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METHODS AND PROTOCOLS

A comparison of two 16S rRNA gene-based PCR primer sets in unraveling anammox bacteria from different environmental samples

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Abstract Two 16S rRNA gene-based PCR primer sets (Brod541F/Amx820R and A438f/A684r) for detecting anammox bacteria were compared using sediments from Mai Po wetlands (MP), the South China Sea (SCS), a freshwater reservoir (R2), and sludge granules from a wastewater treatment plant (A2). By comparing their ability in profiling anammox bacteria, the recovered diversity, community structure, and abundance of anammox bacteria among all these diverse samples indicated that A438f/A684r performed better than Brod541F/Amx820R in retrieving anammox bacteria from these different environmental samples. Five *Scalindua* subclusters (zhenghei-I, SCS-I, SCS-III, arabica, and brodae) dominated in SCS whereas two *Scalindua* subclusters (zhenghei-II and wagneri) and one cluster of *Kuenenia* dominated in MP. R2 showed a higher diversity of anammox bacteria with two new retrieved clusters (R2-New-1 and R2-New-2), which deserves further detailed study. The dominance of *Brocadia* in sample A2 was supported by both of the primer sets used. Results collectively indicate strongly niche-specific community structures of anammox bacteria in different environments, and A438f/A684r is highly recommended for screening anammox bacteria from various environments when dealing with a collection of samples with diverse physiochemical characteristics.

Keywords Anammox · Detection · Diversity · PCR primer · Abundance · Distribution

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Introduction

The anaerobic ammonium oxidizing (anammox) process is a recent addition to the nitrogen cycle. Anammox bacteria have been reported as important players involved in coupling ammonium with nitrate/nitrite to dinitrogen gas (N_2) and contributing to losses of inorganic N species in different ecological niches (Kartal et al. 2007), including freshwater ecosystems (Penton et al. 2006; Wang and Gu 2013b), coastal wetlands (Dang et al. 2010; Li et al. 2010), rice paddy soil (Wang and Gu 2013a), and marine environments (Borin et al. 2013; Kirkpatrick et al. 2012; Lam et al. 2007; Li et al. 2013b, c). So far, five anammox genera have been established, namely *Candidatus* Scalindua (Schmid et al. 2003; van de Vossenberg et al. 2008), *Ca.* Brocadia (Kartal et al. 2008; Oshiki et al. 2011), *Ca.* Anammoxoglobus (Kartal et al. 2007), *Ca.* Jettenia (Quan et al. 2008), and *Ca.* Kuenenia (Schmid et al. 2000; Strous et al. 2006), and they are all affiliated with a deep branch of the *Planctomycetes*.

Since pure culture of anammox bacteria is still hardly available, the diversity and distribution of anammox bacteria in different environments are detected by culture-independent tools, including phylogenetic gene biomarkers through PCR amplification, fluorescence in situ hybridization (FISH) (Schmid et al. 2005), stable isotope probing (SIP) with $^{13}CO_2$ and/or ^{15}N-labeled inorganic N species (Konovalov

et al. 2008; Song and Tobias 2011), and also anammox-specific lipid measurement (Kuypers et al. 2003). Among the phylogenetic biomarkers, the 16S rRNA gene is the most commonly used in the detection of anammox bacteria from various ecosystems, while a suite of functional genes (Li et al. 2011b; Song and Tobias 2011) were also used in revealing the community and abundance of these important *Planctomycetes*. A group of published 16S rRNA gene-based PCR primers is summarized in Table 1. Due to the high sequence divergence among different genera of anammox bacteria (<87.1 % identity) and the low abundance of anammox bacteria in most ecological niches except for sludge and wastewater treatment plants, it is difficult to select 16S rRNA gene-based PCR primers to recover all known genera of anammox bacteria with a high specificity from any given samples (Jetten et al. 2009; Junier et al. 2010; Kartal et al. 2011). Therefore, the efficiency and specificity of those PCR primers were subsequently evaluated and assessed with samples from freshwater (Sonthiphand and Neufeld 2013), estuary/coastal wetlands, and marine environments (Han and Gu 2013; Han et al. 2013). Among recent studies, two 16S rRNA gene-based primer sets (Brod541F/Amx820R and A438f/A684r) were reported as efficient biomarkers for such purpose. The PCR-amplified products with primer sets Brod541F/Amx820R and A438f/A684r cover 279 and 246 bp of the 16S rRNA gene of anammox bacteria, respectively. The PCR primer set Brod541F/Amx820R was widely applied with coastal wetland samples (Li et al. 2011a, c) and marine sediments (Li et al. 2013b) while A438f/A684r showed the ability to recover a more refined community structure of anammox bacteria from a wide range of ecological niches (Han and Gu 2013; Humbert et al. 2012). In previous studies, A438f/A684r amplified the 16S rRNA gene of anammox bacteria from 11 samples with efficiency ranging from 59.3 to 100 %, resulting in six putative new clusters (Han and Gu 2013). In this study, PCR primer set Brod541F/Amx820R was applied for the same 11 samples as previously described, and a comparison of efficiency and specificity of these two primer sets was made and analyzed.

Materials and methods

Sample description

Information on sampling sites and physiochemical parameters of the South China Sea surface sediments collected in 2008 was described in a previous study (Cao et al. 2012). Wastewater treatment plant sample A2, water reservoir sample R2, and wetland sediment samples (1S, 3S, and 3Sm) were collected in July 2012, October 2011, and April 2012, respectively, and described previously (Han and Gu 2013).

DNA extraction and PCR amplification

Total genomic DNAs of these samples were extracted using the Power Soil Isolation Kit (Mo Bio, Carlsbad, CA) according to the manufacturer's instructions, purified, and separately stored at −20 °C for further analysis to be carried out as described in the following (Han and Gu 2013; Li et al. 2013b). For the 16S rRNA gene-based PCR amplification of amplicons in samples, in a final volume of 25 μL, the PCR mixture contained 1 μL of extracted template DNA (1–10 ng), 5 μL of 5× GoTaq Flexi buffer (Promega, Hong Kong) and 2.5 μL of $MgCl_2$ (25 mM, Promega), 0.5 μL of dNTPs (10 mM, Invitrogen, Hong Kong), 1 μL BSA (stock 10 mg mL^{-1}), 0.5 μL of each forward (Brod541F: 5′-GAGCACGTAGGTGGGTTTGT-3′) and reverse primer (Amx820R: 5′- AAAACCCCTCTACTTAGTGCCC- 3′) (20 μM), and 0.2 μL of GoTaq Flexi polymerase (5 U mL^{-1}, Promega, Hong Kong). The PCR program consisted of 95 °C of initial denaturation for 5 min, 33 cycles of 95 °C for 45 s, 54 °C for 30 s, 72 °C for 50 s, followed by

Table 1 A summary of 16S rRNA gene-based PCR primers for detection of anammox bacteria in this study

Primer set	Sequences 5′–3′	*E. coli* position	Specificity group	References
An7f	GGCATGCAAGTCGAACGAGG	7–26	*Ca.* Kuenenia, *Ca.* Brocadia	Penton et al. (2006)
Pla46F	GACTTGCATGCCTAATCC	46–63	*Planctomycetes*	Neef et al. (1998)
Amx368F	CCTTTCGGGCATTGCGAA	368–385	All anammox bacteria	Schmid et al. (2003)
A438f	GTCRGGAGTTADGAAATG	438–455	All anammox bacteria	Humbert et al. (2012)
Brod541F	GAGCACGTAGGTGGGTTTGT	541–560	*Ca.* Scalindua	Penton et al. (2006)
A684r	ACCAGAAGTTCCACTCTC	667–684	All anammox bacteria	Humbert et al. (2012)
Amx820R	AAAACCCCTCTACTTAGTGCCC	820–841	*Ca.* Kuenenia, *Ca.* Brocadia	Schmid et al. (2000)
BS820R	TAATTCCCTCTACTTAGTGCCC	820–841	*Ca.* Scalindua	Kuypers et al. (2003)
An1388r	GCTTGACGGGCGGTGTG	1,372–1,388	*Ca.* Scalindua	Penton et al. (2006)
Brod1260R	GGATTCGCTTCACCTCTCGG	1,241–1,260	*Ca.* Scalindua	Penton et al. (2006)
1392r	ACGGGCGGTGTGTAC	1,392–1,406	Universal bacteria	Ferris et al. (1996)

10 min of final elongation at 72 °C. PCR products were analyzed by electrophoresis in 1 % agarose gels in TAE buffer (20 mM Tris-acetate pH 8.0; 0.5 mM EDTA) at 125 V, 400 mA for 25 min (Amersham Biosciences, Electrophoresis Power Supply 301). The gels were stained by addition of GelRed nucleic acid stain (Biotium) and photographed using a Bio-Rad® GelDoc™ station.

Phylogenetic and quantitative analyses

Clone libraries were constructed with the PCR-amplified products from these samples and the two PCR primer sets. After sequencing, the obtained sequences were aligned, and phylogenetic trees were constructed using MEGA version 5.05 and subjected to phylogenetic inference using the neighbor-joining algorithm followed by 1,000 times of bootstrap.

The 16S rRNA gene abundance of anammox bacteria in all 11 samples was determined in triplicate using an Applied Biosystems StepOnePlus™ Real-Time PCR System. The quantification procedure was based on the fluorescent dye SYBR Green I. Each reaction was performed in a 20-μL volume containing 10 μL of Power SYBR Green PCR Master Mix (Applied BioSystems), 1 μL of DNA template (1–10 ng), 0.2 μL of each primer (20 μM; Brod541F, AMX820R), and 8.8 μL of autoclaved DD water. The PCR profile was 10 min at 95 °C, followed by a total of 40 cycles of 15 s at 95 °C, 1 min at 54 °C, and 15 s at 72 °C. A standard plasmid carrying 16S rRNA gene was generated by amplifying the gene from DNA extracted from samples described above and cloned into the pMD 18-T Vector (Takara). The plasmid DNA concentration was determined, and the gene abundance in terms of copy number of 16S rRNA gene was calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to a quantitative PCR assay in triplicate to generate an external standard curve. The correlation coefficient (r^2) of the standard curve was 0.96. Anammox bacterial community and statistical analyses of all sequences related to anammox bacteria 16S rRNA gene-amplified sequences were analyzed using the Distance-Based OTU and Richness (DOTUR) program to compare their diversity and richness (Schloss and Handelsman 2005). Operational taxonomic units (OTUs) for community analysis were defined at 3 % variation in nucleic acid sequences.

Nucleotide sequence accession numbers

Partial sequences of anammox bacterial 16S rRNA gene amplified with primer set Brod541F/Amx820R from a wastewater treatment plant, mangrove wetland sediments, and a fresh reservoir have been deposited in GenBank under accession numbers KF286707–KF286841. Sequences from the South China Sea were obtained from a previous study (Li et al. 2013b) under accession numbers HQ665558–HQ665699.

Results

PCR and statistical analyses

The successful 16S rRNA gene PCR amplification with primer set Brod541F/Amx820R from all samples resulted in 11 clone libraries, of which diversity and richness indices of anammox bacteria at 3 % difference cutoff were analyzed using the DOTUR program (Table 2). In order to make a comparison, six samples from the South China Sea and three samples from Mai Po wetland were pooled together, respectively, with primer

Table 2 Analysis of DNA sequences obtained by PCR amplification with primer set A438f/A684r and Brod541F/AMX820R, clone libraries, and diversity and richness indices from the 11 samples based on 16S rRNA genes with 3 % nucleotide variation cutoff. Efficiency indicates the percentage of anammox bacteria among all obtained sequences in the relevant clone library

Samples	No. of clones	OTUs	Efficiency (%)	Diversity and richness	
				Shannon–Wiener	Chao index
E702	42/21	11/2	93.3/100	1.78/0.57	29.0/2.0
E704	45/30	9/4	100/100	2.01/1.57	9.0/8.5
E707	35/24	13/4	80.0/100	2.13/1.18	45.0/4.0
E708	30/20	10/3	83.8/100	2.12/0.85	10.5/3.0
E709	21/37	9/3	60.0/100	1.74/0.82	12.0/3.0
E525S	16/20	3/5	59.3/100	0.46/1.37	3.0/5.0
SCS[a]	189/152	19/6	81.5/100	2.13/0.36	29.7/7.0
1S	19/27	6/2	65.5/73.0	1.59/0.26	6.2/2.0
3S	19/30	6/2	86.4/93.8	1.59/0.66	8.00/2.0
3Sm	43/39	7/2	100/100	1.22/0.69	11.5/2.0
MP[b]	81/96	10/4	86.2/88.9	1.58/0.83	18.0/4.0
R2	24/23	10/5	100/85.2	2.02/1.11	12.0/5.25
A2	16/16	2/2	100/100	0.23/0.23	2.0/2.0

[a] Results with the above six SCS samples

[b] Results with the above three MP samples

sets A438f/A684r and Brod541F/Amx820R. For the six South China Sea (SCS) samples, 189 and 152 sequences were obtained with A438f/A684r and Brod541F/Amx820R, respectively. Considering the three Mai Po wetlands (MP) samples, 81 and 96 sequences were retrieved with A438f/A684r and Brod541F/Amx820R, respectively. The efficiencies of these two primer sets in amplifying anammox bacteria 16S rRNA genes are also summarized in Table 2, ranging from 59.3 to 100 % for A438f/A684r and 73.8 to 100 % for Brod541F/Amx820R. At 3 % nucleotide difference cutoff, the calculated numbers of OTUs with primer sets A438f/A684r and Brod541F/Amx820R showed large differences with the same set of samples. Generally, primer set A438f/A684r and Brod541F/Amx820R retrieved 19 and 6 OTUs from SCS samples, 10 and 4 OTUs from MP samples, 10 and 5 OTUs from freshwater reservoir sample, and 2 and 2 OUTs from WWTP samples, respectively. Besides, the Shannon–Wiener and Chao index also varied not only among different samples, but also between the two clone libraries with either of the PCR primers on any of the samples. Rarefaction curves of 16S rRNA gene sequences retrieved from the four types of samples with these two PCR primer sets are shown in Fig. 1. Apparently, the curves with primer set A438f/A684r showed steeper slopes than those of Brod541F/Amx820R, indicating much more diverse anammox bacteria recovered with the former PCR primer set.

Phylogenetic analyses

A phylogenetic tree was constructed using all obtained sequences with primer sets A438f/A684r and Brod541F/Amx820R and also the relevant sequences from NCBI (Fig. 2). A total of 14 clusters can be visualized. Besides the large *Scalindua* group, a combined group consisting of *Jettenia*, *Anammoxoglobus*, *Kuenenia*, and *Brocadia* was also established and at the same time, with two novel clusters (tentatively named as R2-New-1 and R2-New-2) with 48.9 % (23/47) sequences from R2, contributing to Cluster R2-New-1. Two sequences from R2 recovered with PCR primer set A438f/A684r and three with Brod541F/Amx820R fell into Cluster R2-New-2, which was proposed as a novel cluster described previously (Humbert et al. 2010, 2012; Quan et al. 2008; Tal et al. 2005). The similarity of clusters R2-New-1 and R2-New-2 to the closest phylotype species was 78.4–90.0 and 79.5–91.9 %, respectively (Table 3). Furthermore, 10.6 % (5/47) of R2 sequences and one A2 sequence contributed to the *Anammoxoglobus/Jettenia* cluster. Most of the A2 sequences (96.8 %, 31/32) were included in the *Brocadia anammoxidans*/*brasiliensis* cluster, which also contained 1.12 % (2/177) MP sequences. MP and R2 contributed 13.6 % (24/177) and 19.1 % (9/47) of sequences into *Kuenenia* cluster and *Brocadia fulgida/caroliniensis* cluster, respectively. Six reported subclusters (wagneri, arabica, brodae, zhenghei-I, zhenghei-II, and zhenghei-III) of the *Scalindua* group were all obtained in this study with both PCR primer sets, which is in agreement with previous studies considering the diversity of marine anammox bacteria (Hong et al. 2011a; Woebken et al. 2008). In addition, three SCS-specific clusters were also obtained, namely SCS-1, SCS-2, and SCS-3 subclusters. These subclusters in the present study corresponded to S1+S4, S3, and S2 in the previously constructed phylogenetic tree, respectively (Han and Gu 2013). The similarity of the above three putative new subclusters (SCS-1, SCS-2, and SCS-3) to the closest phylotypes was 78.4–94.6, 78.7–95.5, and 80.3–96.0 %, respectively (Table 2). There are 14.9 % (51/341) SCS sequences and 4.3 % (2/47) R2 sequences included in the SCS-1 subcluster. Only 0.3 % (1/341) SCS sequences fell into the SCS-2 subcluster and 5.9 % (20/341) SCS sequences to the SCS-3 subcluster.

Furthermore, consistent with a previous study, no SCS sequence fell into the wagneri subcluster which included 44.6 % (79/177) MP sequences, while only one MP sequence and two SCS sequences were clustered into zhenghei-III. The zhenghei-I subcluster contains 16.1 % (55/341) of sequences from SCS and one from MP. For the zhenghei-II subcluster,

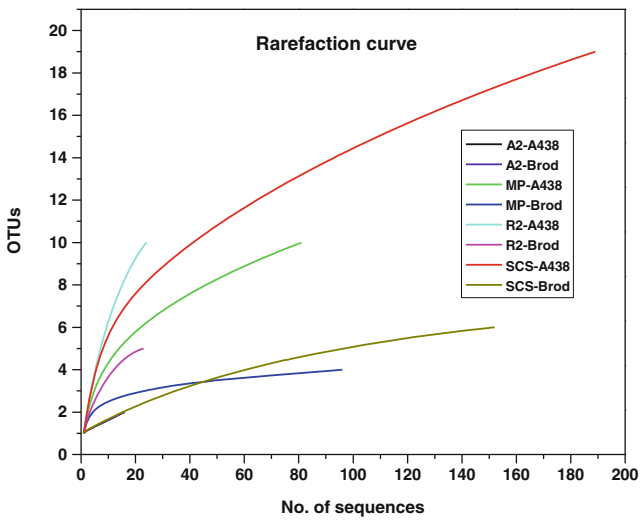

Fig. 1 Rarefaction curves of anammox bacteria 16S rRNA gene PCR-amplified sequences retrieved from different environmental samples with 3 % difference cutoff. *MP* Mai Po Nature Reserve; *SCS* South China Sea; *R2* freshwater reservoir; *A2* wastewater treatment plant. Primer sets used were indicated immediately after the sample name

Fig. 2 A phylogenetic tree constructed with distance and neighbor-joining method from an alignment of 16S rRNA gene sequences with some reference phylotypes from GenBank. PCR primer sets used were indicated after each sample name, and the numbers in brackets refer to the number of clones. The *numbers* at the nodes are percentage indicating the levels of bootstrap support based on 1,000 resampled data sets. Branch lengths correspond to sequence differences as indicated by the *scale bar*. *Red color* represents sequences obtained with primer set A438f/A684r, *blue color* represents sequences obtained with primer set and Brod541F/AMX820R; *square* indicates sequences from MP, *triangle* for R2, *diamond* for SCS, and *circle* for A2

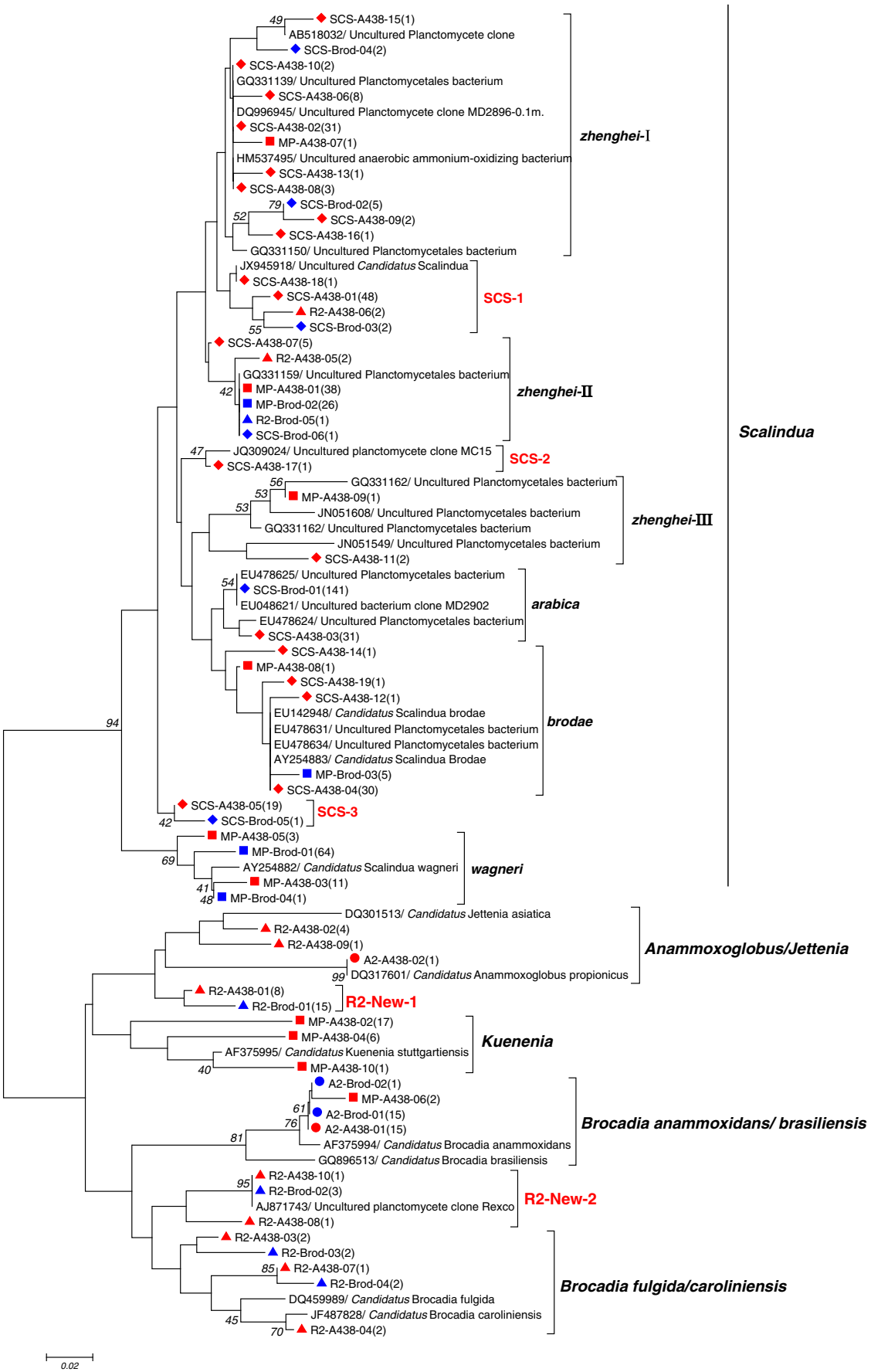

Table 3 Identities (percent) of 16S rRNA gene PCR-amplified sequence between new clusters obtained in this study and the known anammox bacteria phylotypes

New retrieved clusters	Known anammox bacteria phylotypes									
	S. zhenghei-I	*S. zhenghei*-II	*S. zhenghei*-III	*S. wagneri*	*S. brodae*	*S. arabica*	*B. fulgida*/*caroliniensis*	*B. anammoxidans*/*brasiliensis*	*Kuenenia*	*Anammoxoglobus* and *Jettenia*
SCS-1	93.2–93.9	93.2–94.9	90.9–93.9	86.4–90.0	90.9–92.6	94.6–92.0	78.4–80.5	89.0–89.0	82.9–84.3	80.2–81.4
SCS-2	92.0–93.9	95.5–95.5	92.0–92.9	89.8–91.9	92.9–93.9	93.3–93.4	78.7–84.8	83.8–85.4	85.9–87.9	82.8–85.9
SCS-3	93.4–95.5	95.5–96.0	89.8–92.9	92.0–92.4	94.4–94.9	93.6–95.5	80.3–85.9	85.9–86.4	83.8–86.4	84.8–83.8
R2-New-1	78.4–85.4	81.8–87.9	78.4–84.8	80.7–84.8	86.4–87.4	79.5–85.4	82.0–86.7	86.7–87.6	88.6–90.0	89.0–89.5
R2-New-2	79.5–85.4	80.7–86.9	79.5–85.9	81.8–84.8	87.4–87.9	80.7–85.4	85.2–91.9	89.0–90.5	88.1–89.5	85.7–88.1

there are 1.76 % (6/341) sequences from SCS, 36.2 % (64/171) from MP sequences, and 6.38 % (3/47) from R2 sequences. The big arabica subcluster contained 50.4 % (172/341) sequences from all South China Sea samples while the brodae subcluster has 3.5 % (6/171) sequences from MP and 9.68 % (33/341) from SCS sequences.

Quantification of anammox bacteria

The quantity of anammox bacteria in the 11 samples was estimated by the abundance of anammox bacterial 16S rRNA gene with the PCR primer sets Brod541F/Amx820R. The abundance of PCR-amplified anammox 16S rRNA gene ranged from 9.96×10^3 copies/g sediments (dry weight) in MP sample 1S to 2.58×10^7 copies/g granule (dry weight) in the A2 sample and 2.58×10^5 copies/g sediments in SCS sample E702 (dry weight) (Fig. 3). For South China Sea sediment samples, E702 had the highest abundance while the other five samples did not show large differences among them ($6.44–11.5\times10^4$ copies/g). The freshwater reservoir sample R2 had almost equal amounts of anammox bacteria with MP samples. The abundances of anammox bacteria 16S rRNA genes with PCR primer sets A438f/A684r and Brod541F/Amx820R are compared in Fig. 3. The relative abundances among all samples except for A2 and 3Sm with these two primer sets were similar while prime set A438f/A684r resulted in higher copies of 16S rRNA genes.

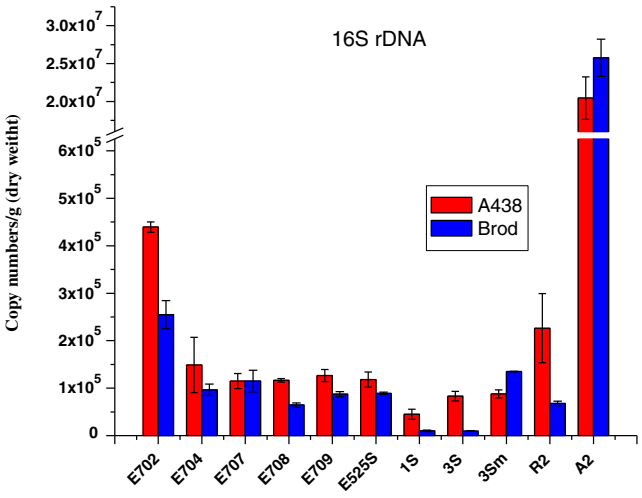

Fig. 3 A comparison of the abundance of anammox bacteria in the 11 samples indicated by abundance of 16S rRNA gene copies with PCR primer set A438 and Brod. *Error bars* represent standard deviations of triplicate experimental analyses

Comparison of anammox bacterial community structures

Based on the phylogenetic analysis, the recovered anammox bacterial community structures of SCS, MP, R2, and A2 samples with primer sets A438f/A684r and Brod541F/Amx820R were compared separately and pooled together (Fig. 4). For SCS samples, five *Scalindua* subclusters (zhenghei-I, SCS-1, SCS-3, arabica, and brodae) comprised the majority of recovered anammox bacteria 16S rRNA gene PCR-amplified sequences with PCR primer sets A438f/A684r, while only the arabica subcluster accounted for >80 % of the retrieved subclusters with PCR primer set Brod541F/Amx820R. Considering MP samples, *Scalindua* subclusters (zhenghei-II, wagneri) and cluster *Kuenenia* comprised the majority of recovered anammox bacteria 16S rRNA gene PCR-amplified sequences with PCR primer sets A438f/A684r, while no *Kuenenia* sequences were retrieved with PCR primer sets Brod541F/Amx820R. Regarding sample R2, two *Scalindua* subclusters zhenghei-II and SCS-1, clusters R2-New-1, R2-New-2 and *B. fulgida/caroliniensis* were found in both A438f/A684r and Brod541F/Amx820R clone libraries, while cluster *Anammoxoglobus/Jettenia* was only detected with A438f/A684r. Considering the WWTP sample A2, among all sequences obtained, the community composition was largely composed of cluster *B. anammoxidans/brasiliensis*, with only one sequence falling into cluster *Anammoxoglobus/Jettenia* by PCR primer sets A438f/A684r. The combined community structure was also analyzed with both clone library sequences among the four types of

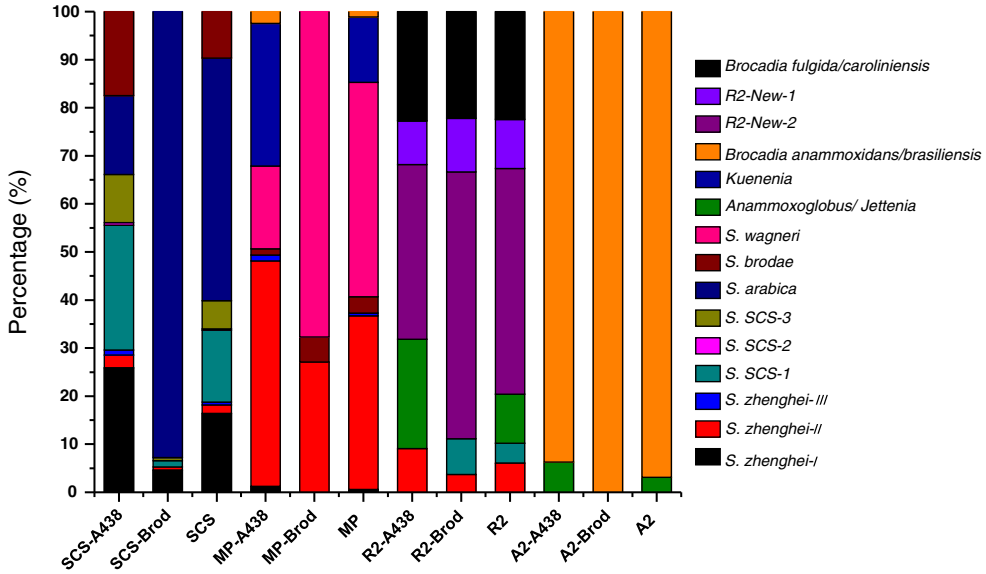

Fig. 4 Community structure of anammox bacteria in different environmental samples based on retrieved sequences amplified with PCR primer sets A438 and Brod, respectively

environmental samples. Generally, all *Scalindua* subclusters except wagneri were found in SCS samples, with arabica as the biggest subcluster. Two *Scalindua* subclusters (zhenghei-II and wagneri) and cluster *Kuenenia* contained the largest portion of the anammox bacterial community in MP samples. R2 showed a diverse anammox bacteria community with *Scalindua* subclusters zhenghei-II and SCS-1, cluster *Anammoxoglobus/Jettenia* and *Brocadia fulgida/caroliniensis,* and also two new clusters R2-New-1 and R2-New-2. The anammox bacterial community of sample A2 was apparently dominated by *B. anammoxidans/brasiliensis*.

Discussions

Comparison of the two 16S rRNA primer sets

The forward primer Brod541F was explored previously (Penton et al. 2006) based on four groups of *Ca.* Scalindua brodae (Schmid et al. 2003) with the reverse primer Brod1260R. These primer sets were considered *Scalindua* specific and successfully applied with 11 both geographically and biogeochemically diverse freshwater and marine sediments in that study (Penton et al. 2006). The reverse primer Amx820R was designed by Schmid et al. (2000) as a FISH probe in detecting anammox bacteria from a trickling filter biofilm in which the anammox bacteria community was dominated by *Ca.* Kuenenia stuttgartiensis. The reverse primer Amx820R usually worked well with primer Amx368F which was also designed by Schmid et al. (2003). The primer set Amx368F/Amx820R was subsequently applied in screening anammox bacteria from freshwater and coastal wetlands (Wang and Gu 2013b; Wang et al. 2013), rice paddy soils (Wang and Gu 2013a, b; Zhu et al. 2011), and marine sediments (Hong et al. 2011a, b). Recently, the combination of PCR forward primer Brod541F and reverse primer Amx820R is frequently adopted in the detection of anammox bacteria in coastal (Li et al. 2011a, c) and marine environments (Li et al. 2013b). Almost all known anammox bacteria could be recovered by this primer set, and the short length of PCR products (279 bp) made it much easier to obtain positive results from samples with low biomass.

PCR primer set A438f/A684r was designed by Humbert based on the retrieved anammox 16S rRNA sequences from a variety of terrestrial ecosystems including marshes, lakeshores, a contaminated porous aquifer, permafrost soil, and agricultural soil (Humbert et al. 2010). This PCR primer set was used to analyze the abundance of anammox bacteria in wetlands (Humbert et al. 2012), and it was subsequently used to analyze the community structure of anammox bacteria from various environments including marine sediments and coastal wetlands (Han and Gu 2013). Furthermore, PCR primer set A438f/A684r was highly recommended for both clone library construction and DGGE analysis based on the results of evaluating PCR primers for profiling anammox bacteria within freshwater environments (Sonthiphand and Neufeld 2013).

In this study, both of the PCR primer sets Brod541F/Amx820R and A438f/A684r showed high efficiency in amplifying the anammox bacteria 16S rRNA gene, indicating their ability in detecting anammox bacteria from various ecological niches. Based on the phylogenetic and community structure analysis, PCR primer sets A438f/A684r could recover more diverse anammox bacteria than primer sets Brod541F/Amx820R. This may be due to the differences in specificity and also the differences in the targeting region of the 16S rRNA gene between these two PCR primer sets. PCR primer Brod541F was designed as *Scalindua* specific, and even

though primer set Brod541F/Amx820R could recover other anammox genera such as *Brocadia* and *Kuenenia* from samples R2 and A2, only *Scalindua* sequences were obtained from MP samples. There were actually *Kuenenia* sequences in A438f/A684r clone libraries, indicating that the PCR primer set Brod541F/Amx820R may have limitations in revealing the complete anammox community information from samples in which *Scalindua* was dominating, but not the only anammox genera. The comparison of the quantitative PCR results with these two PCR primer sets also indicated that Brod541F/Amx820R may insufficiently retrieve the anammox bacteria from various environmental samples. This may be caused by the high specificity of Brod541F/Amx820R to *Scalindua* genera, which may fail to amplify other anammox genera. No big difference in community structure was found in sample R2 with the two PCR primer sets possibly because R2 was not dominated by *Scalindua*, and R2 contained highly diverse anammox bacteria as indicated by the rarefaction curve and also the two recovered new clusters. Results also added further evidences to our hypothesis that high preference of Brod541F/Amx820R targeting *Scalindua* specific 16S rRNA genes occurred in only the *Scalindua*-dominating samples. In addition, even though A438f/A684r was designed based on wetland-related sequences, it showed strong ability in recovering 16S rRNA gene sequences with low identities to known phylotypes from SCS sediment. Moreover, the anammox bacteria community structures of surface and deep marine sediments could only be differentiated in A438f/A684r clone libraries, further indicating a promising phylogenetic biomarker for screening various ecological samples.

Diversity of anammox bacteria in different environments

Based on the phylogenetic analysis, by comparing the community structure of anammox bacteria in Mai Po coastal wetlands, the South China Sea sediments, water reservoir, and wastewater treatment plants, a strong niche-specific distribution of anammox seems to be evident. The SCS sediments were dominated by five subclusters of *Scalindua* (zhenghei-I, SCS-1, SCS-3, arabica, and brodae), while the MP costal wetland was dominated by two subclusters of *Scalindua* (zhenghei-II and wagneri) and *Kuenenia* cluster. Even though both SCS and MP had *Scalindua* detected, they did not share the dominating subclusters, yielding evidence to the hypothesis that the variation of anammox bacteria community structures could serve as bio-indicator for anthropogenic/terrestrial nitrogen inputs in the Pearl River Delta (PRD) and beyond (Li et al. 2013a). The two R2-New clusters draw our special attention to the freshwater reservoir environment, and it also will encourage more research on the anammox community structure in water reservoir in more detailed investigations. Overall, primer set A438f/A684r is highly recommended for screening anammox bacteria from various ecological niches while Brod541F/Amx820R could be selectively used in non-*Scalindua*-dominating ecosystems, such as the wastewater system and anthropogenically impacted freshwater ecosystem.

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References

Borin S, Mapelli F, Rolli E, Song B, Tobias C, Schmid MC, De Lange GJ, Reichart GJ, Schouten S, Jetten M, Daffonchio D (2013) Anammox bacterial populations in deep marine hypersaline gradient systems. Extremophiles 17(2):289–299

Cao H, Hong Y, Li M, Gu J-D (2012) Community shift of ammonia-oxidizing bacteria along an anthropogenic pollution gradient from the Pearl River Delta to the South China Sea. Appl Microbiol Biotechnol 94(1):247–259

Dang HY, Chen RP, Wang L, Guo LZ, Chen PP, Tang ZW, Tian F, Li SZ, Klotz MG (2010) Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. Appl Environ Microbiol 76(21):7036–7047

Ferris MJ, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62(2):340–346

Han P, Gu J-D (2013) More refined diversity of anammox bacteria recovered and distribution in different ecosystems. Appl Microbiol Biotechnol 97(8):3653–3663

Han P, Li M, Gu J-D (2013) Biases in community structures of ammonia/ammonium-oxidizing microorganisms caused by insufficient DNA extractions from Baijiang soil revealed by comparative analysis of coastal wetland sediment and rice paddy soil. Appl Microbiol Biotechnol 97:8741–8756

Hong YG, Li M, Cao H, Gu J-D (2011a) Residence of habitat-specific anammox bacteria in the deep-sea subsurface sediments of the South China Sea: analyses of marker gene abundance with physical chemical parameters. Microb Ecol 62(1):36–47

Hong YG, Yin B, Zheng TL (2011b) Diversity and abundance of anammox bacterial community in the deep-ocean surface sediment from equatorial Pacific. Appl Microbiol Biotechnol 89(4):1233–1241

Humbert S, Tarnawski S, Fromin N, Mallet MP, Aragno M, Zopfi J (2010) Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J 4(3):450–454

Humbert S, Zopfi J, Tarnawski SE (2012) Abundance of anammox bacteria in different wetland soils. Env Microbiol Rep 4(5):484–490

Jetten MS, Niftrik LV, Strous M, Kartal B, Keltjens JT, OpdenCamp HJ (2009) Biochemistry and molecular biology of anammox bacteria. Crit Rev Biochem Mol Biol 44:65–84

Junier P, Molina V, Dorador C, Hadas O, Kim OS, Junier T, Witzel JP, Imhoff JF (2010) Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. Appl Microbiol Biotechnol 85(3):425–440

Kartal B, Rattray J, van Niftrik LA, van de Vossenberg J, Schmid MC, Webb RI, Schouten S, Fuerst JA, Damste JS, Jetten MS, Strous M (2007) *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst Appl Microbiol 30(1):39–49

Kartal B, van Niftrik L, Rattray J, van de Vossenberg JL, Schmid MC, Sinninghe Damste J, Jetten MS, Strous M (2008) *Candidatus*

'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol Ecol 63(1):46–55

Kartal B, Geerts W, Jetten MS (2011) Cultivation, detection, and ecophysiology of anaerobic ammonium-oxidizing bacteria. Methods Enzymol 486:89–108

Kirkpatrick JB, Fuchsman CA, Yakushev E, Staley JT, Murray JW (2012) Concurrent activity of anammox and denitrifying bacteria in the Black Sea. Front Microbiol 3:256

Konovalov SK, Fuchsman CA, Belokopitov V, Murray JW (2008) Modeling the distribution of nitrogen species and isotopes in the water column of the Black Sea. Mar Chem 111(1–2):106–124

Kuypers MM, Sliekers AO, Lavik G, Schmid M, Jorgensen BB, Kuenen JG, Sinninghe Damste JS, Strous M, Jetten MS (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422(6932):608–611

Lam P, Jensen MM, Lavik G, McGinnis DF, Muller B, Schubert CJ, Amann R, Thamdrup B, Kuypers MM (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. Proc Natl Acad Sci U S A 104(17):7104–7109

Li M, Hong YG, Klotz MG, Gu J-D (2010) A comparison of primer sets for detecting 16S rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. Appl Microbiol Biot 86(2):781–790

Li M, Cao H, Hong YG, Gu J-D (2011a) Seasonal dynamics of anammox bacteria in estuarial sediment of the mai po nature reserve revealed by analyzing the 16s rRNA and hydrazine oxidoreductase (*hzo*) genes. Microbes Environ 26(1):15–22

Li M, Ford T, Li X, Gu J-D (2011b) Cytochrome cd_1-containing nitrite reductase encoding gene *nirS* as a new functional biomarker for detection of anaerobic ammonium oxidizing (anammox) bacteria. Environ Sci Technol 45(8):3547–3553

Li M, Hong YG, Cao HL, Gu J-D (2011c) 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(8):1780–1790

Li M, Cao H, Hong Y, Gu J-D (2013a) Using the variation of anammox bacteria community structures as a bio-indicator for anthropogenic/terrestrial nitrogen inputs in the Pearl River Delta (PRD). Appl Microbiol Biotechnol. doi:10.1007/s00253-013-4990-y

Li M, Hong Y, Cao H, Gu J-D (2013b) Community structures and distribution of anaerobic ammonium oxidizing and *nirs*-encoding nitrite-reducing bacteria in surface sediments of the South China Sea. Microb Ecol. doi:10.1007/s00248-012-0175-y

Li M, Hong Y, Cao H, Klotz MG, Gu J-D (2013c) Diversity, abundance, and distribution of NO-forming nitrite reductase-encoding genes in deep-sea subsurface sediments of the South China Sea. Geobiology 11(2):170–179

Neef A, Amann R, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144(Pt 12):3257–3266

Oshiki M, Shimokawa M, Fujii N, Satoh H, Okabe S (2011) Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus* Brocadia sinica'. Microbiology 157(Pt 6):1706–1713

Penton CR, Devol AH, Tiedje JM (2006) Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. Appl Environ Microbiol 72(10):6829–6832

Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. Environ Microbiol 10(11):3130–3139

Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71(3):1501–1506

Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer KH, Wagner M (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 23(1):93–106

Schmid M, Walsh K, Webb R, Rijpstra WI, van de Pas-Schoonen K, Verbruggen MJ, Hill T, Moffett B, Fuerst J, Schouten S, Damste JS, Harris J, Shaw P, Jetten M, Strous M (2003) *Candidatus* "Scalindua brodae", sp. nov., *Candidatus* "Scalindua wagneri", sp. nov., two new species of anaerobic ammonium oxidizing bacteria. SystAppl Microbiol 26(4):529–538

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, Sinninghe Damste JS, Kuypers M, Revsbech NP, Mendez R, Jetten MS, Strous M (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl Environ Microbiol 71(4):1677–1684

Song B, Tobias CR (2011) Molecular and stable isotope methods to detect and measure anaerobic ammonium oxidation (anammox) in aquatic ecosystems. Methods Enzymol 496:63–89

Sonthiphand P, Neufeld JD (2013) Evaluating primers for profiling anaerobic ammonia oxidizing bacteria within freshwater environments. PLoS One 8(3):e57242

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, Medigue C, Collingro A, Snel B, Dutilh BE, Op den Camp HJ, 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 HW, Weissenbach J, Jetten MS, Wagner M, Le Paslier D (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440(7085):790–794

Tal Y, Watts JE, Schreier HJ (2005) Anaerobic ammonia-oxidizing bacteria and related activity in Baltimore inner harbor sediment. Appl Environ Microbiol 71(4):1816–1821

van de Vossenberg J, Rattray JE, Geerts W, Kartal B, van Niftrik L, van Donselaar EG, Sinninghe Damste JS, Strous M, Jetten MS (2008) Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production. Environ Microbiol 10(11):3120–3129

Wang J, Gu J-D (2013a) Dominance of *Candidatus* Scalindua species in anammox community revealed in soils with different duration of rice paddy cultivation in Northeast China. Appl Microbiol Biotechnol 97(4):1785–1798

Wang YF, Gu J-D (2013b) Higher diversity of ammonia/ammonium-oxidizing prokaryotes in constructed freshwater wetland than natural coastal marine wetland. Appl Microbiol Biotechnol 97(15):7015–7033

Wang YF, Feng YY, Ma X, Gu J-D (2013) Seasonal dynamics of ammonia/ammonium-oxidizing prokaryotes in oxic and anoxic wetland sediments of subtropical coastal mangrove. Appl Microbiol Biotechnol 97(17):7919–7934

Woebken D, Lam P, Kuypers MM, Naqvi SW, Kartal B, Strous M, Jetten MS, Fuchs BM, Amann R (2008) A microdiversity study of anammox bacteria reveals a novel *Candidatus* Scalindua phylotype in marine oxygen minimum zones. Environ Microbiol 10(11):3106–3019

Zhu G, Wang S, Wang Y, Wang C, Risgaard-Petersen N, Jetten MS, Yin C (2011) Anaerobic ammonia oxidation in a fertilized paddy soil. ISME J 5(12):1905–1912