Community composition and activity of anaerobic ammonium oxidation bacteria in the rhizosphere of salt-marsh grass...

Article *in* Applied Microbiology and Biotechnology · May 2016

DOI: 10.1007/s00253-016-7625-2

CITATIONS
3

READS
108

10 authors, including:

Yanling Zheng
East China Normal University
28 PUBLICATIONS **234** CITATIONS
SEE PROFILE

Lijun Hou
East China Normal University
98 PUBLICATIONS **1,323** CITATIONS
SEE PROFILE

Juan Gao
East China Normal University
17 PUBLICATIONS **71** CITATIONS
SEE PROFILE

Xiaofei Li
East China Normal University
33 PUBLICATIONS **162** CITATIONS
SEE PROFILE

Appl Microbiol Biotechnol
DOI 10.1007/s00253-016-7625-2

ENVIRONMENTAL BIOTECHNOLOGY

Community composition and activity of anaerobic ammonium oxidation bacteria in the rhizosphere of salt-marsh grass *Spartina alterniflora*

Yanling Zheng[1,2] · Lijun Hou[2] · Min Liu[1] · Guoyu Yin[1,2] · Juan Gao[2] · Xiaofen Jiang[2] · Xianbiao Lin[1] · Xiaofei Li[1] · Chendi Yu[2] · Rong Wang[2]

Received: 20 March 2016 / Revised: 3 May 2016 / Accepted: 7 May 2016

Abstract Anaerobic ammonium oxidation (anammox) as an important nitrogen removal pathway has been investigated in intertidal marshes. However, the rhizosphere-driven anammox process in these ecosystems is largely overlooked so far. In this study, the community dynamics and activities of anammox bacteria in the rhizosphere and non-rhizosphere sediments of salt-marsh grass *Spartina alterniflora* (a widely distributed plant in estuaries and intertidal ecosystems) were investigated using clone library analysis, quantitative PCR assay, and isotope-tracing technique. Phylogenetic analysis showed that anammox bacterial diversity was higher in the non-rhizosphere sediments (*Scalindua* and *Kuenenia*) compared with the rhizosphere zone (only *Scalindua* genus). Higher abundance of anammox bacteria was detected in the rhizosphere (6.46×10^6–1.56×10^7 copies g^{-1}), which was about 1.5-fold higher in comparison with that in the non-rhizosphere zone (4.22×10^6–1.12×10^7 copies g^{-1}). Nitrogen isotope-tracing experiments indicated that the anammox process in the rhizosphere contributed to 12–14 % N_2 generation with rates of 0.43–1.58 nmol N g^{-1} h^{-1}, while anammox activity in the non-rhizosphere zone contributed to only 4–7 % N_2 production with significantly lower activities (0.28–0.83 nmol N g^{-1} h^{-1}). Overall, we propose that the rhizosphere microenvironment in intertidal marshes might provide a favorable niche for anammox bacteria and thus plays an important role in nitrogen cycling.

Keywords Anaerobic ammonium oxidation (anammox) · Rhizosphere · *Spartina alterniflora* · Intertidal sediment · Nitrogen cycle

Introduction

The massive acceleration of nitrogen cycling by human activities has led to many serious environmental issues, ranging from eutrophication of aquatic and terrestrial ecosystems to global acidification and hypoxia (Gruber and Galloway 2008; Diaz and Rosenberg 2008; Kim et al. 2014). Intertidal wetlands are important conduits for reducing the excessive load of reactive nitrogen from agricultural activities and other terrestrial sources transported into marine environments, as they are the direct connections between land and sea (Pennings 2012). Of the nitrogen removal pathways, anaerobic ammonium oxidation (anammox), via oxidizing ammonium (NH_4^+) by reduction of nitrite (NO_2^-), can remove reactive nitrogen permanently from aquatic ecosystems into the atmosphere, as can denitrification (Strous et al. 1999; Hou et al. 2013). Nevertheless, knowledge on the dynamics of anammox bacterial community and their potential role for nitrogen removal in the rhizosphere zone of salt-marsh plants remains scarce in the intertidal environments.

Rhizosphere is a complex environment which can be defined as the zone around plant roots whereby sediment properties are influenced by the presence and activity of the root (Richardson et al. 2009). *Spartina alterniflora*, native to Atlantic and the Gulf Coast of North America, was introduced to China in 1979 and

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7625-2) contains supplementary material, which is available to authorized users.

✉ Lijun Hou
ljhou@sklec.ecnu.edu.cn

✉ Min Liu
mliu@geo.ecnu.edu.cn

[1] College of Geographical Sciences, East China Normal University, Shanghai 200241, China

[2] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200062, China

Published online: 26 May 2016

has rapidly expanded since then and replaced native plants such as *Phragmites australis* and *Suaeda salsa*, becoming one of the dominant species in the coastal marsh ecosystems (Nie et al. 2009; Wan et al. 2009; Wang et al. 2010). *S. alterniflora* exhibits high net primary production rate, reaching up to 3.2 kg cm^{-2} $year^{-1}$, much of which was observed to occur belowground (Giblin and Wieder 1992; Thomas et al. 2014). Additionally, this salt-marsh grass possesses well-developed aerenchyma systems, which confers upon it the ability to transport oxygen down to the belowground parts and establishes a typical oxic-anoxic interface in intertidal sediments (Maricle and Lee 2002; Sousa et al. 2008). Thereby, it was hypothesized that this redox gradient formed in the rhizosphere zone might be a hotspot for anammox (Chu et al. 2015). Through pumping oxygen down to the roots, *S. alterniflora* forms a particular sediment microenvironment where aerobic microorganisms can thrive (Brunea et al. 2000). Aerobic ammonia and nitrite oxidizers might be active in the oxygenated rhizosphere and produce NO_2^- and NO_3^-, respectively. Part of these nitrogen oxides would be expected to diffuse from the oxygenated layer into the adjacent, oxygen-poor rhizosphere zone where they would serve the anammox bacteria as terminal electron acceptors and thereby stimulate the anammox activity (Brunea et al. 2000). However, little is known about the activity and dynamics of anammox community in the rhizosphere or how it differs from its non-rhizosphere counterpart. To extend the understanding, the Yangtze Estuary, one of the largest estuaries in the world where *S. alterniflora* flourishes, was selected as the study area in the present study.

The Yangtze Estuary is situated at one of the most populated as well as most heavily polluted regions of China. Annually, high loads of nutrients from the Yangtze River and great amounts of domestic sewage are discharged into the estuarine and coastal environments, which have caused severely eutrophic status (Deng et al. 2015). Therefore, the biogeochemical transformations of nitrogen are major concerns in the Yangtze Estuary and its adjacent coastal areas. Although the importance of anammox has been recognized, few studies have investigated the influence of salt-marsh roots on the dynamics of anammox in the estuary (Hou et al. 2013). Therefore, the objectives of this study were to investigate the occurrence, diversity, and abundance of anammox bacteria and their contributions to nitrogen removal in the rhizosphere zones of the salt-marsh grass *S. alterniflora* in intertidal marshes. This work improves understandings of the microbial nitrogen transformations in the vegetated environments.

Materials and methods

Sample collection

The study site is located at the Chongming eastern intertidal flat, which is the largest and most extensively developed intertidal wetland in the Yangtze Estuary (Fig. S1). The total area of the *S. alterniflora* community in the eastern intertidal zone of the Chongming Island was up to 1000 hm^2, which accounted for more than 33 % of the total vegetation on the tidal flats (Li et al. 2006; Wang et al. 2010). Field samples were collected every 3 months from April 2014 to January 2015, which spanned the entire growing season. At each field survey, rhizosphere and non-rhizosphere sediments were collected using a five-point sampling method (Chu et al. 2015). Briefly, 30 cm × 30 cm × 30 cm volume of sediments (with plant individuals) was dug at each point of the sampling area using a stainless steel spade. Rhizosphere samples (approximately the first 2 mm of sediments in the closest contact with the root) were collected by eliminating the loosely attached sediments, while the non-rhizosphere samples were taken in 20–30 mm distance from the root mats (Liu et al. 2014). All samples were immediately put into sterile bags on ice and then transported to the laboratory within 2 h. Upon return to the laboratory, sediments from each of the five sampling points were respectively homogenized, which resulted in five replicates of both rhizosphere and non-rhizosphere samples. One part of the sediment samples was preserved at 4 °C for sediment physiochemical analyses and anammox activity measurement, while the remaining part of the samples was stored at −80 °C for genomic DNA extraction and the subsequent molecular analyses.

Determination of environmental parameters

Water content of the sediment was determined by the weight loss of a certain amount of fresh sediment, which was dried under 80 °C to a constant value. Temperature of the sediment was in situ determined using a portable electronic thermometer. The pH values and salinity were measured with pH meter (Mettler-Toledo) and salinity meter (YSI Model 30), respectively, after fresh sediments were mixed with deionized water (CO_2-free) at a ratio of 1:2.5 (volume ratio) (Zheng et al. 2014). Exchangeable NO_2^-, NO_3^-, and NH_4^+ were extracted using 2 M KCl stock solution from fresh sediments and then spectrophotometrically determined using a continuous-flow nutrient analyzer (SAN plus, *Skalar Analytical* B.V., the Netherlands) (Hou et al. 2007). Detailed information on environmental parameters is given in Table S1.

DNA extraction and PCR amplification

Genomic DNA was extracted from approximate 0.25 g of each replicate sediment using Powersoil™ DNA Isolation Kit (MOBIO, USA) according to the instructions. 16S rRNA gene of anammox bacteria was then amplified from pooled sediment DNA extracts using a nested PCR assay (Hou et al. 2013). Briefly, an initial PCR amplification was performed using primer set PLA46f-1390r (Schmid et al. 2000) followed

by a second PCR reaction with primer pair Amx368f–Amx820r (Schmid et al. 2005) (Table S2). The appropriately sized PCR products were purified with Gel Advance-Gel Extraction system (Viogene, China) and then cloned with TOPO-TA cloning kits (Invitrogen, USA). Clones were randomly selected for further sequencing analyses.

Phylogenetic analysis

The selected clones were sequenced with an ABI Prism genetic analyzer (Applied Biosystems, Canada). The qualified sequences with more than 97 % identities were defined as one operational taxonomic unit (OTU) with software mothur (version 1.35.1, USA) by the furthest neighbor method. The anammox bacterial 16S rRNA gene clones and their relatives picked from NCBI were aligned with ClustalX 2.1 (Thompson et al. 1997). MEGA 5.03 software was then used for the construction of phylogenetic trees with neighbor-joining approach (Tamura et al. 2007). Topological confidence of the phylogenetic tree was assessed by conducting 1000 bootstrap replicates. The unique clone sequences acquired in the present study were deposited in GenBank: KU232995 to KU233184.

Quantitative PCR assay

Quantitative PCR (qPCR) analyses were conducted with primer pair AMX-808-F-AMX-1040-R (Hamersley et al. 2007) using SYBR green method on an ABI 7500 Sequence Detection System (Applied Biosystems, Canada) (Table S2). Plasmid Mini Preparation Kit (Tiangen, China) was used to extract the plasmids carrying anammox bacterial 16S rRNA gene. Subsequently, Nanodrop-2000 Spectrophotometer (Thermo, USA) was used to determine the concentrations of the extracted plasmids. Standard curves were then obtained through gradient dilutions of the plasmids containing 16S rRNA gene of anammox bacteria with known copy numbers. Negative controls were performed in all experiments to detect any possible contaminations. Melting curve analyses and gel electrophoreses were further conducted to confirm the specificity of the qPCR amplification. Based on the above constructed standard curves, gene abundances were calculated and then converted into copies per gram of sediment, assuming the extraction efficiency of DNA was 100 %.

Determination of nitrogen transformation rates

Potential rates of anammox and denitrification were determined through sediment slurry experiment in combination with nitrogen isotope-tracing method (Risgaard-Petersen et al. 2004; Engström et al. 2005). Briefly, slurries were prepared with helium-purged tidal water and fresh sediments at a ratio of 5:1 (volume ratio). Subsequently, they were transferred into the 12-ml glass vials (Exetainer, Labco, High Wycombe, Buckinghamshire, UK) and pre-incubated for over 24 h to exhaust residual oxygen, NO_3^-, and NO_2^- under in situ temperature. These vials were divided into three groups after the pre-incubation. Each group was then spiked with helium-purged solutions of (1) $^{15}NH_4^+$ (^{15}N at 99.6 %), (2) $^{15}NH_4^+ + {}^{14}NO_3^-$, and (3) $^{15}NO_3^-$ (^{15}N at 99 %), respectively, with the final ^{15}N concentrations of about 100 μM in each vial. The incubations were inhibited by injecting 300 μl of 50 % $ZnCl_2$ solution after 8 h (Hou et al. 2013; Deng et al. 2015). Concentrations of the $^{29}N_2$ and $^{30}N_2$ produced during the incubation period were determined by membrane inlet mass spectrometry (MIMS) (An and Gardner 2002; Yin et al. 2014). Anammox and denitrification rates could therefore be quantified based on the previously developed methods (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003; Hou et al. 2013).

Statistical analyses

The biodiversity indices (Simpson and Shannon-Wiener) and species richness estimator (Chao1) were estimated for the constructed clone libraries with mothur 1.35.1 (Schloss et al. 2009). Coverages of the clone libraries were calculated by the percentages of the observed OTU number in each clone library divided by Chao1 estimator (Mohamed et al. 2010). Community classifications were conducted by principal coordinates analysis (PCoA) with software Qiime 1.9.0 (Caporaso et al. 2010). The distances between clone libraries were calculated by UniFrac test, and the *P* value has been corrected by Bonferroni correction (Lozupone et al. 2011).

Results

Community dynamics of anammox bacteria

Anammox bacterial communities in the non-rhizosphere and rhizosphere regions of the salt-marsh grass *S. alterniflora* in the Yangtze Estuary were successfully observed with nested PCR assay (Fig. S2). In this study, 462 qualified clones were successfully sequenced from the eight constructed clone libraries. Sequence analysis showed that these retrieved sequences were all related to the known anammox bacterial 16S rRNA genes. In each clone library, 6 to 8 rhizosphere OTUs and 9 to 11 non-rhizosphere OTUs occurred, based on <3 % nucleotide divergence (Table 1). According to Shannon-Wiener and Simpson indicators, diversity of the anammox communities in the rhizosphere was lower than that in the non-rhizosphere (Table 1), and this result was in agreement with the rarefaction analyses (Fig. 1). The estimated coverages of the clone libraries were between 94.7 and 100 %, showing that greater than 90 % of the anammox

Table 1 Diversity characteristics of the anammox bacterial 16S rRNA gene clone libraries

Sampling position	Time	No. of clones	OTUs[a]	Chao1[b]	Shannon-Wiener[c]	1/Simpson[d]	Coverage (%)[e]
Rhizosphere	Apr.	57	6	6.0	1.11	2.31	100
	Jul.	57	8	8.3	1.38	2.87	97.0
	Oct.	62	7	7.3	1.10	2.23	96.6
	Jan.	60	6	6.0	1.20	2.49	100
Non-rhizosphere	Apr.	59	9	9.5	1.87	5.88	94.7
	Jul.	56	9	9.0	1.88	5.81	100
	Oct.	56	11	11.0	2.10	7.44	100
	Jan.	55	11	11.0	2.13	7.50	100

Apr. April, *Jul.* July, *Oct.* October, *Jan.* January

[a] OTUs are defined at 3 % nucleotide acid divergence

[b] Nonparametric statistical predictions of the total richness of OTUs

[c] Shannon-Wiener diversity index. A higher value represents higher diversity

[d] Reciprocal of Simpson's diversity index. A higher value represents higher diversity

[e] Calculated by the percentage of the observed number of OTUs divided by Chao1 estimator

bacterial 16S rRNA gene diversity was captured in each clone library, and the gradually flatting rarefaction curves further proved this point (Fig. 1).

Phylogenetic analysis indicated that two known anammox bacterial genera including *Candidatus Scalindua* and *Candidatus Kuenenia* were detected in this study (Fig. 2). In the phylogenetic tree, 79.9 % of the sequences obtained in this study were affiliated with *Scalindua* with 92.3–96.5 % sequence identity, while only 20.1 % of the sequences showed the closest evolutionary relation (95.6–97.3 %) with the 16S rRNA gene of *Kuenenia*. Results also showed that anammox community in the rhizosphere of *S. alterniflora* was restricted to *Scalindua* at different growing stages of the year, while the non-rhizosphere anammox community was co-dominated by *Scalindua* and *Kuenenia* (Fig. 2).

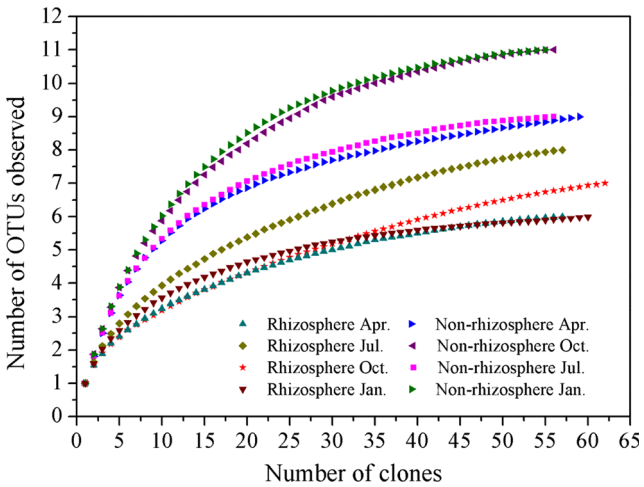

Fig. 1 Rarefaction curves of the anammox bacterial clone libraries. OTUs are defined at 3 % divergence in nucleotides. *Apr.* April, *Jul.* July, *Oct.* October, *Jan.* January

Additionally, community compositions of anammox bacteria between the rhizosphere and non-rhizosphere zones were statistically compared based on the constructed UniFrac distance matrix (Table 2). Results indicated that the anammox communities acquired from the rhizosphere (April, July, and January) were significantly different from those obtained from the non-rhizosphere sites (April, July, October, and January) ($P < 0.05$; Table 2), which was consistent with the results of the phylogenetic analysis (Fig. 2). Furthermore, PCoA analysis showed that the anammox bacterial assemblages were divided into two distinctive groups: the rhizosphere group and the non-rhizosphere group (Fig. 3). However, the seasonal variations of anammox communities in both the non-rhizosphere and rhizosphere zones were not significant ($P > 0.05$), showing that the anammox community compositions were relatively stable in different microecological niches in intertidal marshes.

Abundance of anammox bacteria

A significant linear relationship ($R^2 = 0.9989$) was obtained between the $\log_{10}$ value of the plasmid copy number (2.2×10^1–2.2×10^7 copies μl^{-1}) and the threshold cycle (Ct), with a high amplification efficiency of 96.1 %. In the present study, anammox bacterial 16S rRNA gene abundance was detected between 6.46×10^6 and 1.56×10^7 copies g^{-1} (dry weight, similarly hereinafter) in the rhizosphere and between 4.22×10^6 and 1.12×10^7 copies g^{-1} in the non-rhizosphere (Fig. 4). During the four sampling seasons, anammox bacterial abundance was always higher in the rhizosphere zones than in the non-rhizosphere (Student's *t* test, $P < 0.05$). Relatively higher anammox bacterial numbers were observed in the warmer seasons (July and October,

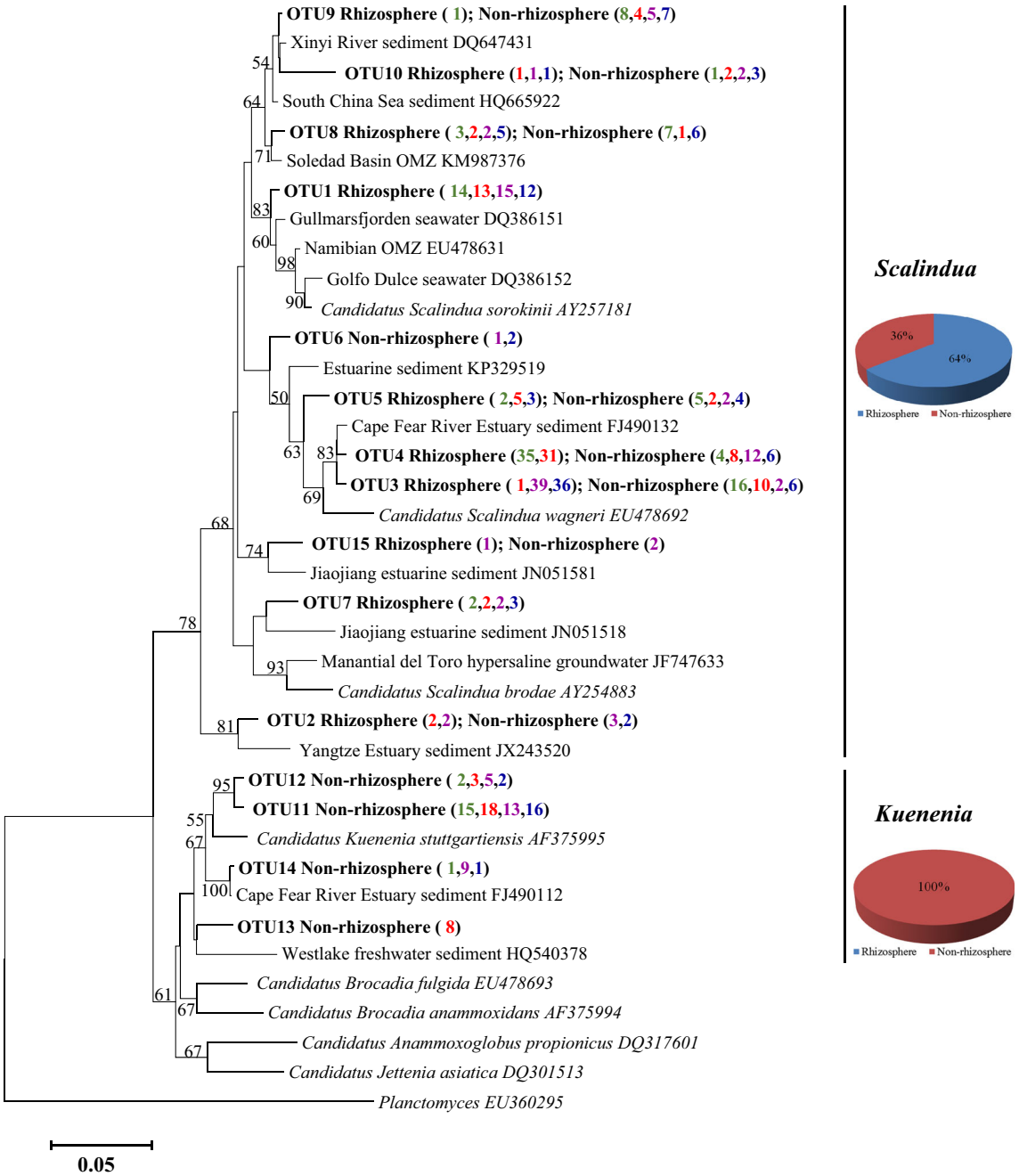

Fig. 2 Neighbor-joining phylogenetic tree based on the 16S rRNA gene of anammox bacteria. Bootstrap values higher than 50 % are shown. GenBank accession numbers of the sequences from other studies are given. Data in parentheses followed each OTU represent the number of sequences retrieved from the rhizosphere and (or) non-rhizosphere of salt-marsh grass *S. alterniflora*. *Green*: April, *red*: July, *violet*: October; *blue*: January

8.65×10^6–1.56×10^7 copies g^{-1}) in comparison with the cooler seasons (January and April, 4.22×10^6–8.23×10^6 copies g^{-1}). Additionally, Pearson correlation analysis further revealed that the abundance of anammox bacteria was positively related to the changes of temperature ($R = 0.799$, $P = 0.017$), as compared with other measured environmental parameters (Table S1).

Anammox activity and contribution to N_2 production

Slurry experiments were performed in combination with a ^{15}N tracer assay to estimate anammox rates and the potential role of anammox in total N_2 production in the non-rhizosphere and rhizosphere sediments. The results revealed that the anammox bacterial activities in the rhizosphere were 0.43–

Table 2 The unweighted UniFrac distance matrix of the anammox bacterial clone libraries

	R_Apr.	R_Jul.	R_Oct.	R_Jan.	N_Apr.	N_Jul.	N_Oct.	N_Jan.
R_Apr.	0.00	0.28	0.45	0.20	0.57**	0.54*	0.66**	0.62**
R_Jul.	0.28	0.00	0.23	0.15	0.54**	0.51**	0.52*	0.48**
R_Oct.	0.45	0.23	0.00	0.32	0.44	0.40	0.40	0.38
R_Jan.	0.20	0.15	0.32	0.00	0.51*	0.48	0.61*	0.57*
N_Apr.	0.57**	0.54**	0.44	0.51*	0.00	0.16	0.19	0.14
N_Jul.	0.54*	0.51**	0.40	0.48	0.16	0.00	0.32	0.27
N_Oct.	0.66**	0.52*	0.40	0.61*	0.19	0.32	0.00	0.06
N_Jan.	0.62**	0.48**	0.38	0.57*	0.14	0.27	0.06	0.00

Data show the unweighted UniFrac distances between each pair of clone libraries

Single asterisk indicates the libraries are drawn from significantly different communities at $P = 0.05$; double asterisks indicate statistical significance at $P = 0.01$. P values have been corrected by Bonferroni correction

R rhizosphere, *N* non-rhizosphere, *Apr.* April, *Jul.* July, *Oct.* October, *Jan.* January

1.58 nmol N g^{-1} h^{-1} and contributed 12–14 % to total N_2 production. In the non-rhizosphere sediments, however, the anammox bacteria exhibited lower activities (0.28–0.83 nmol N g^{-1} h^{-1}; Student's t test, $P < 0.05$) and contributed only 4–7 % to total N_2 production (Fig. 5). The anammox bacteria both in the non-rhizosphere and rhizosphere zones were more active in warmer seasons (July and October, 0.60–1.58 nmol N g^{-1} h^{-1}) than in cooler seasons (January and April, 0.28–0.85 nmol N g^{-1} h^{-1}). Although differences were detected in denitrification activity between the rhizosphere (3.06–11.21 nmol N g^{-1} h^{-1}) and non-rhizosphere (4.88–14.80 nmol N g^{-1} h^{-1}) sediments, denitrification still played a main role in N_2 production in the intertidal marshes (86–88 % in the rhizosphere, 93–96 % in the non-rhizosphere) (Fig. 5). In this study, the cell-specific anammox activities were estimated based on the detected anammox activity and anammox bacterial numbers, assuming each cell exhibited equal activity and only contained one copy of 16S rRNA gene. The calculated cell-specific anammox rates ranged between 1.6–2.5 fmol N d^{-1} in the rhizosphere microenvironment, while they varied from 1.5–2.0 fmol N d^{-1} in the non-rhizosphere region (Fig. S3). Overall, the isotope-tracing experiments revealed that the contribution of anammox process to total N_2 production in the rhizosphere (12–14 %) was approximately twofold higher than that in the non-rhizosphere sediment (4–7 %), suggesting high dissimilarity in nitrogen removal between these two microenvironments in the intertidal marshes.

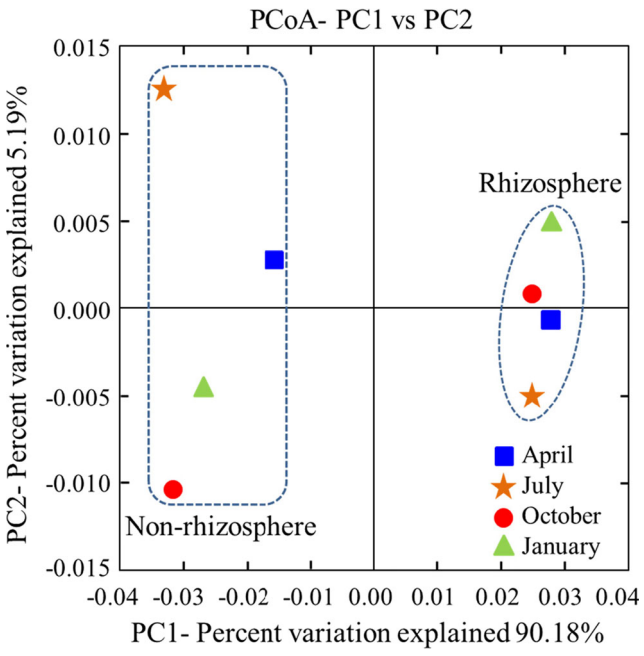

Fig. 3 The unweighted UniFrac PCoA analysis of anammox bacterial communities obtained from the rhizosphere and non-rhizosphere regions of the salt-marsh grass *S. alterniflora*. The first two principal coordinate axes (PC1 and PC2) are shown

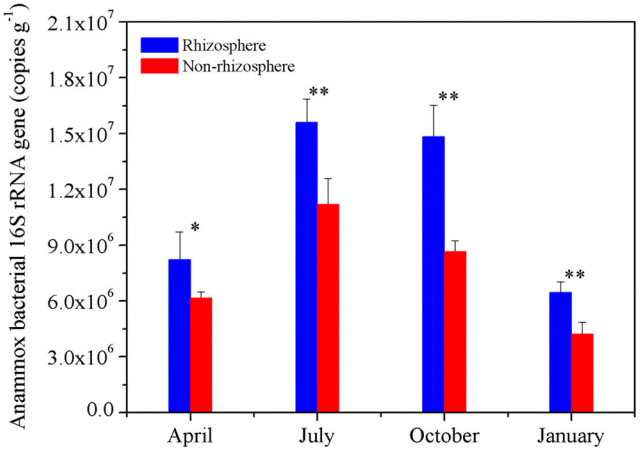

Fig. 4 Anammox bacterial abundance based on 16S rRNA gene in the rhizosphere and non-rhizosphere sediments of *S. alterniflora*. *Vertical bars* represent standard error ($n = 5$). *Single asterisk* represents statistical significance at $P = 0.05$, *double asterisks* represent statistical significance at $P = 0.01$

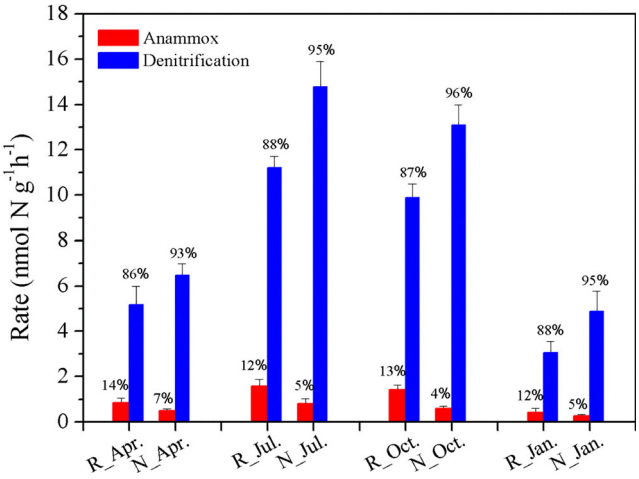

Fig. 5 The rates of anammox and denitrification and their contributions to total N_2 production. *Vertical bars* indicate standard error ($n = 5$)

Discussion

According to our knowledge, the present study is the first report about the community composition, abundance, and activity of anammox bacteria, as well as their potential contributions to total N_2 production in the rhizosphere zone of *S. alterniflora* in intertidal marshes. The findings in this study might shed light on anammox process in salt marshes and indicate that small-scale environmental heterogeneities are important in shaping the abundance, diversity, and potential activity of anammox bacteria.

In this study, communities of anammox bacteria had distinctive spatial heterogeneity between the rhizosphere and non-rhizosphere zones (Figs. 2 and 3). In the rhizosphere sediments, anammox bacterial community was restricted to *Scalindua*, while the non-rhizosphere anammox community was co-dominated by *Scalindua* (account for 59 %) and *Kuenenia* (account for 41 %), which were the two dominant anammox genera in estuarine and intertidal sediments (Wang et al. 2012; Hou et al. 2013). Salinity has been implicated to be an important factor regulating anammox bacterial community compositions (Dale et al. 2009; Fu et al. 2015). However, previous studies also showed that anammox bacteria have a high ability to tolerate changes in salinity (Boran et al. 2006). Thus, correlations between anammox bacterial community and salinity might not be linked to salinity itself, but to other environmental factors affected by salinity, such as the substrate availability (Koop-Jakobsen and Giblin 2009). Indeed, no significant changes in salinity was observed between the rhizosphere and non-rhizosphere regions in the present study (Student's *t* test, $P > 0.05$), which confirmed that the changes in anammox bacterial communities was not caused by salinity. The observed anammox bacterial differences in this study imply that salt-marsh plant roots might influence anammox bacteria by establishing specific rhizosphere microecology (Lee 2003). Previous studies showed that *S. alterniflora* roots can modify the physico-chemical properties and biological compositions of the rhizosphere through oxygen diffusion, rhizodeposition, nutrients uptake, proton extrusion, and exudates release (Richardson et al. 2009; Liu et al. 2014), thereby attributing to the change of anammox bacterial community in the rhizosphere in comparison with the non-rhizosphere zones. However, the underlying mechanisms causing the absence of *Kuenenia* genus in the rhizosphere need to be further explored.

The detected anammox bacterial abundance in the rhizosphere (6.46×10^6–1.56×10^7 copies g^{-1}) was significantly higher than that in the non-rhizosphere sediments (4.22×10^6–1.12×10^7 copies g^{-1}). These numbers of anammox bacteria in this study were in the same range as those retrieved from the paddy soil (Zhu et al. 2011; Nie et al. 2015), Barents marine sediment (Schmid et al. 2007), Pear River riparian sediment (Wang et al. 2012), and the Cape Fear River estuarine sediment (Dale et al. 2009). Anammox bacterial numbers tended to be higher in July and October (8.65×10^6–1.56×10^7 copies g^{-1}) compared with in January and April (4.22×10^6–8.23×10^6 copies g^{-1}). The seasonal fluctuations of anammox bacterial abundance might result from temperature changes (Table S1). It has been reported that the anammox bacterial community size was greatly affected by temperature (Hou et al. 2013). In warmer seasons, anammox bacterial growth and anammox activity are stimulated by substrate NO_2^- which is generated from the enhanced denitrification and nitrification activities (Fig. 5 and Fig. S4). This might also suggest that NO_2^- serves as a limiting factor in the development and activity of anammox bacteria in the marsh sediments (Zhu et al. 2013; Hou et al. 2013; Nie et al. 2015). In the rhizosphere, the abundance of anammox bacteria was approximately 1.5 times higher in comparison with the non-rhizosphere zone, suggesting that the rhizospheric microenvironment provides a suitable niche for anammox bacterial community.

In addition to higher anammox bacterial abundance, higher anammox activities in the rhizosphere (0.43–1.58 nmol N g^{-1} h^{-1}) were also observed in comparison with the non-rhizosphere zone (0.28–0.83 nmol N g^{-1} h^{-1}). The anammox process also played a more important role in total nitrogen removal in the rhizosphere zone (12–14 %) than in the non-rhizosphere sediments (4–7 %). These results showed that anammox activity in the rhizosphere might be an important but overlooked pathway for nitrogen removal from intertidal marshes. According to previous studies, NO_2^- production was a key factor in determining anammox activity in waterlogged ecosystems (Zhu et al. 2013; Hou et al. 2013; Nie et al. 2015). In intertidal sediments, wetland plants could release oxygen through the roots and thus oxygenate the rhizosphere (Armstrong et al. 2000). Therefore, this particular oxygenated microenvironment might allow aerobic microorganisms to thrive. This was confirmed by the studies showing

that the activity and abundance of both ammonia-oxidizing archaea and bacteria were greatly enhanced in the *S. alterniflora*-vegetated sediments (Zhang et al. 2011; Wang et al. 2015). Also, significantly higher (over 3.5 times higher) nitrification rates were observed in the rhizosphere sediments than in the non-rhizosphere sediments in the present study (Student's *t* test, $P < 0.05$; Fig. S4). Consequently, the availability of NO_2^- was greatly enhanced in the oxygenated rhizosphere zone. Part of the substrate would be expected to diffuse from the oxygenated layer into the adjacent, oxygen-poor rhizosphere layer where they would serve the anammox bacteria as terminal electron acceptors and stimulate the anammox activity in the rhizosphere zone (Brunea et al. 2000; Koop-Jakobsen and Giblin 2009). It should also be noted that plant roots could influence nitrogen availability in the rhizosphere via nutrient assimilation and thus might compete NO_2^- with anammox bacteria (Brunea et al. 2000; Liu et al. 2014). However, both anammox bacterial abundance and activity were enhanced in the rhizosphere (Figs. 4 and 5), indicating that despite the potential competitions, the anammox process still benefited from the NO_2^- produced by ammonia oxidizers in the oxygenated rhizosphere (Fig. S4).

The cell-specific anammox rates were estimated to be higher in the rhizosphere microenvironment (1.6–2.5 fmol N d^{-1}) where anammox bacterial community was restricted to *Scalindua* than in the non-rhizosphere region (1.5–2.0 fmol N d^{-1}) where anammox community was co-dominated by *Scalindua* and *Kuenenia* (Fig. S3). The difference of cell-specific anammox activity between the rhizosphere and non-rhizosphere zones might be caused by the availability of NO_2^- provided in different regions (Hou et al. 2013; Nie et al. 2015). Moreover, this difference might also be related to the half-saturation constant (K_s) of different anammox bacteria. It has been reported that the K_s value for nitrite and ammonium of *Scalindua* was lower than that of *Kuenenia* and other known anammox bacteria (Awata et al. 2013). Therefore, *Scalindua* has advantages over other anammox genera in competing substrates, which might consequently result in the relatively higher cell-specific activity of *Scalindua* and the relatively higher cell-specific anammox rate in the rhizosphere where *Scalindua* was more abundant in comparison with the non-rhizosphere zone (Awata et al. 2013). Previous studies also revealed that *Kuenenia* may perform anaerobic respiration of manganese and iron oxides (Strous et al. 2006) and may even grow heterotrophically and perform dissimilatory nitrate reduction to ammonium (DNRA) (Kartal et al. 2008; Humbert et al. 2010). Thus, whether *Kuenenia* observed in the non-rhizosphere indeed performs the classical anammox activity still remains to be explored.

Although denitrification activity was relatively higher (approximately 1.4 times higher) in the non-rhizosphere, which could also provide NO_2^- substrate for anammox bacteria (Meyer et al. 2005; Koop-Jakobsen and Giblin 2009; Hou et al. 2013), it cannot compete with the NO_2^- generated by nitrification in the rhizosphere. Thus, the rhizosphere microenvironment provides a more favorable habitat with higher substrate availability for anammox bacteria. As similar NO_3^- concentrations were detected in both regions (Table S1), the possible reason for the relatively lower denitrification rates in the rhizosphere might be the presence of oxygen. It is in agreement with previous studies that denitrification activity is more sensitive to oxygen than anammox and declines sharply at nanomolar oxygen concentrations (Dalsgaard et al. 2014). Although the observed denitrification rates were different between non-rhizosphere and rhizosphere zones, the results from this study confirmed that denitrification still played a major role in nitrogen removal in intertidal marshes (86–88 % in the rhizosphere and 93–96 % in the non-rhizosphere).

In the present study, our data showed that the diversity, abundance, activity, and role of anammox bacteria were greatly affected by the rhizosphere redox gradient developed by the salt-marsh grass *S. alterniflora* in intertidal marshes. This redox gradient existing in the rhizosphere is quite common for most of the wetland plant type (Pedersen et al. 1998; Armstrong et al. 2000; Lee and Dunton 2000). Thus, the findings in this study might have wide general significance. Taken together, we proposed an anammox hotspot in the rhizosphere microenvironment in intertidal marshes.

Acknowledgments This study was together funded by the National Natural Science Foundation of China (Nos. 41322002, 41271114, 41130525, 41071135, and 41501524), the Program for New Century Excellent Talents in University (NCET), China Postdoctoral Science Foundation (2015M581567), and the State Key Laboratory of Estuarine and Coastal Research. Great thanks are also given to Wayne Gardner and anonymous reviewers for their constructive suggestions on the earlier versions of this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals.

References

An S, Gardner WS (2002) Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Mar Ecol Prog Ser 237:41–50

Armstrong W, Cousins D, Armstrong J, Turner DW, Beckett PM (2000) Oxygen distribution in wetland plant roots and permeability barriers to gas-exchange with the rhizosphere: a microelectrode and modelling study with *Phragmites australis*. Ann Bot 86:687–703

Awata T, Oshiki M, Kindaichi T, Ozaki N, Ohashi A, Okabe S (2013) Physiological characterization of an anaerobic ammonium-

oxidizing bacterium belonging to the "*Candidatus Scalindua*" group. Appl Environ Microbiol 79:4145–4148

Boran K, Kolevab M, Arsovb R, van der Starc W, Jetten MSM, Strous M (2006) Adaptation of a freshwater anammox population to high salinity wastewater. J Biotechnol 126:546–553

Brunea A, Frenzel P, Cypionka H (2000) Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24:691–710

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña 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 Methods 7:335–336

Chu JY, Zhang JP, Zhou XH, Liu B, Li YM (2015) A comparison of anammox bacterial abundance and community structures in three different emerged plants-related sediments. Curr Microbiol 71: 421–427

Dale OR, Tobias CR, Song B (2009) Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River Estuary. Environ Microbiol 11:1194–1207

Dalsgaard T, Stewart FJ, Thamdrup B, De Brabandere L, Revsbech NP, Ulloa O, Canfield DE, DeLong EF (2014) Oxygen at nanomolar levels reversibly suppresses process rates and gene expression anammox and denitrification in the oxygen minimum zone off northern Chile. mBio 5(6):e01966–e01914

Deng F, Hou LJ, Liu M, Zheng Y, Yin G, Li X, Lin X, Chen F, Gao J, Jiang X (2015) Dissimilatory nitrate reduction processes and associated contribution to nitrogen removal in sediments of the Yangtze Estuary. J Geophys Res Biogeosci 120:1521–1531

Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321:926–929

Engström 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 Acta 69:2057–2065

Fu B, Liu J, Yang H, Hsu TC, He B, Dai M, Kao SJ, Zhao M, Zhang XH (2015) Shift of anammox bacterial community structure along the Pearl Estuary and the impact of environmental factors. J Geophys Res Oceans 120:2869–2883

Giblin AE, Wieder RK (1992) Sulphur cycling in marine and freshwater wetlands. In: Howarth RW, Steward JWB, Ivanov MV (eds) Sulphur cycling on the continents: wetlands, terrestrial ecosystems, and associated water bodies. Wiley, Chichester, pp. 85–117

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. Nature 451:293–296

Hamersley MR, Lavik G, Woebken D, Rattray JE, Lam P, Hopmans EC, Damsté JSS, Krüger S, Graco M, Gutiérrez D, Kuypers MMM (2007) Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. Limnol Oceanogr 52:923–933

Hou LJ, Liu M, Xu SY, Ou DN, Yu J, Cheng SB, Lin X, Yang Y (2007) The effects of semi-lunar spring and neap tidal change on nitrification, denitrification, and N_2O vertical distribution in the intertidal sediments of the Yangtze Estuary, China. Estuar Coast Shelf Sci 73: 607–616

Hou LJ, Zheng YL, Liu M, Gong J, Zhang XL, Yin GY, You LL (2013) Anaerobic ammonium oxidation (anammox) bacterial diversity, abundance, and activity in marsh sediments of the Yangtze Estuary. J Geophys Res Biogeosci 118:1237–1246

Humbert S, Tarnawski S, Fromin N, Mallet M, Aragno M, Zopfi J (2010) Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J 4:450–454

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

Kim IN, Lee K, Gruber N, Karl DM, Bullister JL, Yang S, Kim T (2014) Increasing anthropogenic nitrogen in the North Pacific Ocean. Science 346:1102–1106

Koop-Jakobsen K, Giblin AE (2009) Anammox in tidal marsh sediments: the role of salinity, nitrogen loading, and marsh vegetation. Estuar Coasts 32:238–245

Lee RW (2003) Physiological adaptations of the invasive cordgrass *Spartina anglica* to reducing sediments: rhizome metabolic gas fluxes and enhanced O_2 and H_2S transport. Mar Biol 143:9–15

Lee KS, Dunton KH (2000) Diurnal changes in pore water sulfide concentrations in the seagrass *Thalassia testudinum* beds: the effects of seagrasses on sulfide dynamics. J Exp Mar Biol Ecol 255:201–214

Li HP, Zhang LQ, Wang DH (2006) Distribution of an exotic plant *Spartina alterniflora* in Shanghai. Biodivers Sci 14:114–120

Liu D, Fang S, Tian Y, Chang SX (2014) Nitrogen transformations in the rhizosphere of different tree types in a seasonally flooded soil. Plant Soil Environ 60:249–254

Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. ISME J 5:169–172

Maricle BR, Lee RW (2002) Aerenchyma development and oxygen transport in the estuarine cordgrasses *Spartina alterniflora* and *S. anglica*. Aquat Bot 74:109–120

Meyer RL, Risgaard-Petersen N, Allen DE (2005) Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. Appl Environ Microbiol 71:6142–6149

Mohamed NM, Saito K, Tal Y, Hill RT (2010) Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges. ISME J 4: 38–48

Nie M, Wang M, Li B (2009) Effects of salt marsh invasion by *Spartina alterniflora* on sulfate-reducing bacteria in the Yangtze River estuary, China. Ecol Eng 35:1804–1808

Nie SA, Li H, Yang XR, Zhang ZJ, Weng BS, Huang FY, Zhu GB, Zhu YG (2015) Nitrogen loss by anaerobic oxidation of ammonium in rice rhizosphere. ISME J 9:2059–2067

Pedersen O, Borum J, Duarte CM, Fortes MD (1998) Oxygen dynamics in the rhizosphere of *Cymodocea rotundata*. Mar Ecol Prog Ser 169: 283–288

Pennings SC (2012) The big picture of marsh loss. Nature 400:352–353

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305–339

Risgaard-Petersen N, Meyer RL, Schmidt M, Jetten MSM, Prast AE, Rysgaard S, Revsbech NP (2004) Anaerobic ammonia oxidation an estuarine sediment. Aquat Microb Ecol 36:293–304

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541

Schmid MC, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten MSM, Metzger J, Schleifer K, Wagner M (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 23:93–106

Schmid MC, Maas B, Dapena A, van de Pas-Schoonen K, van de Vossenberg J, Kartal B, van Niftrik L, Schmidt I, Cirpus I, Kuenen JG, Wagner M, Damste JSS, Kuypers M, Revsbech NP, Mendez R, Jetten MSM, Strous M (2005) Bio-markers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl Environ Microbiol 71:1677–1684

Schmid MC, Risgaard-Petersen N, van de Vossenberg J, Kuypers MMM, Lavik G, Petersen J, Hulth S, Thamdrup B, Canfield D, Dalsgaard T, Rysgaard S, Sejr MK, Strous M, Op den Camp HJM, Jetten MSM (2007) Anaerobic ammonium-oxidizing bacteria in marine

environments: widespread occurrence but low diversity. Environ Microbiol 9:1476–1484

Sousa AI, Lillebø AI, Caçador I, Pardal MA (2008) Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environ Pollut 156:628–635

Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MSM (1999) Missing lithotroph identified as new *Planctomycete*. Nature 400:446–449

Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW, Horn M, Daims H, Bartol-Mavel D, Wincker P, Barbe V, Fonknechten N, Vallenet D, Segurens B, Schenowitz-Truong C, Médigue C, Collingro A, Snel B, Dutilh BE, Op den Camp HJM, van der Drift C, Cirpus I, van de Pas-Schoonen KT, Harhangi HR, van Niftrik L, Schmid M, Keltjens J, van de Vossenberg J, Kartal B, Meier H, Frishman D, Huynen MA, Mewes H, Weissenbach J, Jetten MSM, Wagner M, Le Paslier D (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440:790–794

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

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

Thomas F, Giblin AE, Cardon ZG, Sievert SM (2014) Rhizosphere heterogeneity shapes abundance and activity of sulfur-oxidizing bacteria in vegetated salt marsh sediments. Front Microbiol 5:309

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

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

Wan SW, Qin P, Liu JN, Zhou HX (2009) The positive and negative effects of exotic *Spartina alterniflora* in China. Ecol Eng 35:444–452

Wang RZ, Yuan L, Zhang LQ (2010) Impacts of *Spartina alterniflora* invasion on the benthic communities of salt marshes in the Yangtze Estuary, China. Ecol Eng 36:799–806

Wang S, Zhu GB, Peng YZ, Jetten MSM, Yin C (2012) Anammox bacterial abundance, activity, and contribution in riparian sediments of the Pear River Estuary. Environ Sci Technol 46:8834–8842

Wang HT, Su JQ, Zheng TL, Yang XR (2015) Insights into the role of plant on ammonia-oxidizing bacteria and archaea in the mangrove ecosystem. J Soils Sediments 15:1212–1223

Yin GY, Hou LJ, Liu M, Liu ZF, Gardner WS (2014) A novel membrane inlet mass spectrometer method to neasure $^{15}NH_4^+$ for isotope-enrichment experiments in aquatic ecosystems. Environ Sci Technol 48:9555–9562

Zhang QF, Peng JJ, Chen Q, Li XF, Xu CY, Yin HB, Yu S (2011) Impacts of *Spartina alterniflora* invasion on abundance and composition of ammonia oxidizers in estuarine sediment. J Soils Sediments 11: 1020–1031

Zheng YL, Hou LJ, Newell S, Liu M, Zhou JL, Zhao H, You LL, Cheng XL (2014) Community dynamics and activity of ammonia-oxidizing prokaryotes in intertidal sediments of the Yangtze Estuary. Appl Environ Microbiol 80:408–419

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

Zhu GB, Wang S, Wang W, Wang Y, Zhou L, Jiang B, Op den Camp HJM, 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