₄⁺

Table 1 Rates of anammox and denitrification measured by ^{15}N tracing technique and the relative contribution to total denitrogen production

Sites	Anammox rate ($\text{nmol N g}^{-1} \text{ dw h}^{-1}$)	Denitrification rate ($\text{nmol N g}^{-1} \text{ dw h}^{-1}$)	Anammox contribution (%)
LN-LH	1.72 ± 0.97	3.25 ± 0.46	34.61
LN-BLH	1.35 ± 0.32	17.98 ± 1.56	6.98
HB-LH	0.57 ± 0.03	15.63 ± 9.58	3.52
TJ-YDXH	1.32 ± 0.23	25.61 ± 3.40	4.90
ZJ-QTJ	0.29 ± 0.15	0.31 ± 0.06	48.33
ZJ-YJ	1.55 ± 0.31	30.00 ± 1.97	4.91
ZJ-JJ	1.10 ± 0.51	15.31 ± 1.88	6.70
ZJ-OJ	2.09 ± 0.48	0.44 ± 0.02	82.61
FJ-HTX	4.18 ± 1.86	22.85 ± 3.04	15.46
FJ-MJ	2.51 ± 0.46	36.11 ± 9.53	6.50
FJ-JJ	0.97 ± 0.97	26.95 ± 2.11	3.47
FJ-JLJ	3.24 ± 0.45	56.63 ± 17.29	5.41
GD-HJ	1.09 ± 0.46	33.81 ± 4.01	3.12
GD-LJ	1.11 ± 0.70	21.52 ± 2.70	4.90
GD-ZJ	13.68 ± 3.98	50.9 ± 2.46	21.18
GX-NLJ	1.66 ± 0.43	4.76 ± 1.02	25.86
GX-QJ	2.83 ± 0.43	43.3 ± 8.59	6.13
GX-FCJ	1.15 ± 0.12	47.01 ± 4.93	2.39

concentration ($P < 0.01$). As revealed by RDA analysis, NH_4^+ concentration, pH, latitude, and temperature were found to be the main factors affecting the anammox bacterial diversity (Fig. 5).

4 Discussion

In the present study, the occurrence of anammox in coastal estuarine sediments in China was corroborated by using both molecular and isotope-tracing experiments. Four known genera of anammox bacteria (*Candidatus Scalindua*, *Candidatus Brocadia*, *Candidatus Kuenenia*, and *Candidatus Jettenia*) were identified, illustrating a relatively diverse set of anammox bacteria in the selected estuarine sediments. Similarly, diverse anammox bacteria have been observed in other estuarine and coastal wetlands (Dale et al. 2009; Hong et al. 2014; Lisa et al. 2014). This indicates that the fresh water-seawater interface may provide diverse habitats and eco-niches for a higher diversity of anammox bacteria. In contrast, anammox community diversity in oceans (Schmid et al. 2007), rivers (Zhang et al. 2007; Hu et al. 2012), and lakes (Hamersley et al. 2009) was low, limited primarily to *Scalindua* or *Brocadia*. Specifically, *Candidatus Scalindua* species were reported to be dominant in marine (Schmid et al. 2007; Dale et al. 2009; Cao et al. 2011) and fresh water ecosystems (Schubert et al. 2006). Furthermore, the microbial diversity of anammox bacteria exhibited a latitudinal gradient along the coastal wetlands of China, which was consistent with previous results (Hou et al. 2015). This implies that

temperature is a key environmental factor shaping the distribution and diversity of anammox bacteria in the coastal estuaries of China, and was further supported with the RDA analysis (Fig. 5). Therefore, the distribution pattern based on latitude underlines the significance of temperature in regulating the biogeographical distribution of anammox bacterial community structure and diversity over a large spatial scale.

Our results exhibited a clear group-specific biogeographical distribution (Fig. 5 and Fig. S1, Electronic Supplementary Material). *Candidatus Scalindua* was detected at all sites and had the highest relative abundance (up to 43%) among all anammox genera. *Candidatus Scalindua* has been found in both marine (Schmid et al. 2007) and fresh water ecosystems (Schubert et al. 2006), and this flexibility could be the reason for its high abundance in most of our estuarine sites. *Candidatus Brocadia* accounted for 19.32% of the total anammox bacteria across all sites. It was reported that *Brocadia* possess a diverse metabolism (Gori et al. 2011). This may explain its ubiquitous distribution, where high organic loading is imported from river water. Interestingly, four novel clusters were also detected in our samples, suggesting that unknown anammox bacteria are yet to be discovered and investigated.

A wide range of *hzsB* copy numbers from anammox bacteria was detected (ranging from $1.80 \times 10^5 \pm 3.4 \times 10^4$ to $3.6 \times 10^8 \pm 7.5 \times 10^7$ copies $\text{g}^{-1} \text{ dw}$), indicating that the overall abundance of anammox bacteria was highly variable in sediments at a continental scale. To our knowledge, the highest anammox abundance (2×10^9 copies per gram dry weight) recorded in natural environments was detected in

Fig. 1 Abundance of anammox bacteria in the 18 estuarine sediments in China. The base map used is from the National Fundamental Geographic Information System of China. The columns in the box chart represent the

log number of the *hzsB* gene copy (copies g^{-1} dry soil). For all boxplots, black center lines represent the median and box edges are the first and third quartiles

riparian sediments of the Pearl River Estuary in winter (Wang et al. 2012a, b). The *hzsB* abundance in our study, measured in the summer, was comparable to the Pearl River Estuary in summer (1.3×10^6 – 1.2×10^7 copies g^{-1} dw) (Wang et al. 2012a, b), China coastal wetlands (1.17×10^7 – 4.25×10^7 copies g^{-1} dw) (Hou et al. 2015), and interface sediments (8×10^6 – 2×10^7 copies g^{-1} dw) (Zhu et al. 2013). Additionally, the abundance of the *hzsB* gene was significantly related to salinity, and it was positively but not significantly correlated with temperature (Fig. 4). One should expect that copy numbers of anammox functional genes will be a significant factor in controlling anammox activity; however, here the anammox activity was not positively correlated with the *hzsB* gene copy number. This was probably due to

quantification based on DNA rather than RNA. Similar results by Etchebehere and Tiedje (2005) and Metz et al. (2003) demonstrated that no correlation was observed between copy numbers of functional gene and relative functions. Additionally, the abundances of anammox-related genera were significantly correlated with the latitude (Fig. 5), indicating that temperature was likely a key environmental factor shaping the biogeographical distribution and diversity of the anammox bacterial community.

Our results suggest a ubiquitous distribution of anammox bacteria in estuarine sediments along 4000 km of coast in China. On average, it was estimated that the anammox process contributed 15.94% to the total N loss from the coastal wetland sediments of China. This approximate value was

Fig. 2 The relative abundance of each cluster in the Planctomycetes phylum in the 18 estuarine sediment samples. The colors indicate different taxa. The horizontal axis is the relative abundance, and the vertical axis is the sampled estuaries

comparable to that reported in rivers (Zhao et al. 2013), lakes (Schubert et al. 2006; Wenk et al. 2014), paddy soils (Zhu et al. 2011; Yang et al. 2015), and other estuaries (Risgaard-Petersen et al. 2004; Rysgaard et al. 2004; Engström et al. 2005), but lower than that generally detected in marine ecosystems (Tamdrup and Dalsgaard 2002; Brabandere et al. 2014). Based on our results, N loss attributed to anammox

was estimated to reach an average value of 9.70×10^5 t N per year on the basis of sediment weight, study area, and anammox average rate obtained from slurry incubations. This removal suggests approximately 40.4% of the total average terrigenous inorganic nitrogen (2.4×10^6 t N per year) transported into the coastal wetlands of China. However, our results from the slurry incubations in lab might overestimate

Fig. 3 The locations of the 18 sampling sites (a) and the correlation analysis between relative abundance of the predominant genera **b** *Candidatus Scalindua*, **c** *Candidatus Brocadia*, and **d** *Candidatus Kuenenia* to the Planctomycetes phylum and the latitude of sampling sites

Fig. 4 Pearson correlation analyses of anammox rate, *hzsB* gene abundance, *hzsB*/16S rRNA, and the soil properties (including TN, TC, C/N, pH, NH_4^+ , NO_3^- , temperature, dissolved oxygen, and salinity) in the collected paddy soils. Red and blue denote positive and negative correlations, respectively. **P* value of <0.05 and ***P* value of <0.01. *Tem* temperature; *DO* dissolved oxygen; *sal* salinity; *TN* total nitrogen; *TC* total carbon

the in situ anammox activity for three reasons. First, the anammox activity might be enhanced since excess substrates were amended. Second, labile organic carbon could be depleted after the prolonged pre-incubation, leading to favorable conditions for anammox rather than denitrification. Finally, co-denitrification may be an additional pathway for N_2 production in sediments. Co-denitrification can generate $^{29}\text{N}_2$ by reducing $^{45}\text{N}_2\text{O}$, which is produced by using $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-/^{15}\text{NO}_2^-$ in ^{15}N isotope pairing experiments (Long et al. 2013). Nevertheless, the ubiquitous detection of anammox bacteria indicated that anammox must play an important role in N_2 production. Therefore, our current study on the roles of anammox bacteria on N cycling at a continental

scale is an important step for the estimation N loss in the vast coastal estuaries which span over 4000 km of coastline and cover a range of climatic and geological zones. Furthermore, this study also emphasizes the importance of protecting coastal estuaries due to their non-negligible removal capacity of terrigenous inorganic nitrogen via both denitrification and anammox.

5 Conclusions

In conclusion, our results suggested that anammox bacteria are ubiquitous in estuarine sediments across coastal China.

Fig. 5 Redundancy analysis (RDA) to measure the relationship between the anammox community composition, physicochemical properties, and relative abundance of *Candidatus Scalindua*, *Candidatus Brocadia*, *Candidatus Kuenenia*, and *Candidatus Jettenia*. Only those factors that significantly describe variance in the composition of the anammox bacterial community determined by variation inflation factors (VIFs) calculated during RDA are shown here

The anammox community was dominated by the genera of *Candidatus Scalindua*, *Candidatus Kuenenia*, and *Candidatus Brocadia* and showed a clear biogeographic variation pattern from north to south. The anammox activity contributed 2.39–82.61% of total N_2 production, suggesting the important role of anammox in controlling N cycling across ecosystems.

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