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Nitrogen loss by anaerobic ammonium oxidation in a mangrove wetland of the Zhangjiang Estuary, China

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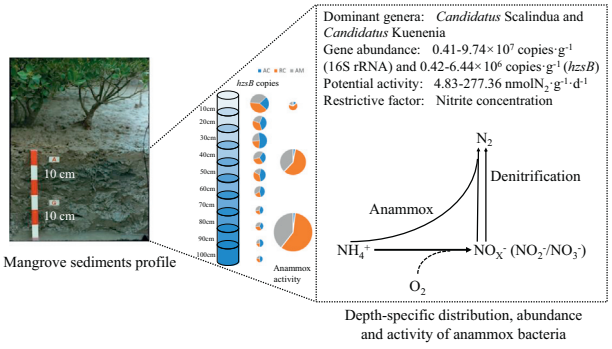
HIGHLIGHTS

- Anammox bacteria were widespread across mangrove sediment profiles.
- *Candidatus* Scalindua and *Candidatus* Kuenenia were the dominant anammox bacteria genera.
- Potential anammox rates were higher deep in the sediment profile.
- Nitrite concentrations limited anammox bacteria activity in mangrove sediments.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic ammonium oxidation (anammox), a microbial process in which NH_4^+ is oxidized to N_2 gas, is considered a significant nitrogen cycle process, but its significance in mangrove wetland sediments, particularly its depth- and genus-specific distribution and activity have remained uncertain. Here we report the vertical distribution, abundance, activity and role of anammox bacteria in mangrove sediments of Zhangjiang Estuary, China. We used stable isotope-tracer techniques, 16S rRNA and anammox bacterial functional gene (Hydrazine synthase B: *hzsB*) clone libraries and quantitative polymerase chain reaction (qPCR) assays, along with an assessment of nutrient profiles of sediment core samples. We observed a widespread occurrence of anammox bacteria at different depths of mangrove sediments. The abundance of anammox bacterial 16S rRNA and *hzsB* genes ranged from 0.41×10^7 to 9.74×10^7 and from 0.42×10^6 to 6.44×10^6 copies per gram of dry soil and peaked in the upper layer of mangrove sediments. We also verified the co-occurrence of different genera of anammox microorganisms in mangrove sediments, with *Candidatus* Scalindua and *Candidatus* Kuenenia being the dominant genera. Potential anammox rates ranged from 4.83 to 277.36 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$ at different depths of sediment cores, and the highest rates were found in the deeper layer (70–100 cm) of mangrove sediments. Scaling our findings up to the entire mangrove system, we estimated that anammox hotspots accounted for a loss of 751 $gN \cdot m^{-2} \cdot y^{-1}$, and contributed to over 12% of the nitrogen lost from the deeper layer of mangrove sediments in this region.

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1. Introduction

For decades, heterotrophic denitrification was considered the major nitrogen removal process in natural ecosystems (Ward et al., 2009; Li et al., 2015; Zhu et al., 2013; Kuypers et al., 2005). However, this concept has gradually changed by the discovery of anaerobic ammonium oxidation (anammox) (Strous et al., 1999), in which ammonium was oxidized to nitrogen gas with nitrite as an electron acceptor. The anammox process is performed by the phylum Planctomycetes (Strous et al., 1999), which are always found in anoxic environments and play a key role in the global nitrogen cycle. Some studies at local to regional indicated that this process accounted for as much as 13% of nitrogen gas production in freshwater lakes (Schubert et al., 2006) and over 50% in marine ecosystems (Kuypers et al., 2005). Lansdown et al. found that anammox could contribute over 50% of *in situ* N_2 production in riverbed sediments (Lansdown et al., 2016).

Thus far, phylogenetically different anammox bacteria, including the genera *Candidatus* Brocadia (Kartal et al., 2008), *Candidatus* Kuenenia (Schmid et al., 2000), *Candidatus* Scalindua (van de Vossenberg et al., 2013), *Candidatus* Anammoxoglobus (Kartal et al., 2007), *Candidatus* Anammoximicrobium (Khramenkov et al., 2013) and *Candidatus* Jettenia (Hu et al., 2012a), have been discovered in various ecosystems, and the geographic distributions of these anammox bacteria reveals that they have genus-specific or species-specific habitats. According to previous research, *Candidatus* Scalindua genera mainly exist in the marine environment (Hong et al., 2011; Borin et al., 2013), and only a small portion are distributed in freshwater habitats (Zhao et al., 2013; Shen et al., 2016), whereas the other five genera of anammox bacteria are mostly distributed in freshwater ecosystems (Wenk et al., 2013; Sun et al., 2014).

Until now, anammox bacteria have been detected in various marine ecosystems (Shao et al., 2014; Bale et al., 2014), freshwater ecosystems (Zhu et al., 2013; Bl et al., 2012), and man-made environments (Zhu et al., 2011). Nevertheless, the existence and significance of anammox bacteria in natural ecosystems remain to be investigated further. Additional studies in different natural habitats are also required to estimate the quantitative contribution of this process to promote nitrogen loss and reduce eutrophication.

It is well-known that mangrove wetlands are an important nitrogen sink, which can remove approximately 6% of anthropogenic nitrogen inputs to the environment (Jordan et al., 2011). Moreover, mangrove wetlands located in the land/ocean interface are periodically flooded by seawater, which could create an anaerobic environment in the sediment, and the anoxic conditions of the sediments could provide suitable habitats for anammox bacteria. In addition, the mangrove wetland ecosystem displays highly dynamic nitrogen cycling, and a significant vertical gradient of nutrients (like ammonium and nitrite) in mangrove sediments (Li et al., 2011a; Xiao et al., 2018; Zhang et al., 2018). Hence, the mangrove wetland system might act as a model system for studying the ecology of anammox bacteria. However, the published literature has mainly focused on the distribution and phylogenetic diversity of anammox bacteria (Schmid et al., 2000; Hong et al., 2011). Additionally, the depth-specific abundance, activity and relationship between environmental factors and the relative contributions of anammox bacteria to the nitrogen cycle in mangrove ecosystems have remained unclear. Moreover, previous studies of anammox bacteria in mangrove sediments have mainly concentrated on surface layers (Xiao et al., 2018; Li et al., 2011b; Han and Gu, 2015) (0–20 cm), and the situation for these organisms in deep layers (20–100 cm) has remained unknown. Consequently, the hotspots of anammox bacteria in mangrove sediments might be omitted, and their contributions in the mangrove ecosystem to nitrogen removal underestimated. To date, the role of anammox bacteria in mangrove wetlands is not well understood due to a lack of sufficient scientific data. Simultaneously, because anammox bacteria are strictly anaerobic microorganisms with a unique structure, the anammoxosome (de Almeida et al., 2015; Oshiki et al., 2016), in which enzymatic reactions of anammox such as hydrazine biosynthesis and dehydration take place, we propose the hypothesis that anammox bacteria are more active in deeper mangrove sediments.

The primary objective of the present study was to investigate the occurrence, biodiversity, activities and contributions of anammox bacteria to nitrogen loss in mangrove sediments using stable isotope measurements, quantitative PCR assays, and 16S rRNA and hydrazine synthase gene clone library analyses. The Yunxiao Mangrove National Nature Reserve (YMNNR) positioned at the Zhangjiang Estuary in Southeast China was chosen as the study site, where land uses in the upstream area include residential land, cropland and aquaculture ponds that produce domestic wastewater, agricultural runoff and aquaculture water (Wang et al., 2019), which must have important implications on ecological environments of mangrove sediments. We have had a lot of research work about microbial community biodiversity and function for more than a decade in YMNNR (Zhang et al., 2018). This study contributed to expanding our knowledge of the diversity and distribution of anammox bacteria in natural ecosystems and highlighted their potential contribution to nitrogen loss in mangrove ecosystems.

2. Materials and methods

2.1. Site background and sample collection

The study site is located at the YMNNR China (N 23°55′51.31″, E 117°24′50.57″), with a total area of 2360 hm^2 (Fig. 1). This area experiences a subtropical monsoon climate, with an annual mean air temperature of 21.2 °C and a mean annual rainfall of 1715 mm. The estuary has a semidiurnal tide with a large range (0.43–4.67 m). The habitats of the mangrove plants *Aegiceras corniculatum* (AC), *Rhizophora candel* (RC) and *Avicennia marina* (AM) were selected in our study because they are the dominant species in the YMNNR. Three habitats were distributed in different regions of the mangrove wetland with distinct soil hydrological condition. The *Aegiceras corniculatum* species has underdeveloped aerial roots and grows in silty sediment (0–1 m) near sewage drainage; The *Rhizophora candel* and *Avicennia marina* species both have better developed aerial roots and the sediment environment is mud at 0 to 50 cm and sandy at 50 to100 cm. The site background of three habitats was described previously (Zhang et al., 2018). Five cruises were conducted on July 8 (summer) and October 22 (autumn) 2015 and January 3 (winter), April 15 (spring) and July 13 (summer) 2016. In each habitat, three sediment cores were collected using a stainless steel ring sampler (HYDRO-BIOS, Germany). All sediment cores were sliced at 10-cm intervals and the same layer sediment samples in each habitat were mixed and divided into three parts. One part was incubated to conduct the stable isotope tracer experiments, another part was stored at 4 °C for later physicochemical analyses and the residual samples were stored at −80 °C for molecular experiments. All the samples were immediately transferred into a 4 °C cooler for transport to the laboratory.

2.2. Physical and chemical parameters analyses

The pH and temperature were measured *in situ* using a pH meter (Extech Instruments, A FLIR Company, USA). The redox potential of the sediment samples was determined *in situ* using an ORP meter (Extech Instruments, A FLIR Company, USA). The salinity of the sediment was estimated using a salinity meter (Bellingham-Stanley, UK). Methane gas was quantified using an Agilent 7890B gas chromatograph (Agilent Technologies, USA). Nitrite and nitrate were evaluated using a nutrient automatic analyzer AA3 (Bran-Luebbe, Germany), and ammonium was measured using Tir-223 (Bran-Luebbe, Germany) following the manufacturer's instructions. The total nitrogen content, total organic carbon concentration and total sulfur content were determined using a CN Elemental analyzer (Elementar, Germany). Methods to

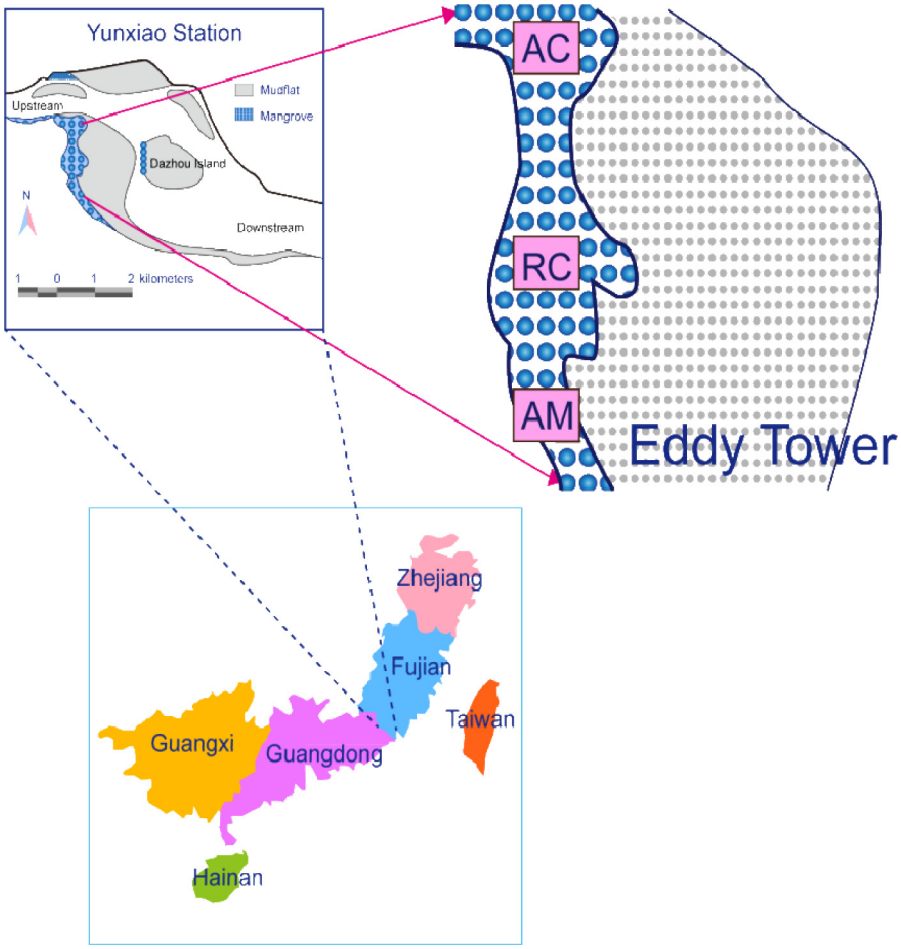

Fig. 1. Sampling sites located in the Zhangjiang Estuary mangrove wetland, Fujian Province, China. AC, RC, and AM represent sample sites of *Aegiceras corniculatum*, *Rhizophora candel* and *Avicennia marina* habitats in the mangrove wetland.

measure and calculate the water content, methane concentration, ammonium, nitrite and nitrate concentration, total nitrogen, total organic carbon and total sulfur concentration of mangrove sediments are detailed in the supplementary materials.

2.3. DNA extraction and PCR amplification

Sediment samples were grouped at 20 cm intervals for molecular analyses. DNA was extracted using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, California, USA) according to the manufacturer's instructions. A nested-PCR assay was conducted to detect anammox bacteria based on the 16S rRNA gene sequences. For the anammox bacteria functional gene *hzsB*, the hzsB396F-hzsB742R primer pair was used. The primers and corresponding reaction profiles are shown in Table S1.

2.4. Cloning, sequencing and phylogenetic analysis

The PCR-amplified anammox bacterial 16S rRNA gene products were cloned using the pMD19-T vector following the manufacturer's instructions. The sequences of the 16S rRNA and *hzsB* genes of the anammox bacteria were determined using Illumina high-throughput sequencing technology. Phylogenetic analysis of the recovered sequences was performed using MEGA 6.0 software (Tamura et al., 2013) and the neighbor-joining method. A bootstrap analysis with 1000 replicates was applied to estimate the confidence values of the tree nodes.

2.5. Quantitative real-time PCR

Quantitative PCR of anammox 16S rRNA and *hzsB* genes was performed to verify the abundance of anammox bacteria in different layers of mangrove sediments. Detailed experimental procedures are described in the supplementary materials. The primer sequences and corresponding reaction profiles are listed in Table S1.

2.6. ^{15}N tracer experiments

The potential anammox and denitrification rates were determined using ^{15}N tracer experiments (Risgaard-Petersen et al., 2003; Thamdrup and Dalsgaard, 2002). Sediments sampled from the three different habitats at 0–30, 30–70, and 70–100 cm depth were selected. The experiment included three treatment groups that received different amendments: $^{15}NH_4^+$ (a), $^{15}NH_4^+ + NO_2^-$ (b), and $^{15}NO_3^-$ (c). Three independent experiments were performed for each treatment. Treatment (a) was used to investigate whether the preincubation process had consumed the residual NOx^- and oxygen; treatment (b) was used to determine the potential rates of anammox bacteria; and treatment (c) was utilized to measure the potential activity of the denitrification process. The potential rates and contribution of each process to N_2 formation were calculated from the production of $^{29}N_2$ and $^{30}N_2$, measured using a Thermo Scientific Mat 253 (Hu et al., 2014). The production rates of $^{29}N_2$ and $^{30}N_2$ were acquired from the slopes of linear regressions of changes in the amount of $^{29}N_2$ and $^{30}N_2$ with incubation time. Detailed experimental procedures were described in supplementary materials.

2.7. Statistical analyses

Analysis of variance (ANOVA) followed by the Duncan test ($P < 0.05$) was used to check quantitative differences among groups. Operational taxonomic units (OTUs) for determining the 16S rRNA and *hzsB* genes diversity of anammox bacteria were defined using 3% and 5% differences in nucleotide sequences based on the Mothur program, respectively. R software was used to perform hierarchical cluster analysis, principal coordinate analysis (PCoA) and redundancy analysis (RDA) of the anammox bacteria using the 16S rRNA gene sequences. Pearson correlation analysis (significance level $\alpha = 0.05$) was used to test potential correlations between the anammox bacterial abundance, activity, diversity and different environmental factors using SPSS 19.0 software (Chicago, Illinois, USA).

2.8. Nucleotide sequence accession numbers

The sequences of anammox bacteria obtained in this study are available from the GenBank database under accession numbers KY967020-KY967052 for the anammox bacteria 16S rRNA gene and KY967018-KY967019 for the *hzsB* gene.

3. Results

3.1. Physiochemical profiles of mangrove sediments

The basic physiochemical parameters of the mangrove sediments sampled from different seasons are shown in Fig. S1. For ammonium and nitrite, the substrates for anammox bacteria, the highest concentrations (16.43 mg kg^{-1} for ammonium and 121.99 μg kg^{-1} for nitrite) were both present in the 0–20 cm layer (NH_4^+: *t*-test, $p<0.01$, $p = 0.002$, df = 8; NO_2^-: *t*-test, $p<0.01$, $p = 0.003$, df = 11). However, the maximum value of the ammonium concentration appeared in the *Aegiceras corniculatum* (AC) habitat in summer (U), and the highest nitrite concentration originated from the *Rhizophora candel* (RC) habitat in spring (S). Concurrently, the ammonium concentration varied from 1.41 to 16.43 mg kg^{-1} in four seasons (S: Spring, U: Summer, A: Autumn, W: Winter) of three habitats, whereas the nitrite concentration ranged from 21.12 to 121.99 μg kg^{-1}. Overall, the ammonium concentrations were almost 1–3 orders of magnitude higher than those of the nitrite concentration.

3.2. Diversity and phylogenetic analysis of anammox bacteria 16S rRNA and hzsB genes in mangrove sediments

To investigate the diversity and phylogeny of anammox bacteria, the anammox 16S rRNA and *hzsB* gene sequences were obtained from different types of mangrove sediments. A total of 33 operational taxonomic units (OTUs) (97% cutoff) were obtained from mangrove sediments (Table S2), and the phylogenetic analysis showed that these OTUs were all clustered into three different anammox genera, including *Candidatus* Brocadia, *Candidatus* Kuenenia and *Candidatus* Scalindua. Furthermore, some OTUs fell into a new group associated with planctomycetes (Fig. 2). The *Candidatus* Brocadia genus contained 2 OTUs and 2388 sequences of the anammox 16S rRNA gene, *Candidatus* Kuenenia included 7 OTUs and 372,197 sequences, and *Candidatus* Scalindua embodied 10 OTUs and 53,832 sequences. Based on the retrieved anammox 16S rRNA gene sequences, *Candidatus* Kuenenia and *Candidatus* Scalindua were the dominant genera in mangrove sediments.

The phylogenetic tree of anammox *hzsB* gene was constructed from five representative depths (0–20, 20–40, 40–60, 60–80 and 80–100 cm) of the mangrove sediments cores (Fig. 3). The 63,789 sequences of anammox *hzsB* gene were retrieved and affiliated with the *Candidatus* Kuenenia and *Candidatus* Scalindua genera. Simultaneously, these sequences were assigned to 2 OTUs (95% cutoff). 1 OTU and 61,533 sequences of anammox *hzsB* gene belonged to *Candidatus* Kuenenia genus, 1 OTU and 2253 sequences belonged to *Candidatus* Scalindua genus.

3.3. Community structure variations of anammox bacteria in mangrove sediments

To elucidate the community structure of anammox bacteria in mangrove sediments, the hierarchical cluster and principal coordinates analysis (PCoA) based on anammox 16S rRNA gene sequences were constructed, and anammox bacteria could be assigned to three groups (Fig. 4a and b). Samples AC1, RC1 and AM1 (1: 0–20 cm) showed a similar 16S rRNA gene community structure, whereas AC2, AC3, RC2, RC3, AM2 and AM3 (2: 20–40 cm, 3: 40–60 cm) shared a similar community structure. Moreover, samples AC4, AC5, RC4, RC5, AM4 and AM5 (4: 60–80 cm, 5: 80–100 cm) had the similar community structure. These results coincided with the stratification of mangrove sediments (0–30 cm, 30–70 cm and 70–100 cm), which was utilized to conduct the rate analysis of anammox bacteria.

According to the redundancy analysis (RDA) using anammox 16S rRNA gene sequences, the sediment pH, potential redox (Eh), water content (WC), nitrite concentration, nitrate concentration, total nitrogen (TN) and total organic carbon (TOC) appeared to have the most pivotal influence on the spatial variation of anammox community structures in the examined samples among the investigated environmental factors (Fig. 4c).

To elucidate the analogous community structure of anammox bacteria between this study site and other habitats, PCoA analyses were carried out based on anammox 16S rRNA gene sequences. The results demonstrated that the community structure of anammox bacteria in mangrove sediments was most similar to that of the bay sediments (Rich et al., 2008), while there were some similarities with the rhizosphere soils (Li et al., 2015) and Taihu sediments (Wu et al., 2012) (Fig. 4d). The bay habitat was located at the boundary of the sea and land, similar to the situation of the mangrove wetland. This feature may explain the high community structure similarity of anammox bacteria in these two environments.

3.4. Abundance of anammox bacteria in mangrove sediments

The quantitative PCR results for the anammox bacteria 16S rRNA and *hzsB* genes further confirmed the occurrence of anammox bacteria in different depths of the examined mangrove sediments. The copy numbers of anammox bacteria 16S rRNA genes varied from 1.90×10^7 to 5.49×10^7, 1.55×10^7 to 9.32×10^7 and 1.39×10^7 to 9.74×10^7 copies per gram of dry soil in the AC, RC and AM habitats, respectively (Fig. 5a), whereas the abundance of *hzsB* genes in the three sampling sites (AC, RC and AM) ranged from 0.80×10^6 to 5.54×10^6, 0.50×10^6 to 6.44×10^6 and 0.42×10^6 to 6.05×10^6 copies per gram of dry soil, respectively (Fig. 5b). The abundance of *hzsB* genes displayed the same trend as the 16S rRNA genes. However, the anammox bacterial abundance was observed over a wide range with high spatial heterogeneity, and it was much higher in upper compared with deeper layers (*t*-test, $p<0.01$, $p = 0.002$, df = 19).

3.5. Depth-specific significance of anammox in mangrove sediments

To measure the anammox rate and contribution to nitrogen loss, laboratory incubations were performed with homogenized sediment using a ^{15}N isotope-tracing technique in four seasons. The results showed that, in the sediment samples amended with $^{15}NH_4^+$ alone, no significant accumulation of ^{15}N-labeled gas ($^{29}N_2$ and $^{30}N_2$) could be detected, suggesting that the oxidizing substance had been consumed during the preincubation period (Fig. S2a). When both $^{15}NH_4^+$ and $^{14}NO_2^-$ were added, $^{29}N_2$ but not $^{30}N_2$ was detected, indicating the presence of the anammox process (Fig. S2b). However, $^{29}N_2$ and $^{30}N_2$ were all detected

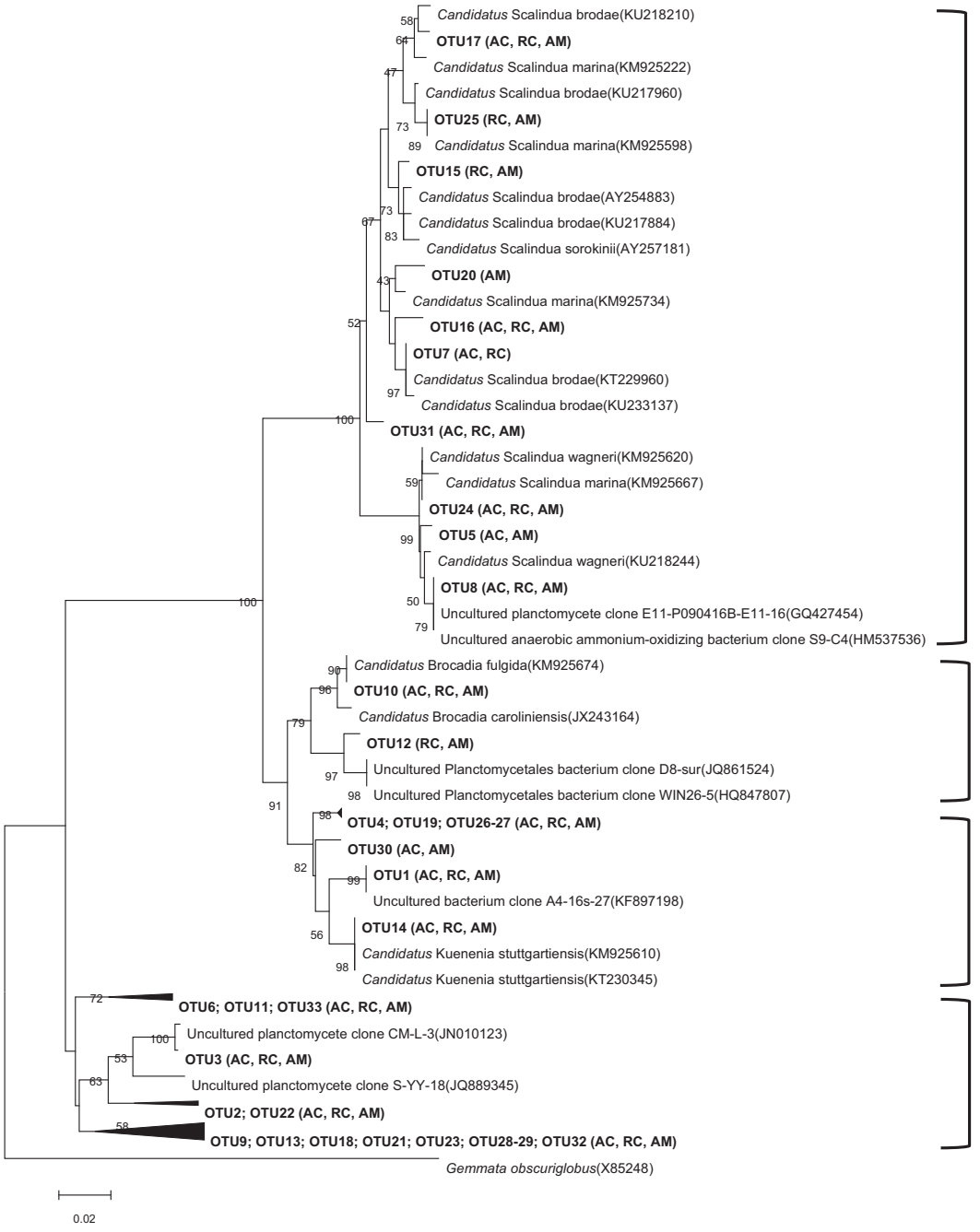

Fig. 2. Phylogenetic tree reconstructed with the distance and neighbor-joining methods of the anammox 16S rRNA gene sequences recovered from the *Aegiceras corniculatum* (AC), *Rhizophora candel* (RC) and *Avicennia marina* (AM) habitats in the mangrove wetland. Bootstrap values for 1000 replicates are shown at the branch points. The scale bar represents 2% estimated sequence divergence.

in the incubations amended with $^{15}NO_3^-$ alone, suggesting the co-occurrence of anammox and denitrification processes (Fig. S2c). The potential anammox rates varied from 4.83 to 93.88, 7.57 to 277.36, and 12.83 to 258.51 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$ in the AC, RC and AM habitats, respectively (Fig. 6a, b and c). The potential anammox activity in the RC and AM habitats was much higher than that in the AC habitat (*t*-test, $p<0.01$, $p = 0.001$, $df = 7$). Concurrently, the rates of anammox bacteria clearly increased between 0 and 30 cm and 30–70 cm (RC: *t*-test, $p<0.05$, $p = 0.016$, $df = 5$; AM: *t*-test, $p<0.05$, $p = 0.026$, $df = 6$) in the RC and AM habitats. Above all, the spatiotemporal analysis showed that mangrove wetlands provided a more favorable habitat for anammox bacteria, and the deeper layers of mangrove sediments contained a hotspot of the anammox process. In the meantime, a similar trend was observed for the denitrification rates in mangrove sediments in summer. The potential denitrification rates ranged from 191.66 to 343.29, 572.64 to 890.88, and 464.39 to 799.08 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$ in the AC, RC and AM habitats, respectively (Fig. 6d). Obviously, the anammox were much lower than the denitrification rates. Additionally, anammox contributed approximately 1.61%–16.70% of the nitrogen gas produced, while the remainder was produced by denitrification bacteria, indicating that denitrification was still the main contributor of nitrogen gas production in mangrove sediments.

The potential contribution of anammox in mangrove sediments was investigated with respect to nitrogen removal. On the basis of the anammox rates and sediment density data (2.65 g cm^{-3}), the nitrogen gas flux caused by anammox bacteria was calculated. The flux varied

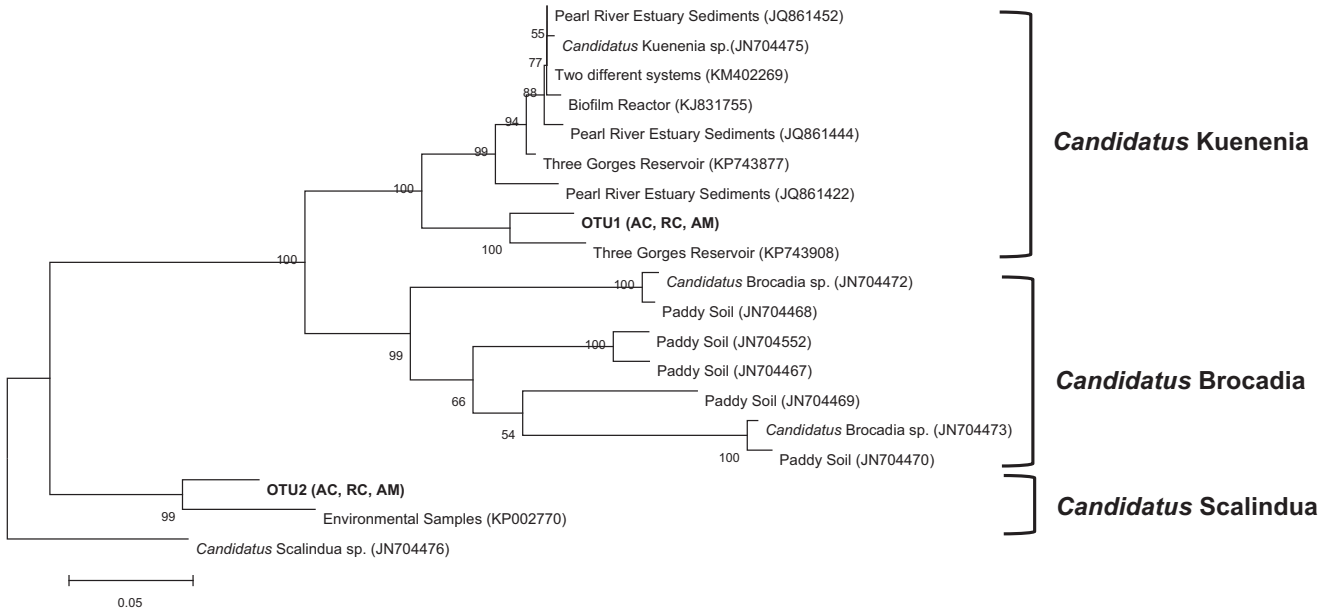

Fig. 3. Neighbor-joining phylogenetic tree showing the phylogenetic affiliations of anammox *hzsB* gene sequences in sediment cores collected from the three habitats (*Aegiceras corniculatum* (AC), *Rhizophora candel* (RC) and *Avicennia marina* (AM)) in the mangrove wetland. Bootstrap values based on 1000 replicates are shown at the branch points. The scale bar represents 5% of the sequence divergence.

from 13.09 to 254.26, 20.51 to 751.17, and 34.74 to 700.11 $gN \cdot m^{-2} \cdot y^{-1}$ in the AC, RC and AM habitats, respectively (Fig. S3a, S3b and S3c). To conservatively estimate the ecological benefits mediated by anammox process in the mangrove wetland ecosystem, the minimum activity of anammox bacteria was selected to conduct the analysis. It was discovered that the lowest rate of anammox bacteria was 4.83 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$ in the 30–70 cm layer of the AC habitat in winter and the nitrogen gas flux was 13.09 $gN \cdot m^{-2} \cdot y^{-1}$. Furthermore, the intensity of the exogenous nitrogen load in wetland ecosystems varied greatly; the range was 2.0×10^{-3}-9.0×10^{3} $gN \cdot m^{-2} \cdot y^{-1}$, and the average exogenous nitrogen load was 241.2 $gN \cdot m^{-2} \cdot y^{-1}$ (Jordan et al., 2011). Therefore, the contribution rate of anammox bacteria in mangrove wetland to exogenous nitrogen load was >5.4%. Nevertheless, it should be noted that sediment samples were only collected at low tide and the overall nitrogen fluxes with the atmosphere were expected to be constrained by diffusion limitations during high tide, which was likely important in macro-tidal regions. As the nitrogen flux estimates assumed low tide conditions 24 h per day over 365 days per year, it was possible that rates could be overestimated.

3.6. Correlations between the anammox bacteria process and environmental factors in mangrove sediments

To clarify potentially influential factors in the anammox process, statistical analyses were performed between anammox bacterial abundance, activity and diversity, and different environmental factors using SPSS (Statistical Package for the Social Sciences). Among the various physicochemical variables, the Eh, nitrite concentration and TOC significantly correlated with the diversity of anammox bacteria ($p < 0.01$) (Table S3). Notably, the WC, nitrate concentration and total nitrogen concentration (TN) showed a significant correlation with the abundance of anammox bacteria ($p < 0.01$) (Table S4). In addition, the TOC and TN had an important impact on the rate of anammox bacteria ($p < 0.05$) (Table S4).

4. Discussion

To the best of our knowledge, this was the first study to investigate the depth-specific physiochemical profiles of YMNNR sediments in four seasons. It was also a systematic study to explore the diversity of anammox bacteria and to quantify the number of anammox 16S rRNA and *hzsB* genes in mangrove sediments. Concurrently, ^{15}N stable isotope tracer experiments were used to determine the depth-specific rates of anammox bacteria in mangrove sediments. The results would improve our understanding of the nitrogen cycle in the mangrove ecosystem.

In the current study, the anammox communities were closely related to three genera, *Candidatus* Brocadia, *Candidatus* Kuenenia and *Candidatus* Scalindua, based on analyses of the anammox 16S rRNA genes. *Candidatus* Kuenenia and *Candidatus* Scalindua were the dominant genera, in accordance with a study in the Yangtze Estuary sediments, China (Hou et al., 2013). Interestingly, our results showed that *Candidatus* Kuenenia was mainly distributed in surface sediment layers (0–40 cm), while *Candidatus* Scalindua occurred in deeper layers of mangrove sediments (40–100 cm). Hence, it might be reasonable to draw the conclusion that the *Candidatus* Scalindua genera made up the main microorganisms with anammox activity in mangrove sediments. However, it was remarkable that the *Candidatus* Scalindua genera was mainly distributed in marine habitats (Hong et al., 2011; Borin et al., 2013) and the *Candidatus* Kuenenia genera was mostly discovered in freshwater environments (Wenk et al., 2013; Sun et al., 2014), reflecting the particularity of the mangrove wetland, connecting the land and sea. Additionally, some features can be proposed to explain why these two genera were the dominant microorganisms in mangrove sediments. A recent physiological study has revealed that the abundance of *Candidatus* Scalindua 16S rRNA gene sequences increases with increasing salinity (Dale et al., 2009), and when the salt concentration is lower than 1.5% (*w*/*v*), *Candidatus* Scalindua cells lose anammox activity, indicating that they are halophilic bacteria (Awata et al., 2013). In contrast, most research has reported that some members of *Candidatus* Scalindua can survive under low salinity conditions (Wang, 2013; Hendrickx et al., 2014). In addition, *Candidatus* Kuenenia has also been detected in saline environments (Mohamed et al., 2010). In this study, the salinity of mangrove sediments varied from 10‰ to 30‰, which represented a higher level. However, research has suggested that TN and TOC have an important impact on the community structure of anammox bacteria in upland-cropping soil (Hui et al., 2017), whereas another study has shown that plant species of mangrove have a great influence on the distribution of anammox bacteria

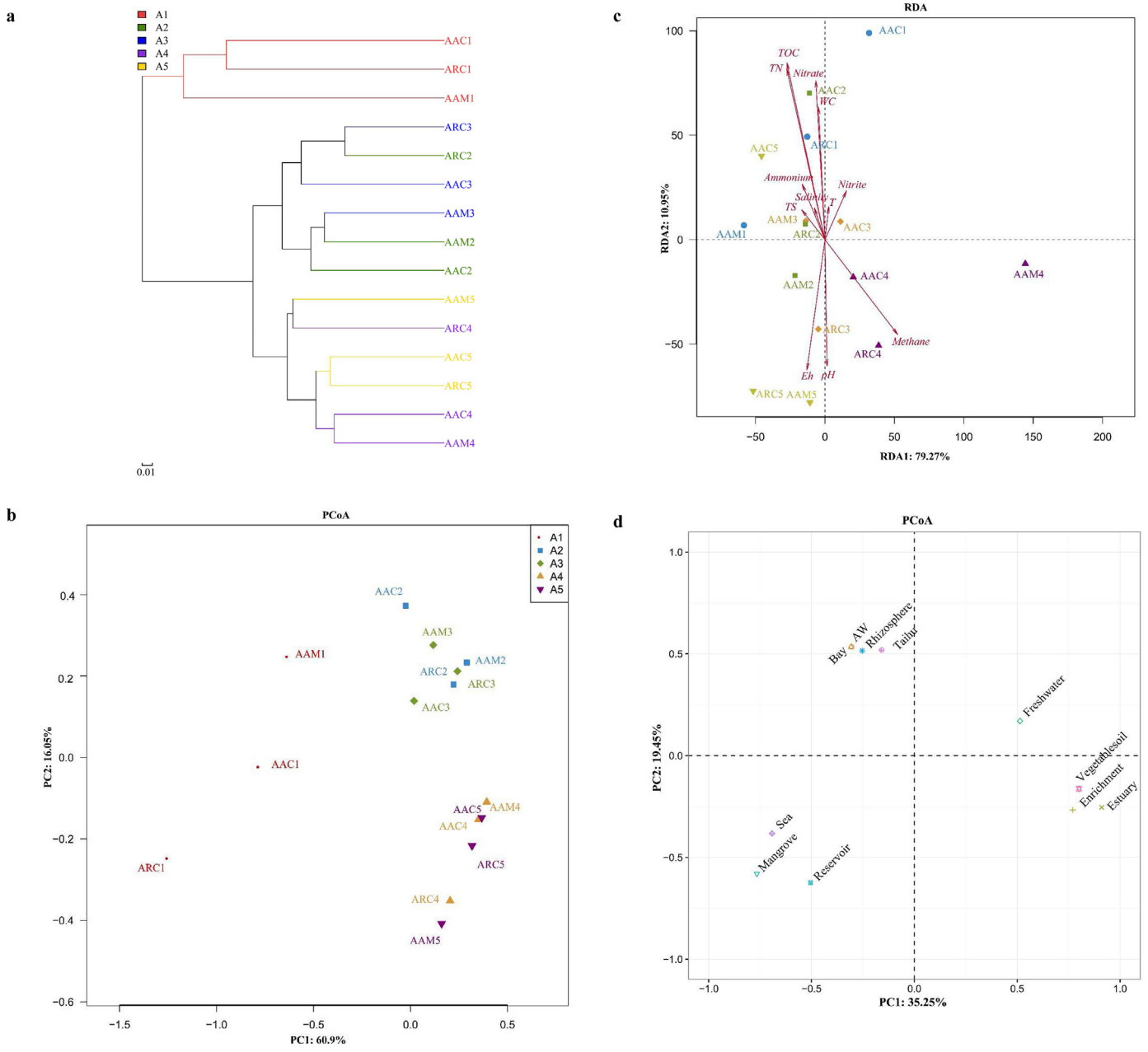

Fig. 4. Community structure variations of anammox bacteria in mangrove sediments. (a), Hierarchical cluster analysis based on the unweighted unifrac metric of anammox bacteria diversity using 16S rRNA gene amplified sequences. The hierarchical clustering method was average. (b), Ordination diagram of the unweighted unifrac normalized PCoA analysis of the anammox bacterial communities using the 16S rRNA gene sequences. (c), Redundancy analysis (RDA) of the physicochemical parameters and anammox bacteria community using the 16S rRNA gene sequences. The calculation method was the Euclidean distance metric. (d), PCoA ordination diagram of anammox bacteria assemblages calculated using the 16S rRNA gene sequences from mangrove sediments and other habitats. The calculation method was the unweighted unifrac metric. AW represented the anammox bacterial 16S rRNA gene sequences in mangrove sediments.

(Li et al., 2011b). Our work revealed that Eh, WC and nitrite concentrations had a prominent influence on the spatial variation of anammox community structures in a mangrove wetland. The redox potential could indicate the oxygen concentration in the habitat and that the anammox bacteria are strictly anaerobic microbes (Oshiki et al., 2016), Hence, Eh may have a great impact on these bacteria. It is well-known that when the water content rises, the dissolved oxygen in the water will increase, demonstrating that, like Eh, WC might greatly influence the occurrence of the anammox process in mangrove wetlands. Moreover, nitrite can be utilized as a substrate for anammox bacteria, and the nitrite concentration is very low in mangrove sediments. Therefore, the community structures of anammox bacteria would demonstrate a vital effect from nitrite.

Hydrazine synthase B is a key gene in the metabolic process of anammox bacteria and is responsible for converting ammonium and nitric oxide to hydrazine in anammoxosome (Van Niftrik et al., 2010). Hence, the diversity of the anammox *hzsB* gene was investigated in mangrove sediments, and only 2 OTUs were discovered. One OTU belonged to *Candidatus* Kuenenia, and the other OTU was affiliated with *Candidatus* Scalindua. Nevertheless, some studies have also promulgated the diversity of the anammox *hzsB* gene in different habitats. The sequences of *hzsB* genes have been detected in the anammox reactor system (Meng et al., 2014) and unconfined aquifer soils (Wang et al., 2017), and they can be classified into 7 and 10 OTUs, respectively. Furthermore, a relatively higher diversity of the *hzsB* genes has been reported in upland-cropping soil (Hui et al., 2017), for which a total of

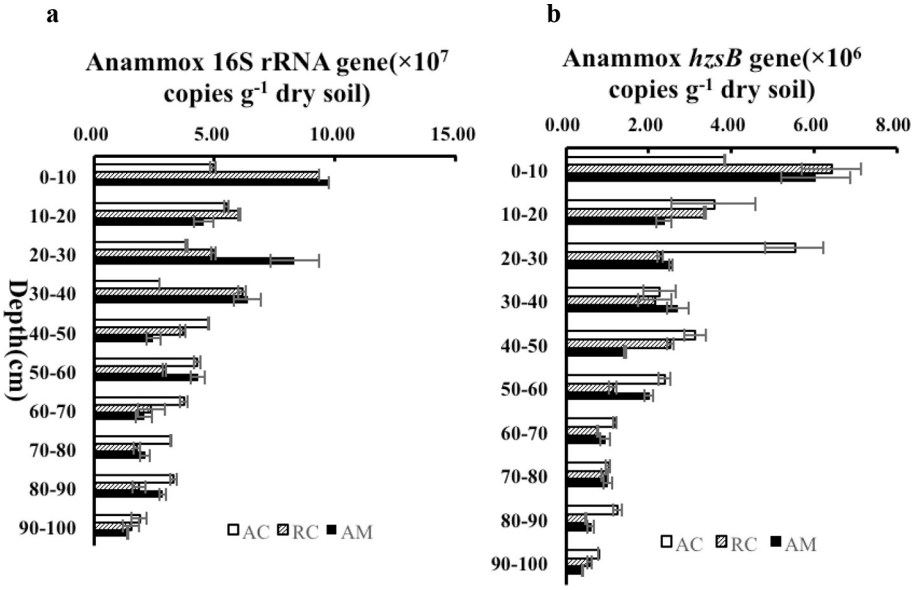

Fig. 5. Spatiotemporal distribution patterns of anammox bacterial abundance in sediment cores collected from *Aegiceras corniculatum* (AC), *Rhizophora candel* (RC) and *Avicennia marina* (AM) habitats in the mangrove wetland. (a), The copy number of the anammox bacteria 16S rRNA gene in AC, RC and AM habitats. (b), Abundance of *hzsB* genes in the three sampling sites. Error bars indicate the standard deviation ($n = 3$).

55 OTUs were discovered. Thus, the diversity of the anammox *hzsB* gene in the examined wetland is clearly lower than that detected in other environments.

The qPCR assays revealed the widespread occurrence of anammox bacteria in the mangrove wetland. The abundance of the 16S rRNA gene ranged from 1.39×10^7 to 9.74×10^7 copies per gram of dry soil, which was higher than the values reported in paddy soil (Wang et al., 2012a) (6.5×10^3–7.5×10^4 copies g^{-1} ds) but equivalent to unconfined aquifer soils (Wang et al., 2017) (0.75×10^7–1.40×10^7 copies g^{-1} ds). The quantification of *hzsB* genes varied from 0.42×10^6 to 6.44×10^6 copies per gram of dry soil, which was much higher than that in vegetable field (Shen et al., 2017) and upland-cropping soil (Hui et al., 2017) (3.16×10^3–1.10×10^6 copies g^{-1} ds). However, some researchers have reported a higher abundance of *hzsB* genes in Pearl River estuary sediments (Wang et al., 2012b), with abundances ranging from 1.4×10^8 to 2.0×10^9 copies per gram of dry soil. Furthermore, the vertical distribution patterns of the anammox bacteria abundance was also surveyed in the present study. The abundance of anammox 16S rRNA and *hzsB* genes were all decreased with the depth of the mangrove sediments, indicating that the upper layers were the preferred habitats for the growth of anammox bacteria.

The occurrence of anammox bacteria in the examined sediment cores was confirmed based on our stable isotope experiments. The potential anammox rates ranged from 4.83 to 277.36 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$ in our study, which was about one or two orders of magnitude higher than that in riparian sediments of Pearl River Estuary and marsh sediments of the Yangtze Estuary (0.84–31.20 $nmolN_2 \cdot g^{-1} \cdot d^{-1}$) (Hou et al., 2013; Wang et al., 2012b), suggesting that mangrove wetlands could provide favorable conditions for the metabolism of anammox bacteria. Some researchers have also reported increased activity of anammox bacteria in land-freshwater interfaces of Baiyangdian Lake (Zhu et al., 2013) (84–240 nmol $N_2 \cdot g^{-1} \cdot d^{-1}$), which were similar to the values investigated in this study. However, it is noteworthy that the potential anammox rates might overestimate the actual *in situ* activity because ammonium and nitrite may limit the *in situ* process. The *in situ* average concentrations of ammonium and nitrite were 5–8 $mg \cdot kg^{-1}$ and 0.05 $mg \cdot kg^{-1}$, but these two physicochemical parameters used as substrates for anammox bacteria were 7.2 $mg \cdot kg^{-1}$ and 19 $mg \cdot kg^{-1}$ in the stable isotope tracer experiment, which suggested that the ammonium concentrations were similar in our study and the *in situ* environment, but the nitrite concentrations were two orders of magnitude higher in the experiment than that in the mangrove sediments. Moreover, the nitrite concentration did not reach the threshold value to inhibit the growth of anammox bacteria in the experiment. In contrast, the nitrite concentration might promote the metabolism of anammox bacteria. Nevertheless, anammox bacteria showed a high affinity for nitrite when ammonium was supplied in excess (Oshiki et al., 2016; Shen et al., 2015; Luesken et al., 2011; Haroon et al., 2013); hence, the anammox activity could not be seriously overestimated. In addition, the anammox bacteria are clearly obligate anaerobes (Strous et al., 1999), which metabolize in a specialized structure-the anammoxosome. The membrane surrounding the anammoxosome in anammox bacteria contains unique lipids, termed ladderane lipids, which consist of linearly concatenated cyclobutane moieties (Rattray et al., 2008).

In our research, the redox potential indicating the concentration of dissolved oxygen in mangrove sediments varied from −126 to −31 mV, which was much lower than that in the paddy field and Xiazhuhu wetland (Hu et al., 2014), hinting that mangrove sediments are in a reducing state. It is worth noting that the quantity and activity of anammox bacteria in mangrove sediments varied inconsistently with increasing depth. The quantity of anammox bacteria showed a decreasing trend whereas the activity of anammox bacteria showed an upward trend with increasing depth. Additionally, the depth-specific distribution of *Candidatus* Kuenenia and *Candidatus* Scalindua could be used to explain this phenomenon in mangrove sediments, and a more detailed mechanism requires further study. Simultaneously, a majority of studies have also observed the same consequence in different habitats, such as Pearl River estuary sediments (Wang et al., 2012b), unconfined aquifer soils (Wang et al., 2017), rice rhizosphere (Li et al., 2015) and so on.

The microbial process is regulated by multifarious environmental factors, and a large number of studies have investigated major environmental factors influencing the anammox bacteria distribution, abundance and activity, including the ammonium concentration (Shen et al., 2017), nitrate concentration (Wu et al., 2012) and total organic carbon content (Hu et al., 2012b). In our study, Pearson moment correlation analysis indicated that the distribution, abundance and activity of anammox bacteria significantly correlated with the sediment nitrate concentration, total organic carbon content and redox potential in the examined sediment cores. It is common knowledge that nitrate can be

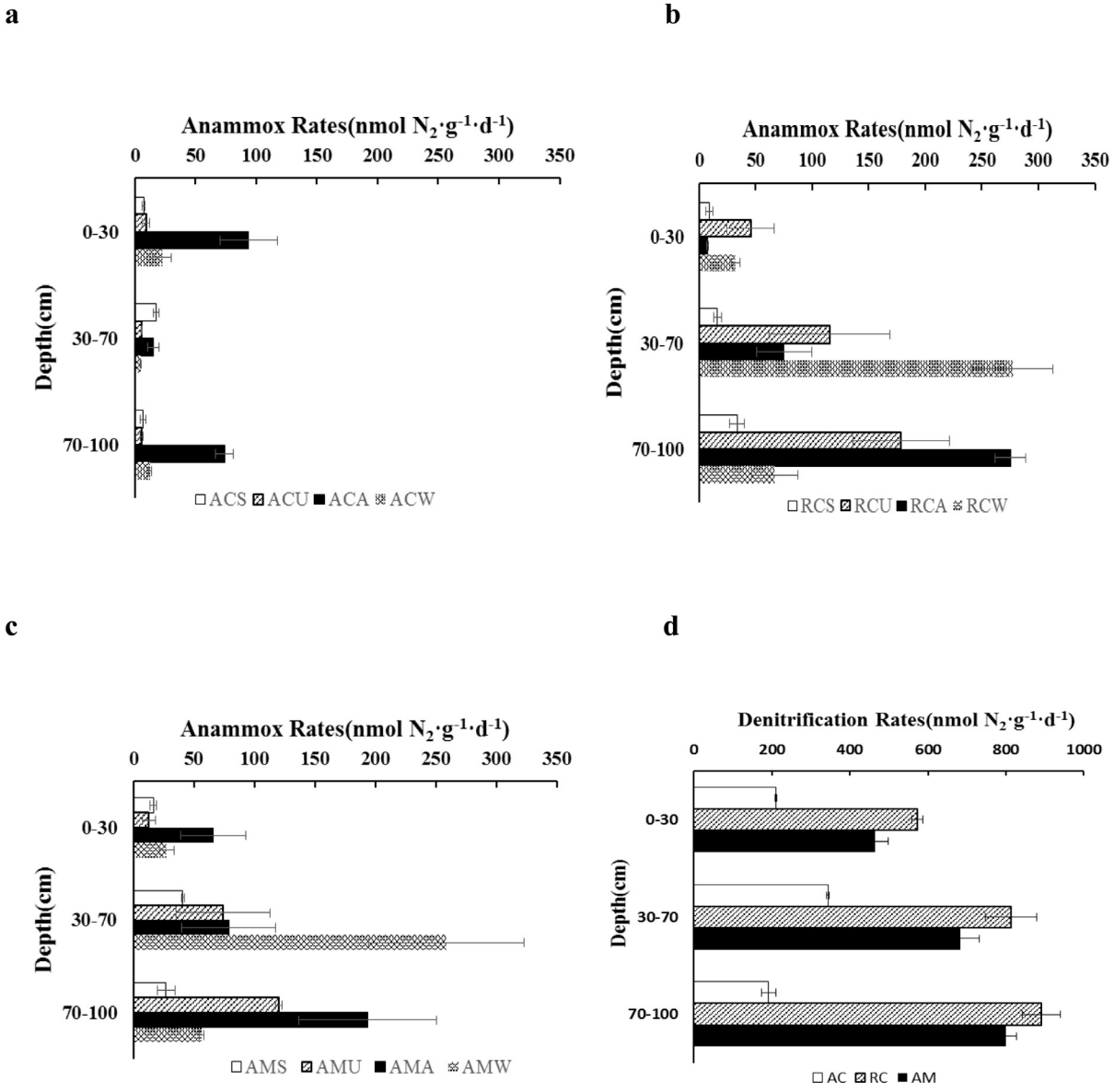

Fig. 6. Spatiotemporal distribution patterns of the potential anammox and denitrification rates in sediment cores collected from *Aegiceras corniculatum* (AC), *Rhizophora candel* (RC) and *Avicennia marina* (AM) habitats in the mangrove wetland. (a), The potential rates of anammox bacteria in AC habitats in four seasons. (b), The potential rates of anammox bacteria in RC habitats in four seasons. (c), The potential rates of anammox bacteria in AM habitats in four seasons. (d), The potential rates of denitrification in mangrove sediments in summer. Error bars indicate the standard deviation (n = 3).

converted to nitrite through the denitrification process, so the denitrifiers could supply nitrite for the anammox process; *i.e.*, denitrifiers reduce nitrate to nitrite, and the formed nitrite is subsequently utilized in the anammox process (Zhao et al., 2013; Zhou et al., 2014). Additionally, anammox bacteria are classified as gram-negative chemolithoautotrophic organisms (Strous et al., 1999), and they fix carbon using carbon dioxide as a carbon source.

Overall, there has been a rise in anthropogenic nitrogen inputs in wetlands (Finlay et al., 2013), and thus the anammox process could play a significant part in removing excess N originated from human activities and mitigating the global greenhouse effect in mangrove wetlands (Strous et al., 1999). However, more studies are needed to confirm the role of anammox in mangroves at regional and global scales. Extremely few studies have addressed anammox in mangroves so far, and these ecosystems occur on a large variability of climatic and hydrologic conditions, and of resource availability across the globe. Therefore, it is possible that not all mangroves exhibit the same patterns regarding anammox.

5. Conclusions

Our study provided direct evidence for the presence of anammox bacteria in mangrove wetlands and the results indicated that the upper layer of mangrove sediments provided a comfortable environment for the growth of anammox bacteria, but the deeper layer supplied a biogeographical hotspot for the metabolism of this organism in mangrove wetlands, which may act as an important and overlooked sink of inorganic nitrogen. Furthermore, the anammox process was an important pathway alleviating nitrogen pollution in the studied mangroves, also likely contributing to reducing undesirable nitrous oxide fluxes from these ecosystems.

Author contributions

YT and MPZ designed the study; MPZ, PLD and LAL performed the experiments; MPZ, PLD, XLL, LAL, BH, YMZ and YT analyzed the data; YT and MPZ wrote the paper and all co-authors substantially

contributed to commenting and revising it. All authors read and approved the final manuscript.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134291.

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