SEDIMENTS, SEC 2 • PHYSICAL AND BIOGEOCHEMICAL PROCESSES • RESEARCH ARTICLE

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

Xiao-Ru Yang[1] • Bo-Sen Weng[1] • Hu Li[1] • Christopher W. Marshall[2,3] • Hong Li[4] • Yong-Shan Chen[1] • Shen Yu[1] • Gui-Bing Zhu[5] • Yong-Guan Zhu[1]

[1]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China

[2]Department of Surgery, University of Chicago, Chicago IL 60637, U.S.A

[3]Biosciences Division, Argonne National Laboratory, 9700, S. Cass Ave. Lemont, IL 60439, U.S.A.

[4]Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, United Kingdom

[5]Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

X.-R. Yang, B.-S. Weng and H. Li contributed equally to this work.

✉ Yong-Guan Zhu

Tel.: +86-592-6190997; fax: +86-592-6190977

ygzhu@iue.ac.cn

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 4,000 kilometers. Experiments using an ^{15}N-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 were also determined.

Results and discussion The abundance of the anammox-specific hydrazine synthase (*hzs*B) gene ranged from $1.8\times10^{5} \pm 3.4 \times10^{4}$ copies g^{-1} dw to $3.6\times10^{8} \pm 7.5 \times10^{7}$ 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 N_2 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 first proposed the hypothesis that a group of unknown microorganisms might exist in anoxic marine sediments that could carry out anammox (Richard 1965). 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; De 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. 2012; 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 (De 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 have 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. 2012; 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, 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

82 estimated that nearly 20% of the total nitrogen loss, mostly in the form of nitrate, was transported into
83 estuarine and coastal ecosystems in the past three decades through riverine discharge and atmospheric
84 deposition (Cui et al. 2013), resulting in water pollution (e.g., coastal eutrophication, hypoxia, harmful
85 algae blooms) (Deegan et al. 2012). Thus, estuaries are thought to be a potential sink of nitrogen,
86 especially nitrate, which is the substrate for many nitrogen cycling processes (e.g., denitrification and
87 anammox). Several studies have reported anammox processes in estuarine sediments in China (Dang et
88 al. 2010; Li et al. 2011; Hu et al. 2012; Dang et al. 2013; Zhu et al. 2013), but understanding of N
89 cycling and the release of fixed N as dinitrogen gas (N_2) is lacking due to the high heterogeneity of the
90 anammox activity in different estuarine and coastal sediments. Therefore, it is crucial to estimate the
91 contributions of N_2 from anammox in different estuarine environments and discover the key factors
92 governing activity and microbial diversity at a large scale.
93 On the basis of previous studies (Dang et al. 2010; Li et al. 2011; Hu et al. 2012; Zhu et al. 2013;
94 Hou et al. 2015), we hypothesize that coastal estuaries are hotspots of anammox processes. Therefore,
95 we sampled estuarine sediments from 18 rivers (from north to south China) in order to: (i) determine
96 anammox abundance *via* qPCR of the hydrazine synthase (*hzs*B) gene and investigate the community
97 composition of anammox bacteria; (ii) evaluate the contribution of denitrification and anammox to N_2
98 production using an ^{15}N-tracer technique; and (iii) elucidate the impact of environmental factors on
99 anammox process in estuarine sediments along the coastal zone in China.
100
101 **2 Materials and methods**
102 **2.1 Sample collection**
103 Surface sediment samples (0-10 cm) of five replicates (*ca.* 500 g each subsample) were collected
104 from 18 estuaries during July 2013 (total of 90 subsamples), spanning over 4,000 kilometers of the
105 coastline and covering a range of climatic and geological zones. The study area and sampling sites are
106 presented in Table S1. The overlaying water was also collected and kept on ice during transportation.
107 Sediment samples were stored in sterile plastic bags, sealed and transported to the laboratory on ice.
108 Each replicate sample was partitioned into three subsamples. One subsample was incubated to
109 determine denitrification and anammox activities immediately after arrival and another subsample was
110 used for analysis of chemical properties. The remainder was stored at -80 °C for genomic DNA
111 extraction and further molecular analysis.

112

113 **2.2 Chemical properties of sediment samples**

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

121

122 **2.3 Anammox and denitrification activity measurement with ^{15}N labeled ammonium and nitrate**

123 The rates of anammox and denitrification were measured using the ^{15}N-tracer technique (Thamdrup
124 and Dalsgaard 2002; Risgaard-Petersen et al. 2004) with slight modifications, and their relative
125 contributions to N_2 production were then calculated based on the rates. Briefly, ~3.5 g sediment (wet
126 weight) was transferred to a 12.0 mL glass vial (Extainer, Labco, High Wycombe, Buckinghamshire,
127 UK) and filled with N_2-purged water. The resulting sediment slurries were pre-incubated for 24 h to
128 remove intrinsic NO_x^- and oxygen. After the pre-incubation step, both NO_2^- and NO_3^- were under the
129 detection limit of ion chromatograph (0.05~0.1 ppm and 0.075~0.1 ppm for NO_2^- and NO_3^-,
130 respectively). Subsequently, the vials were portioned into three treatments, which were spiked through
131 the stopper of each vial with 100 μL of N_2-purged stock solution of (1) $^{15}NH_4^+$ (^{15}N-$(NH_4)_2SO_4$, ^{15}N
132 at.%: 99.14), (2) $^{15}NH_4^+$+ $^{14}NO_3^-$, and (3) $^{15}NO_3^-$ (^{15}N-KNO_3, ^{15}N at.%: 98.15%), resulting in a final
133 concentration of about 100 μM N. The incubations were performed at a temperature of 25 ± 1°C and
134 blocked at schedule time intervals (0, 3, 6, 12, 24 h) by injecting $ZnCl_2$ solution (200 μL, 7 M) to stop
135 microbial activity. Concentrations of the produced $^{29}N_2$ and $^{30}N_2$ were measured by continuous flow
136 isotope ratio mass spectrometry (MAT253 with Gasbench II and autosampler (GC-PAL), Bremen,
137 Thermo Electron Corporation, Finnigan, Germany). The rate and potential contribution to N_2
138 production of either anammox or denitrification were calculated as described before (Thamdrup and
139 Dalsgaard 2002; Trimmer et al. 2003).

140 **2.4 DNA extraction and clone library construction**

141 Approximately 0.5 g soil was used for genomic DNA extraction using a FastDNA™ SPIN Kit for

Soil (MP Biomedicals, USA) according to the protocols provided by manufacturer's instructions. A nested PCR approach was used to amplify the anammox 16S 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 (MajorBio 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. 2013) software and manually checked and trimmed.

2.5 Quantitative PCR (qPCR) assay

The abundance of *hzsB* gene was determined in triplicate using a Light Cycler 480 with the primer set HSBeta396F-HSBeta742R (Wang et al. 2012). The 20 μL qPCR reaction contained 10 μL 2×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 5-fold diluted DNA as a template. The standard curve was obtained using 10-fold serial dilutions of plasmid DNA with 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 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 1,000 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. 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. Concentration of NH_4^+-N ranged between 0.18±0.1 mg kg^{-1} dw (ZJ-QTJ) and 12.54±9.59 mg kg^{-1} dw (TJ-YDXH). NOx^--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 N_2 production

No significant accumulation of $^{15}N_2$-labeled gas ($^{29}N_2$ and/or $^{30}N_2$) was detected in any of the sample slurries amended with only $^{15}NH_4^+$ (Fig. S1A, Supplemental material), indicating that all residual $^{14}NO_x^-$ had been consumed during pre-incubation. When both $^{15}NH_4^+$ and $^{14}NO_3^-$ were added, only $^{29}N_2$ accumulated in each soil (Fig. S1B, Supplemental material), indicating that anammox must have been occurring. When amended with $^{14}NH_4^+$ and $^{15}NO_3^-$, significant production of both $^{29}N_2$ and $^{30}N_2$ were detected, as a result of both anammox and denitrification (Fig. S1C, Supplemental 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 rate 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 N_2 production was calculated based on these rates, with anammox contributing between 2.39 % (GX-FCJ) and 82.61% (ZJ-OJ) of total N_2 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 *hzs*B genes in the sediments was from $1.8\times10^5 \pm 3.4\times10^4$ copies g^{-1} dw to $3.6\times10^8 \pm 7.5\times10^7$ copies g^{-1} dw. The ratio of *hzs*B gene copies to total bacterial 16S rRNA gene copies ranged from 0.005% (ZJ-YJ) to 3.72% (GD-ZJ).

A total of 2,930 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 (4 Clusters, I-IV) (Fig. S2). The most abundant genus was *Candidatus Scalindua,* followed by *Candidatus Brocadia, Candidatus Kuenenia* and *Candidatus Jettenia* (Fig. S2). 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 (Fig. 2, Fig. 3). In contrast, the abundance of *Candidatus Brocadia* and *Candidatus Kuenenia* exhibited a general pattern which increased from north to south (Fig. 2, Fig.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), NO_3^- concentration (P<0.05), *hzsB* abundance (P<0.05), *hzsB/16S rRNA* (P<0.05), and salinity (P<0.05), whereas negatively correlated with DO (P<0.01). The *hzsB* abundance was positively correlated with salinity (P<0.01), NO_3^- concentration (P<0.05), and negatively with NH_4^+ 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-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, Fig. S1). *Candidatus Scalinduas* was detected at all sites and had the highest relative abundance (up to 43%) among all anammox genera. *Candidatus Scalinduas* 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 $1.80\times10^5 \pm 3.4\times10^4$ copies g^{-1} dw to $3.6\times10^8 \pm 7.5\times10^7$ copies g^{-1} 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 riparian sediments of the Pearl River Estuary in winter (Wang et al. 2012). 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. 2012), 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 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 et al. (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 4,000 kilometers 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 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; De 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 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 $^{29}N_2$ by reducing $^{45}N_2O$, which is produced by using $^{14}NH_4^+$ and $^{15}NO_3^-$/$^{15}NO_2^-$ 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 4,000 kilometers 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. 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.

Acknowledgements

This study was financially supported by the Natural Science Foundation of China (41401297, 41571130063), the China Postdoctoral Science Foundation (2014M551846) and the International Science & Technology Cooperation Program of China (2011DFB91710).

References

Brabandere LD, Canfield DE, Dalsgaard T, Friederich GE, Revsbech NP, Ulloa O, Thamdrup B (2014) Vertical partitioning of nitrogen-loss processes across the oxic-anoxic interface of an oceanic oxygen minimum zone. Environ Microbiol 16:3041-3054

Broda E (1977) Two kinds of lithotrophs missing in nature. Z Allg Mikrobiol 17: 491–493

Cao HL, Hong YG, Li M, Gu JD (2011) Diversity and abundance of ammonia-oxidizing prokaryotes in sediments from the coastal Pearl River estuary to the South China Sea. Anton. Leeuw. Int J G 100:545-556

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7(5):335-336

Cui SH, Shi YL, Groffman PM, Schlesinger WH, Zhu Y G (2013) Centennial-scale analysis of the creation and fate of reactive nitrogen in China (1910–2010). Proc Natl Acad Sci USA 110:2052–2057

Dale OR, Tobias CR, Song BK (2009) Biogeographical distribution of diverse anaerobic ammonium

oxidizing (anammox) bacteria in Cape Fear River Estuary. Environ Microbiol 11:1194-1207

Dang HY, Chen RP, Wang L, Guo LZ, Chen PP, Tang ZW, Tian F, Li SZ, Klotz M (2010) Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. Appl Environ Microbiol 76:7036-7047

Dang HY, Zhou HX, Zhang ZN, Yu ZS, Hua E, Liu CS, Jiao NZ (2013) Molecular Detection of Candidatus *Scalindua pacifica* and environmental responses of sediment anammox bacterial community in the Bohai Sea, China. PLoS ONE 8: e61330

Dang HY, Huang RF, Jiao NZ (2016) Inspirations from the scientific discovery of the anammox bacteria: A classic example of how scientific principles can guide discovery and development. Sci China Earth Sci 59:449-455

Deegan LA, Johnson DS, Warren RS, Peterson BJ, Fleeger JW, Fagherazzi S, Wollheim WM (2012) Coastal eutrophication as a driver of salt marsh loss. Nature 490:388–392

Engstrom P, Dalsgaard T, Hulth S, Aller RC (2005) Anaerobic ammonium oxidation by nitrite (anammox): Implications for N_2 production in coastal marine sediments. Geochim Cosmochim Ac 69:2057-2065

Etchebehere C, Tiedje J (2005) Presence of two different active nirS nitrite reductase genes in a denitrifying *Thauera* sp. from a high-nitrate-removal-rate reactor. Appl Environ Microbiol 71(9):5642-5645

Gori F, Tringe SG, Kartal B, Marchiori E, Machiori E, Jetten M (2011) The metagenomic basis of anammox metabolism in Candidatus' *Brocadia fulgida*'. Biochem Soc T 39(6):1799-1804

Hamersley MR, Woebken D, Boehrer B, Schultze M, Lavik G, Kuypers MMM (2009) Water column anammox and denitrification in a temperate permanently stratified lake (Lake Rassnitzer,Germany). Systematic Appl Microbiol 32:571-582

Hong YG, Xu XR, Kan JJ, Chen F (2014) Linking seasonal inorganic nitrogen shift to the dynamics of microbial communities in the Chesapeake Bay. Appl Microbiol Biotechnol 98:3219-3229

Hou LJ, Zheng YL, Liu M, Li XF, Lin, XB, Yin GY, Gao J, Deng FY, Chen F, Jiang XF (2015) Anaerobic ammonium oxidation and its contribution to nitrogen removal in China's coastal wetlands. Sci Rep 5:15621

Hu BL, Shen LD, Zheng P, Hu AH, Chen TT, Cai C, Liu S, Hou LP (2012) Distribution and diversity of anaerobic ammonium-oxidizing bacteria in the sediments of the Qiantang River. Environ

Microbiol Rep 4:540-547

Jetten MSM, Op den Camp HJM, Kuenen JG, Strous M (2010) Description of the order Brocadiales. In Krieg NR, Staley JT, Hedlund BP, Paster BJ, Ward N, Ludwig W, Whitman WB (ed.), Bergey's Manual of Systematic Bacteriology. Springer, Heidelberg, Germany

Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pommerening-Roser A, Koops HP, Wagner M (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl Environ Microb 64:3042-3051

Kartal B, Kuypers MMM, Lavik G, Schalk J, den Camp HJMO, Jetten MSM, Strous M (2007) Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. Environ Microbiol 9:635-642

Kartal B, Maalcke WJ, de Almeida NM, Cirpus I, Gloerich J, Geerts W, den Camp HJMO, Harhangi HR, Janssen-Megens EM, Francoijs KJ, Stunnenberg HG, Keltjens JT, Jetten MSM, Strous M (2011) Molecular mechanism of anaerobic ammonium oxidation. Nature 479:127-U159

Kartal B, van Niftrik L, Rattray J, de Vossenberg JLCMV, Schmid MC, Damste JSS, Jetten MSM, Strous M (2008) *Candidatus 'Brocadia fulgida'*: an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol Ecol 63:46-55

Kuypers MM, Lavik G, Woebken D, Schmid M, Fuchs BM, Amann R, Jørgensen BB, Jetten MS (2005) Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proc Natl Acad Sci USA 102(18):6478-6483

Li M, Hong YG, Cao HL, Gu JD (2011) Mangrove trees affect the community structure and distribution of anammox bacteria at an anthropogenic-polluted mangrove in the Pearl River Delta reflected by 16S rRNA and hydrazine oxidoreductase (HZO) encoding gene analyses. Ecotoxicology 20:1780-1790

Lisa J, Song B, Tobias CR, Duernberger KA (2014) Impacts of freshwater flushing on anammox community structure and activities in the New River Estuary, USA. Aquat Microb Ecol 72:17-31

Long A, Heitman J, Tobias C, Philips R, Song B(2013) Co-occurring anammox, denitrification, and codenitrification in agricultural soils. Appl Environ Microb 79:168-176

Malte K, Max W, Vanessa D, Martin W, Cordula AJ, Andreas SR, Rolf B, David M, Marie F, Wolfgang SS, Roland S (2015) Cell type specific gene expression analysis of prostate needle biopsies

410 resolves tumor tissue heterogeneity. Oncotarget 6:1302–1314
411 Metz S, Beisker W, Hartmann A, Schloter M (2003) Detection methods for the expression of the
412 dissimilatory copper-containing nitrite reductase gene (*DnirK*) in environmental samples. J
413 Microbiol Meth 55(1):41-50
414 Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation
415 discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol 16:177–184
416 Nie SA, Li H, Yang XR, Zhang ZJ, Weng BS, Huang FY, Zhu GB, Zhu YG (2015) Nitrogen loss by
417 anaerobic oxidation of ammonium in rice rhizosphere. ISME J 9:2059-2067
418 Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH (2008) Diversity of
419 ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox)
420 reactor. Environ Microbiol 10:3130-3139
421 Richard F A (1965) Anoxic basins and fjords. In: Ripley J P, Skirrow G, eds. Chemical Oceanography.
422 Manhattan: Academic Press. 1: 611–645
423 Risgaard-Petersen N, Meyer RL, Schmid M, Jetten MSM, Enrich-Prast A, Rysgaard S, Revsbech NP
424 (2004) Anaerobic ammonium oxidation in an estuarine sediment. Aquat Microb Ecol 36:293-304.
425 Rysgaard S, Glud RN, Risgaard-Petersen N, Dalsgaard T (2004) Denitrification and anammox activity
426 in Arctic marine sediments. Limnol Oceanogr 49(5):1493-1502
427 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB,
428 Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009)
429 Introducing mothur: Open-Source, Platform-independent, community-supported software for
430 describing and comparing microbial communities. Appl Environ Microbiol 75(23):7537-7541
431 Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer KH,
432 Wagner M (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing
433 anaerobic ammonium oxidation. Syst Appl Microbiol 23:93-106
434 Schmid M, Walsh K, Webb R, Rijpstra WIC, van de Pas-Schoonen K, Verbruggen MJ, Hill T, Moffett
435 B, Fuerst J, Schouten S, Damste JSS, Harris J, Shaw P, Jetten M, Strous M (2003) *Candidatus*
436 *"Scalindua brodae*", sp. nov., *Candidatus "Scalindua wagneri"*, sp. nov., two new species of
437 anaerobic ammonium oxidizing bacteria. Syst Appl Microbiol 26:529-538
438 Schmid MC, Maas B, Dapena A, de Pas-Schoonen KV, de Vossenberg JV, Kartal B, van Niftrik L,
439 Schmidt I, Cirpus I, Kuenen JG, Wagner M, Damste JSS, Kuypers M, Revsbech NP, Mendez R,

Jetten MSM, Strous M (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl Environ Microb 71:1677-1684

Schmid MC, Risgaard- Petersen N, Van De Vossenberg J, Kuypers MM, Lavik G, Petersen J, Hulth S, Thamdrup B, Canfield D, Dalsgaard T (2007) Anaerobic ammonium- oxidizing bacteria in marine environments: widespread occurrence but low diversity. Environ Microbiol 9(6):1476-1484

Schubert CJ, Durisch- Kaiser E, Wehrli B, Thamdrup B, Lam P, Kuypers MM (2006) Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika) Environ Microbiol 8(10):1857-1863

Seitzinger SP, Mayorga E, Bouwman AF, Kroeze C, Beusen AHW, Billen G, Van Drecht G, Dumont E, Fekete BM, Garnier J, Harriso JA (2010) Global river nutrient export: A scenario analysis of past and future trends. Glob Biogeochem Cycles 24: GB0A08

Shao SD, Luan XW, Dang HY, Zhou HX, Zhao YK, Liu HT, Zhang YB, Dai LQ, Ye Y, Klotz M (2014) Deep-sea methane seep sediments in the Okhotsk Sea sustain diverse and abundant anammox bacteria. FEMS Microbiol Ecol 87:503-516

Tamura KD, Peterson N, Peterson G, Stecher M, Nei, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10): 2731-2739

Thamdrup B, Dalsgaard T (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl Environ Microb 68:1312-1318

Trimmer M, Nicholls JC, Deflandre B (2003). Anaerobic ammonium oxidation measured in sediments along the Thames Estuary, United Kingdom. Appl Environ Microb 69:6447-6454

Trimmer M, Nicholls JC (2009) Production of nitrogen gas via anammox and denitrifcation in intact sediment cores along a continental shelf to slope transect in the North Atlantic. Limnol Oceanogr 54, 577–589

Wang S, Zhu G, Peng Y, Jetten MSM, Yin C (2012) Anammox bacterial abundance, activity, and contribution in riparian sediments of the Pearl River estuary. Environ Sci Technol 46:8834-8842

Wang Y, Zhu G, Harhangi HR, Zhu B, Jetten MSM, Yin C, Op den Camp HJM (2012) Co-occurrence and distribution of nitrite-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil. FEMS Microbiol Lett 336:79-88

Wenk CB, Zopfi J, Gardner WS, McCarthy MJ, Niemann H, Veronesi M, Lehmann MF (2014) Partitioning between benthic and pelagic nitrate reduction in the Lake Lugano south basin. Limnol Oceanogr 59:1421–1433

Yang XR, Li H, Nie SA, Su JQ, Weng BS, Zhu GB, Yao HY, Gilbert JA, Zhu YG (2015) Potential contribution of anammox to nitrogen loss from paddy soils in Southern China. Appl Environ Microbiol 81:938-947

Zhang Y, Ruan XH, Camp HJMOd, Smits TJ, Jetten M, Schmid M (2007) Diversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in freshwater sediments of the Xinyi River (China). Environ Microbiol 9:2375-2382

Zhu G, Wang S, Wang W, Wang Y, Zhou L, Jiang B, Camp HJMOd, Risgaard-Petersen N, Schwark L, Peng Y, Hefting MM, Jetten MSM, Yin C(2013) Hotspots of anaerobic ammonium oxidation at land–freshwater interfaces. Nat Geosci 6:103-107

Zhu G, Wang S, Wang Y, Wang C, Risgaard-Petersen N, Jetten MSM, Yin C (2011) Anaerobic ammonia oxidation in a fertilized paddy soil. ISME J 5:1905-1912

Zhu GB, Wang SY, Zhou LL., Wang Y, Zhao SY, Xia C, Wang WD, Zhou R, Wang CX, Jetten MSM, Hefting MM, Yin CQ, Qu JH (2015) Ubiquitous anaerobic ammonium oxidation in inland waters of China: an overlooked nitrous oxide mitigation process. Sci Rep 5:17306

Figure legends

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 *hzs*B 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.

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

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*, (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 correlation, respectively. *denotes a P value of <0.05 and **denotes a P value of <0.01. Tem=temperature; DO= dissolved oxygen; sal=salinity; TN=total nitrogen; TC=total carbon.

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.

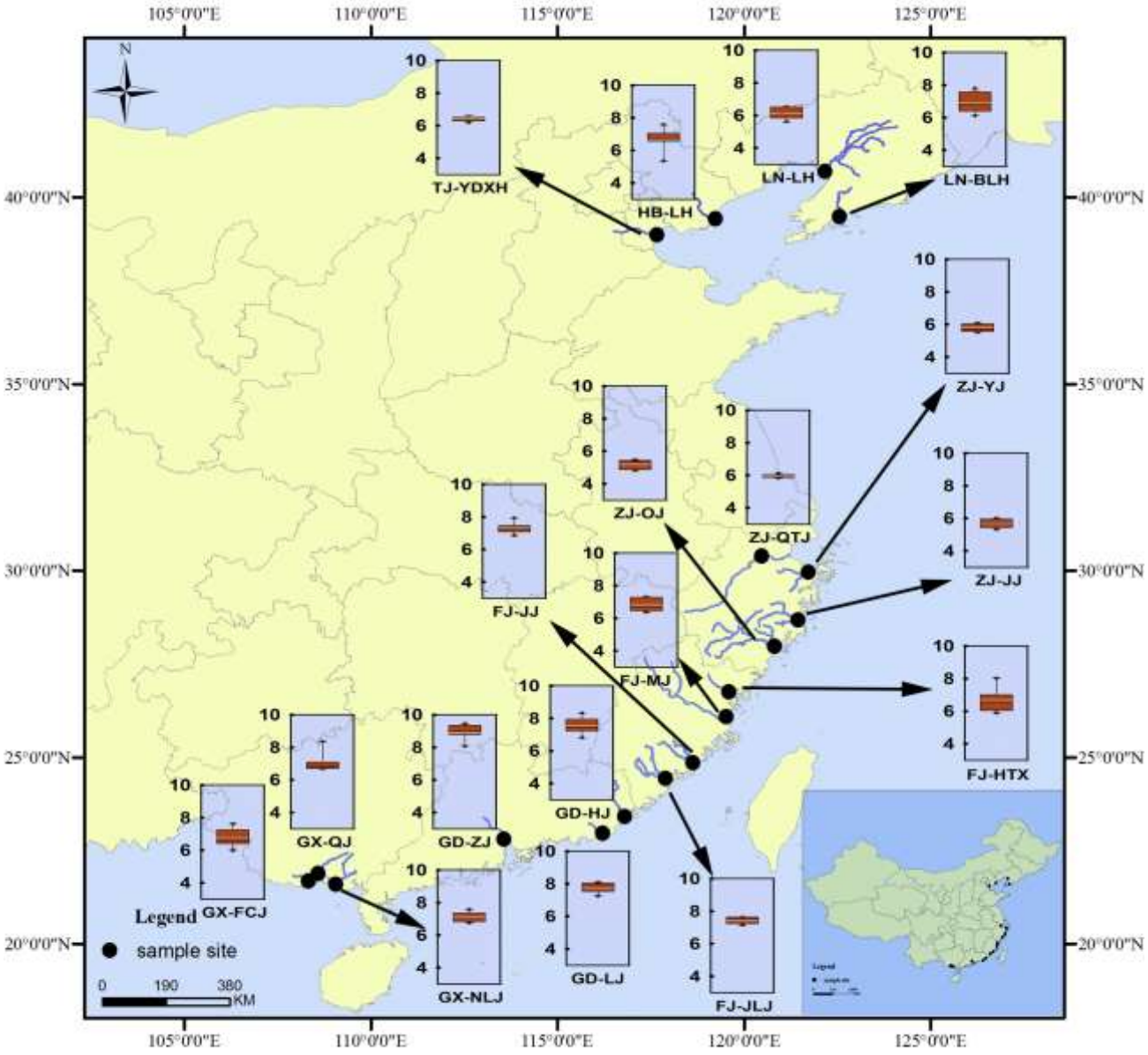

105°0'0"E
110°0'0"E
115°0'0"E
120°0'0"E
125°0'0"E
40°0'0"N
35°0'0"N
30°0'0"N
25°0'0"N
20°0'0"N
N
TJ-YDXH
HB-LH
LN-LH
LN-BLH
ZJ-YJ
ZJ-OJ
ZJ-QTJ
ZJ-JJ
FJ-JJ
FJ-MJ
FJ-HTX
GD-HJ
GD-ZJ
GD-LJ
GX-QJ
GX-FCJ
GX-NLJ
FJ-JLJ
Legend
sample site
0 190 380
KM

Fig.1

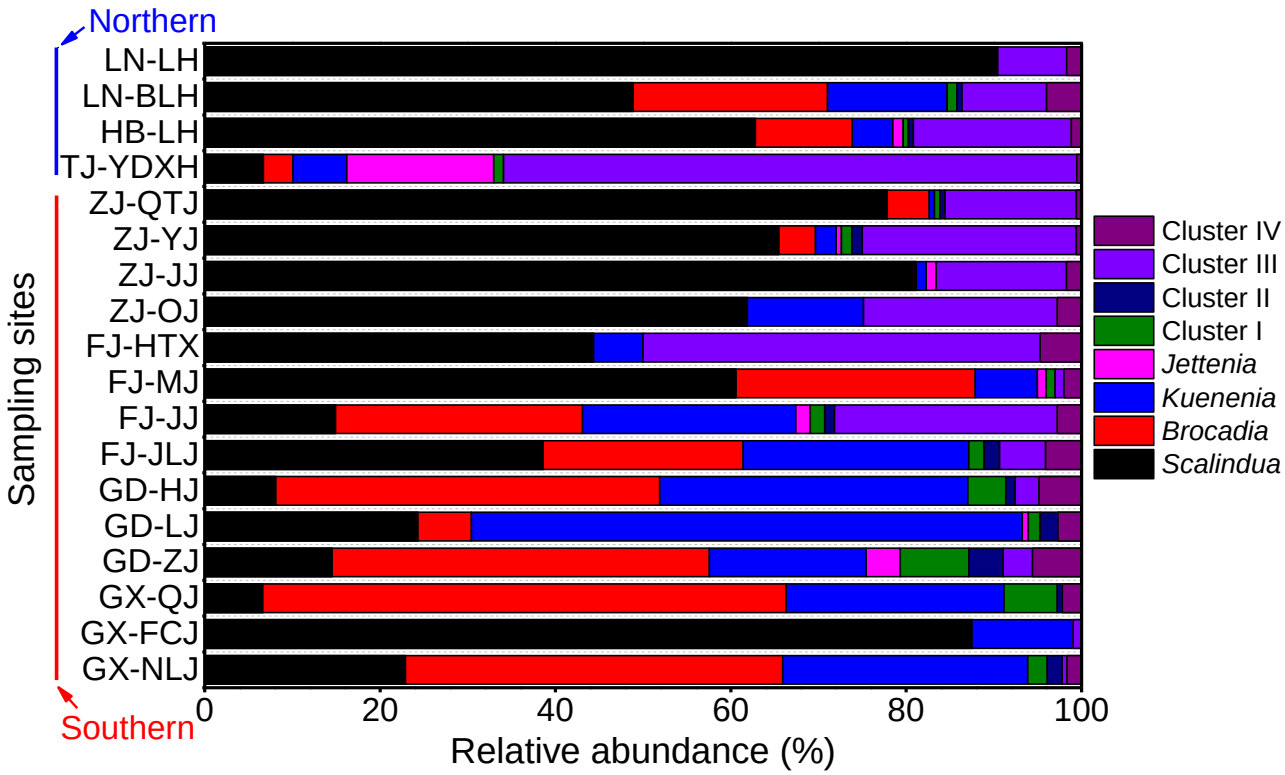

Northern
LN-LH
LN-BLH
HB-LH
TJ-YDXH
ZJ-QTJ
ZJ-YJ
ZJ-JJ
ZJ-OJ
FJ-HTX
FJ-MJ
FJ-JJ
FJ-JLJ
GD-HJ
GD-LJ
GD-ZJ
GX-QJ
GX-FCJ
GX-NLJ
Southern
Sampling sites
0
20
40
60
80
100
Relative abundance (%)
Cluster IV
Cluster III
Cluster II
Cluster I
Jettenia
Kuenenia
Brocadia
Scalindua

Fig. 2

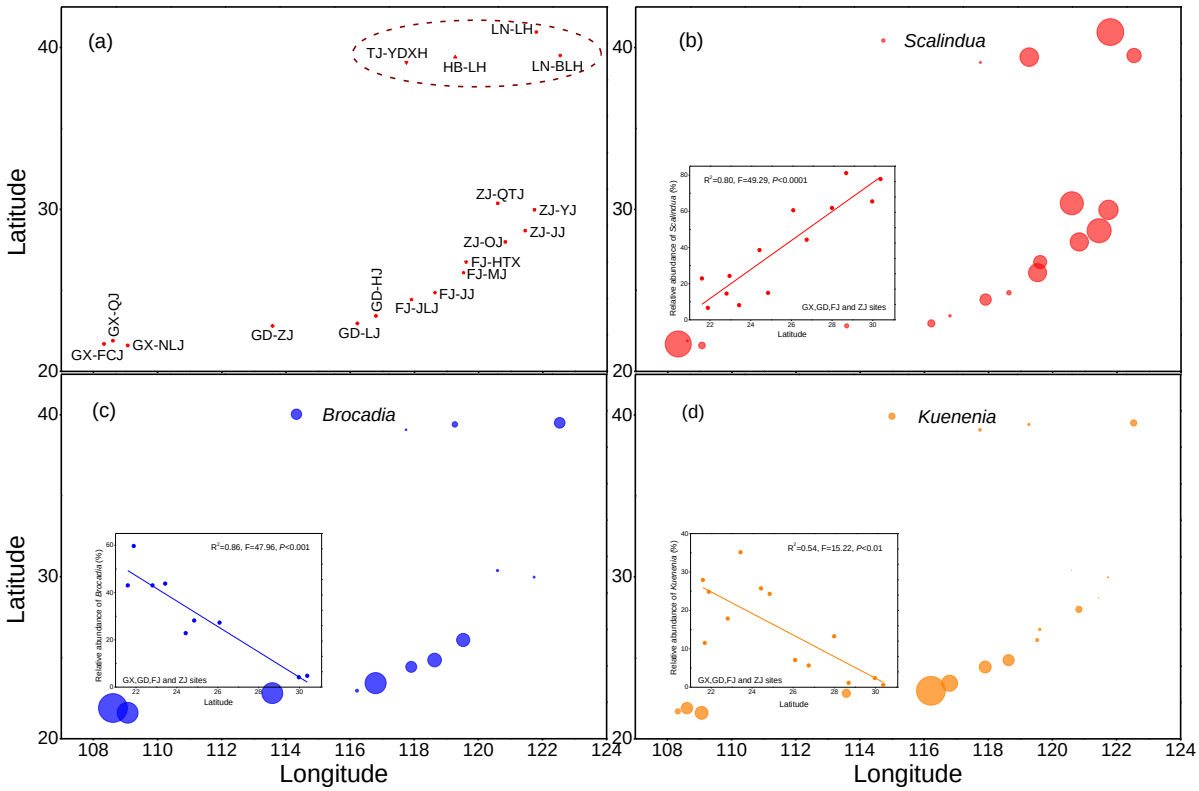

(a)
LN-LH
TJ-YDXH
HB-LH
LN-BLH
ZJ-QTJ
ZJ-YJ
ZJ-JJ
ZJ-OJ
FJ-HTX
FJ-MJ
FJ-JJ
FJ-JLJ
GD-HJ
GD-ZJ
GD-LJ
GX-QJ
GX-NLJ
GX-FCJ
(b)
Scalindua
R²=0.80, F=49.29, P<0.0001
Relative abundance of Scalindua (%)
GX,GD,FJ and ZJ sites
Latitude
(c)
Brocadia
R²=0.86, F=47.96, P<0.001
Relative abundance of Brocadia (%)
(d)
Kuenenia
R²=0.54, F=15.22, P<0.01
Relative abundance of Kuenenia (%)
Latitude
Longitude

Fig. 3

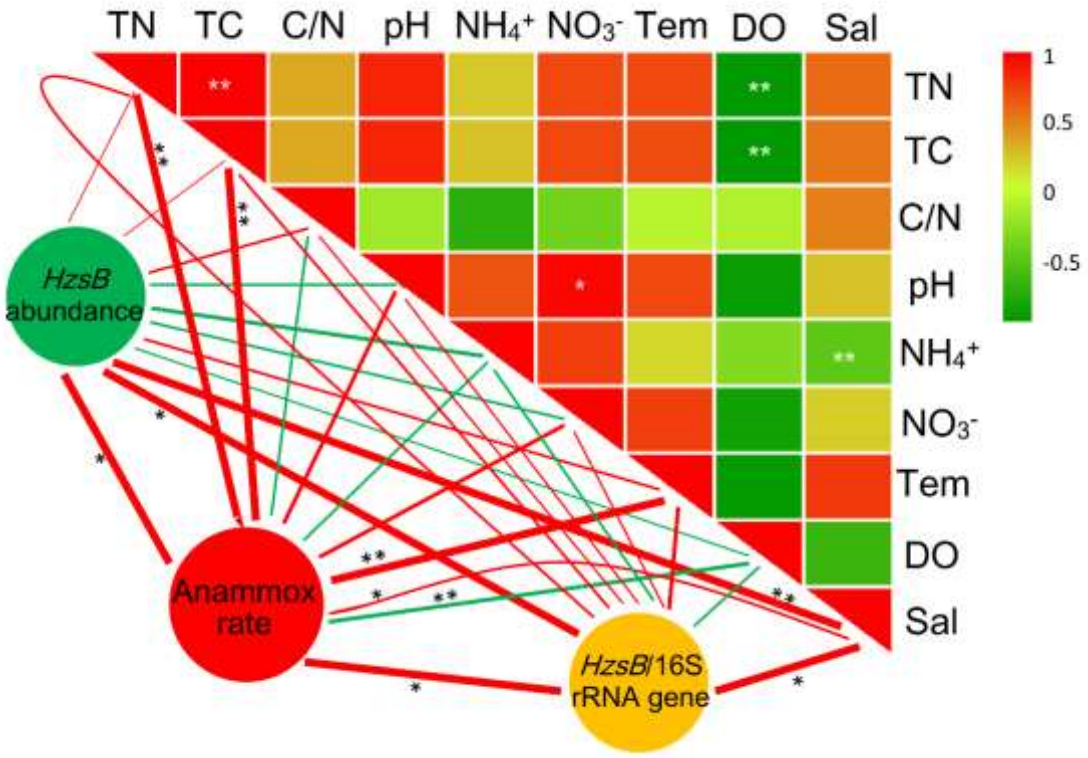
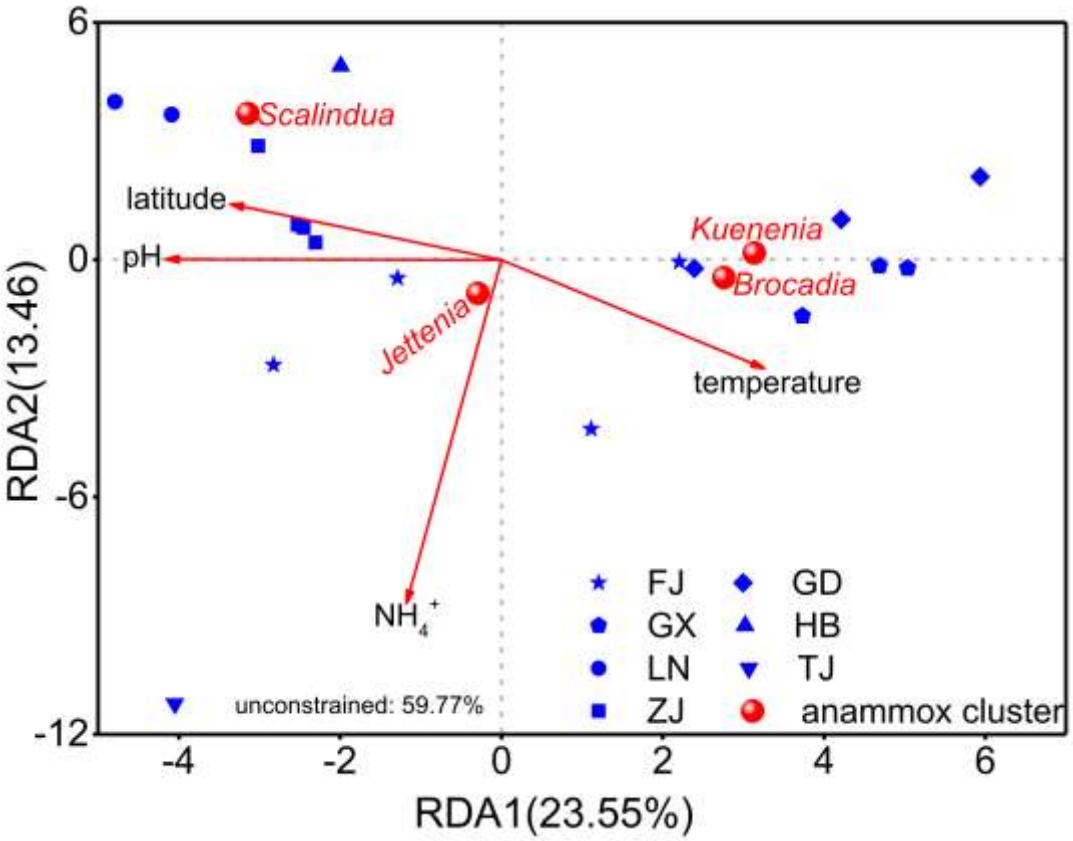

TN
TC
C/N
pH
NH4+
NO3-
Tem
DO
Sal
1
0.5
0
-0.5
HzsB abundance
Anammox rate
HzsB/16S rRNA gene

Fig. 4



572

573 **Fig. 5**

574

575 **Table 1** Rates of anammox and denitrification measured by ^{15}N tracing technique and the relative

576 contribution to total dinitrogen production

Sites	Anammox rate (nmol N g^{-1} dw h^{-1})	Denitrification rate (nmol N g^{-1} 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

577