Appl Microbiol Biotechnol (2013) 97:9875–9883
DOI 10.1007/s00253-013-4990-y

ENVIRONMENTAL BIOTECHNOLOGY

Using the variation of anammox bacteria community structures as a bio-indicator for anthropogenic/terrestrial nitrogen inputs in the Pearl River Delta (PRD)

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Received: 9 April 2013 / Revised: 1 May 2013 / Accepted: 9 May 2013 / Published online: 2 June 2013

Abstract The variation of anammox bacteria community composition was evaluated in sediments collected from the Pearl River Delta area with an anthropogenic/terrestrial input gradient. Results indicated that the community composition of anammox bacteria shifted from estuarine environment to the South China Sea deep ocean along with the anthropogenic/terrestrial input gradient, where *Scalindua* genus of anammox bacteria predominated in the area with less anthropogenic/terrestrial influences, such as in the open oceanic area, while genera of *Kuenenia*/*Brocadia* anammox bacteria have higher proportions in the area with higher anthropogenic/terrestrial impacts. The canonical correspondence analysis demonstrated that salinity, organic matter contents, and ratio of NH_4^+ to (NO_2^-+NO_3^-) strongly affected the shifting of anammox bacterial community compositions within the same gradients. The results obtained in this study, together with the similar variation of anammox bacteria community structures in other several estuaries in the world, indicated that anammox bacteria might have a habitat-specific distribution pattern according to their living habits, and their community composition could be served as a bio-indicator to monitor the anthropogenic/terrestrial N inputs in coastal environments.

Keywords Anammox bacteria · Community structure · Bio-indicator · Anthropogenic/terrestrial nitrogen inputs · Pearl River Delta

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-4990-y) contains supplementary material, which is available to authorized users.

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Introduction

Estuaries, connecting the freshwater and marine environment, are among the most dynamic and the productive ecosystems on Earth. However, they are under threat from anthropogenic N loading, resulting in various environmental problems. The Pearl River Delta (PRD), the low-lying area surrounding the Pearl River Estuary (PRE) where the Pearl River flows into the South China Sea (SCS), is one of the fast-growing economic regions and is a major manufacturing center of the world with a population of approximately 20 million. In the PRD, many important cities in China, such as Hong Kong, Macau, Guangzhou, Shenzhen, and Dongguan, are located in this area; thus, the long-term sustainability of the regional economy and environmental quality is the central focus in urban planning. However, as one of the most densely urbanized regions in the world, PRD is notoriously polluted by sewage and industrial wastewater as observed on the steadily deterioration of water quality in recent years (Yin and Harrison 2007). It has been reported that 4.7~8.3 billion tons of sewage were discharged into the ocean off the coast of PRD area in each year (http://www.gdofa.gov.cn/index.php/Catagories/index/id/247). Pollutions are evident in the PRD, and among them, reactive N species (NO_3^-, NO_2^-, NO_x, etc.) are a class of the most important pollutants in the PRE. In the water column, the inorganic N is high accounting for 85 % of total N, while NO_3–N is 95 % of inorganic N, but both NO_2–N

and NH_4–N are low. In contrast, the content of total N is relatively high in the surface sediment on average 1,649 mg kg^{-1}, in which 83 % are organic N that averaged at 1,374 mg kg^{-1}. NH_4–N is the main form of inorganic N on average of 209 mg kg^{-1}, with NO_3–N and NO_2–N together at 54 mg kg^{-1} (http://www.gdofa.gov.cn/index.php/Catagories/index/id/247). According to the available evidence, the enrichment of N in subtropical PRE coastal water and sediment has resulted in various symptoms of eutrophication and other many associated consequences, including the low diversity of benthic infauna (Shen et al. 2010) and the increasing frequency of algal blooms (Yin and Harrison 2007; Yin et al. 2004). Given that the demand for N in food production is continuing to increase, the intensity of pollution from N use may increase by N discharges associated with human activities, such as untreated domestic and industrial wastewaters, into estuarine and coastal environments (Galloway et al. 2008). The additional anthropogenic reactive nitrogen is expected to affect the chemistry of the atmosphere, and the composition and function of the terrestrial and aquatic ecosystems, with possible implications even for global climate change (Mulder et al. 1995; Vandegraaf et al. 1995). Although limited reports have been involved in the N cycle in the PRD (Dai et al. 2008; Wang et al. 2012; Yin et al. 2001; Zhang et al. 1999), no mechanism for the progressively worsening environmental conditions has been formulated.

Current knowledge on N cycle has not been fully accounted for the N flux in estuarine ecosystems, but the intensity and duration of estuarine eutrophication and the rate of estuarine recovery strongly depend on microbial N removal processes, including anammox and denitrification (Nicholls and Trimmer 2009). Denitrification has been widely studied in various estuaries, while anammox is much less studied in estuarine ecosystems. Existing studies indicate that anammox rates and its contribution to total N_2 production (anammox significance) are system specific and controlled in part by reaction-scale substrate limitations and by environmental parameters (Dale et al. 2009). Furthermore, the community structure of anammox bacteria, linking to their activities, has shown a strong variation along with environmental gradients in natural ecosystems, such as the estuaries (Nicholls and Trimmer 2009; Rich et al. 2008; Risgaard-Petersen et al. 2004; Wang et al. 2012). Several recent reports also indicated that the structures of anammox bacterial communities might be strongly affected by anthropogenic pollution inputs (Amano et al. 2011; Dang et al. 2010; Li et al. 2010, 2011a), and our previous studies have evaluated the responses of aerobic and anaerobic ammonia/ammonium-oxidizing microorganisms to anthropogenic pollution in coastal environments, providing some hints that anammox bacteria may serve as bio-indicators for environmental quality (Cao et al. 2011; Li et al. 2011a); however, more evidence is needed to more comprehensively elucidate this hypothesis.

To better understand the relationship of anammox bacteria communities and the anthropogenic/terrestrial nitrogen inputs, this study will investigate the variation of anammox bacterial community structures from PRD coastal sediments to SCS open ocean surface and subsurface sediments, which is representing an anthropogenic or terrestrial input gradient. In addition, the results of this study will also be compared with several other estuarine coastal ecosystems, including Yodo River Estuary, Cape Fear River Estuary, and Colne Estuary. The results indicate that the anammox bacteria community structures shifted along with the anthropogenic/terrestrial input gradients, and the variation of their community composition could serve as a bio-indicator to monitor the anthropogenic or terrestrial inputs to marine coastal environments.

Materials and methods

Data collection and description Three different ecosystems selected in this study are the estuary, the mangrove wetland, and the SCS, and the details of the sampling sites are shown in Fig. 1. Based on the previous studies, the anammox bacteria community structures have been independently described base on the 16S rRNA and *hzo* genes in three ecosystems (Hong et al. 2011; Li et al. 2010, 2011a, b, 2013a, b). Sequences obtained from these three ecosystems were divided into five groups, representing as summer estuarine sediments (MP-5), winter estuarine sediments (MP-11), mangrove sediments (mangrove), and surface (SCS-SS) and subsurface sediments (SCS-SB) of the SCS. Based on the basic physicochemical characteristics and their geological location, five group sediment samples form an anthropogenic/terrestrial input gradient with different influences (Table 1). In addition, variations of anammox bacteria community structures in several estuarine ecosystems, including Yodo River Estuary in Japan, Cape Fear River Estuary in USA, and Colne Estuary in UK, are also analyzed to parallel confirm the influences of anthropogenic or terrestrial inputs on the anammox bacterial community structures.

Phylogenetic and statistical analyses All sequences of anammox bacterial 16S rRNA (nucleic acids) and *hzo* (amino acids) genes retrieved from the selected sampling sites of the present study are aligned using the ClustalW program (Thompson et al. 1994). Phylogenetic trees of 16S rRNA and *hzo* genes were constructed by MEGA 5.0 with the neighbor-joining method with 1,000 bootstrap replicate resampling method to estimate the confidence intervals of the tree nodes. Sequences of 16S rRNA and *hzo* genes were analyzed using the DOTUR program to compare their diversity and richness (Schloss and Handelsman 2005). Three percent cutoff for nucleotides (16S rRNA gene) and 5 % amino acid (*hzo* gene) sequence variations to define an

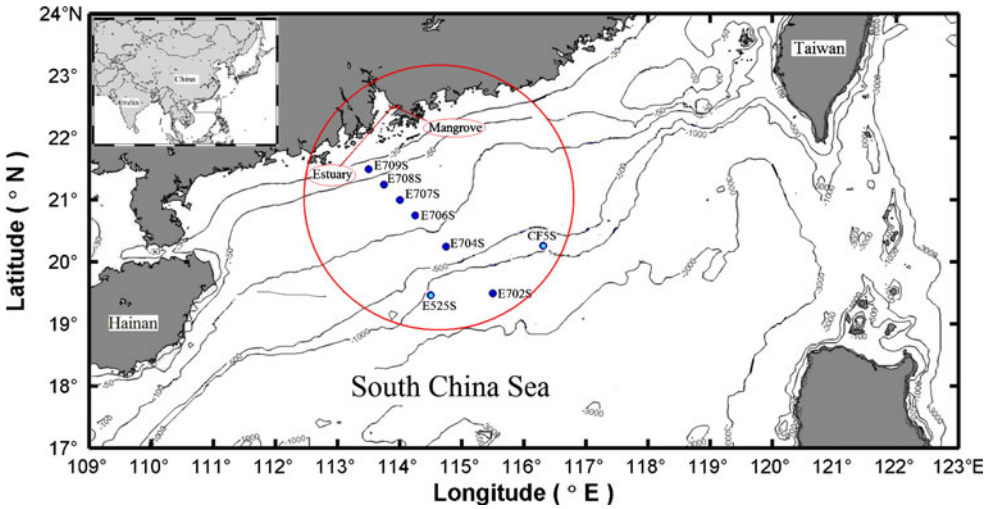

Fig. 1 Sampling locations of this study

operational taxonomic unit (OTU) (Schloss and Handelsman 2005). The anammox bacterial community compositions were analyzed based on the sequences of phylogenetic affiliation and their proportions in each sequence groups. Canonical correspondence analysis (CCA) was performed in CANOCO 4.5 for Windows to identify the relationships between anammox bacteria community structures and environmental parameters.

Variation of anammox bacterial community structures in other estuarine ecosystems The variation of anammox bacteria community compositions in several other estuarine ecosystems was also calculated based on the available 16S rRNA gene sequences in the database. These estuarine ecosystems include the Yodo River Estuary in Japan (AB522738-AB522761), the Cape Fear River Estuary in USA (Dale et al. 2009), and the Colne Estuary in UK (Dong et al. 2009), and the proportions of different phylogenetic groups of anammox bacteria were calculated along with the anthropogenic or terrestrial input gradients in each ecosystem.

Nucleotide sequence accession numbers The GenBank accession numbers of sequences reported in the PRD are the following: GQ427230 to GQ427485 and HM209472 to HM209609 for MP-5 and MP-11, HQ665558 to HQ66592 for SCS-SS, GQ331139 to GQ331201 and GQ1202 to GQ331244 for SCS-SB (16S rRNA gene), GQ427486 to GQ427673 and HM209610 to HM209725 for MP-5 and MP-11, GQ849414 to GQ849421 and HQ665927 to HQ666219 for SCS-SS, and GQ331245 to GQ331332 for SCS-SB (*hzo* gene).

Results

Phylogenetic diversity analysis Diversity of anammox bacteria in the PRD was evaluated through the phylogenetic analyses of 16S rRNA and *hzo* genes, and two biomarkers of anammox bacteria obtained 41 and 136 OTUs from all selected sample groups, respectively. The OTU numbers showed similar variation trends with the values of Shannon, Chao, and Simpson diversity indices, indicating samples from the SCS-SS and MP-5 have a relatively higher diversity of anammox bacteria, while the lowest diversity of anammox bacteria was found in the SCS-SB samples (Table 2). From the phylogenetic relationship of anammox bacteria, 16S rRNA genes could be divided into six different phylogenetic groups, including *Kuenenia* cluster, *Scalindua wagneri* cluster, *Scalindua zhenghei* clusters I to III, and *Scalindua arabica*/*brodae* cluster, while the *hzo* genes were

Table 1 Physicochemical characteristic of sediment samples used in this study

Sampling sites	Sample numbers	Sequences groups	Salinity (‰)	pH	NH_4^+ (mM)[a]	(NO_2^- + NO_3^-) (μM)[a]	NH_4^+/(NO_2^- + NO_3^-)	Organic matter (%)
Estuary (summer)	5	MP-5	3.52–7.05	6.24–7.18	11.01–12.28	1.15–9.38	10.90–142.9	10.0–17.9
Estuary (winter)	5	MP-11	13.82–26.36	7.41–7.55	0.95–2.65	0.94–3.66	126.9–326.1	9.2–13.7
Mangrove (wetland)	6	Mangrove	17.10–20.62	5.68–7.24	0.25–0.68	3.29–28.24	12.57–205.6	11.6–17.2
SCS (surface)	12	SCS-S	34.21–34.58	7.50–8.21	0.12–0.66	2.21–12.88	9.34–513.2	1.74–9.29
SCS (subsurface)	4	SCS-SB	34.55–34.56	8.13–8.15	0.16–0.20	1.39–1.51	0.12–0.32	5.40–7.31

[a] The concentrations in pore water

Table 2 Diversity characteristics of anammox bacteria in present study

Markers	Groups	Sequence numbers	OTUs	Shannon	Chao	Simpson
16S rRNA	MP-5	207	16	2.01	19.3	0.17
	MP-11	187	10	1.58	11.5	0.26
	Mangrove	152	8	1.68	8.0	0.23
	SCS-SS	230	16	1.94	16.6	0.20
	SCS-SB	31	5	1.31	5.0	0.30
	Total	807	41	2.78	45.0	0.09
hzo	MP-5	185	29	2.48	46.5	0.12
	MP-11	119	18	2.18	21.0	0.17
	Mangrove	212	31	2.15	58.3	0.23
	SCS-SS	199	56	3.65	101.9	0.03
	SCS-SB	38	8	1.85	8.5	0.15
	Total	753	136	3.90	224.2	0.04

grouped into five different phylogenetic clusters representing as *Kuenenia* cluster and *Scalindua* clusters 1 to 4, respectively. Furthermore, different phylogenetic groups of anammox bacteria also showed that habitat-specific characteristics, such as *S. zhenghei* I of 16S rRNA and *Scalindua* cluster 2 of *hzo*, were only detected in the SCS surface and subsurface sediments (Figs. 2 and 3).

Community structure variation After the phylogenetic diversity analysis, anammox bacteria in each phylogenetic group were calculated to clearly understand the community compositions and their variation in the five selected sample groups (Fig. 4). From the community compositions, it clearly showed that different phylogenetic groups of anammox bacteria in five sample groups have quite different proportions. In 16S rRNA gene database, the proportion of *S. arabica*/*brodae* cluster decreased from the SCS-SB (65.8 %) to the MP-5 (13.1 %) along with the increasing anthropogenic/terrestrial inputs, while *S. zhenghei* II group has relatively higher proportions in estuary and mangrove sediment samples than that in the SCS surface and subsurface sediments (Fig. 4a). However, the proportion of anammox bacterial *hzo* gene in *Kuenenia* cluster increased from under detection to 87.8 % from the SCS to the MP-5 sediment samples, and *hzo Scalindua* cluster 4 also has much higher proportions in the SCS samples than that in mangrove and estuary sediment samples (Fig. 4b).

Furthermore, the anammox community compositions in the Cape Fear River Estuary of USA (Dale et al. 2009), the Yodo River Estuary of Japan, and the Colne Estuary of UK (Dong et al. 2009) were also analyzed. Although no 16S rRNA gene sequences were found to be related to the *S. zhenghei* I–III in these estuaries, the relative proportion of *S. wagneri* cluster also decreased from the marine sites to the terrestrial sites along with the salinity decreasing gradients, while the *Brocadia*/*Kuenenia* clusters showed the opposite variation patterns at the same gradient (Fig. S1, results of Yodo River Estuary and the Cole Estuary were not shown in here). The variations of anammox bacteria community in these estuaries were consistent with that of our study.

Correlations between anammox bacterial community compositions and environmental factors The CCA results showed that the CCA axes in 16S rRNA gene and hydrazine oxidoreductase (HZO) sequence-deduced schemes could explain more than 43.6 and 65.0 % of the cumulative variance of the correlation between the environmental factors and the anammox bacterial community distribution, respectively (Fig. 5). Furthermore, both 16S rRNA and HZO sequences indicated that all of the anammox bacterial assemblages fell into three groups, representing as MP-11 and mangrove, MP-5, and SCS (Fig. 5). From the diversity of anammox bacteria, it could be found that salinity negatively correlated with the anammox bacteria in *Kuenenia*, *S. wagneri*, and *S. zhenghei* II clusters and positively correlated with *S. arabica*/*brodae* and *S. zhenghei* I clusters in the 16S rRNA CCA plot, while in the HZO CCA plot, salinity negatively correlated with the anammox bacteria in *Kuenenia* and *Scalindua* cluster 1 and positively correlated with *Scalindua* clusters 2 and 4, indicating *S. arabica*/*brodae* and *S. zhenghei* I clusters of 16S rRNA gene as possible coordinate with *Scalindua* clusters 2 and 4 of HZO sequences. Furthermore, the ratio of NH_4^+ to (NO_2^- + NO_3^-) and organic matter also positively correlated with anammox bacteria in the *Kuenenia* cluster and *S. zhenghei* II and *S. wagneri* clusters, respectively (Fig. 5). From the correlations between the distribution of anammox bacteria and environmental factors, both 16S rRNA and HZO CCA plots also indicated the same results that salinity and pH are positively correlated with anammox bacteria distribution in the SCS samples and organic matter, concentrations of $NH4^+$ and (NO_2^- + NO_3^-) positively correlated with the distribution in MP-11 and mangrove samples, and the ratio of NH_4^+ to (NO_2^- + NO_3^-) positively correlated with the distribution in MP-5 samples (Fig. 5).

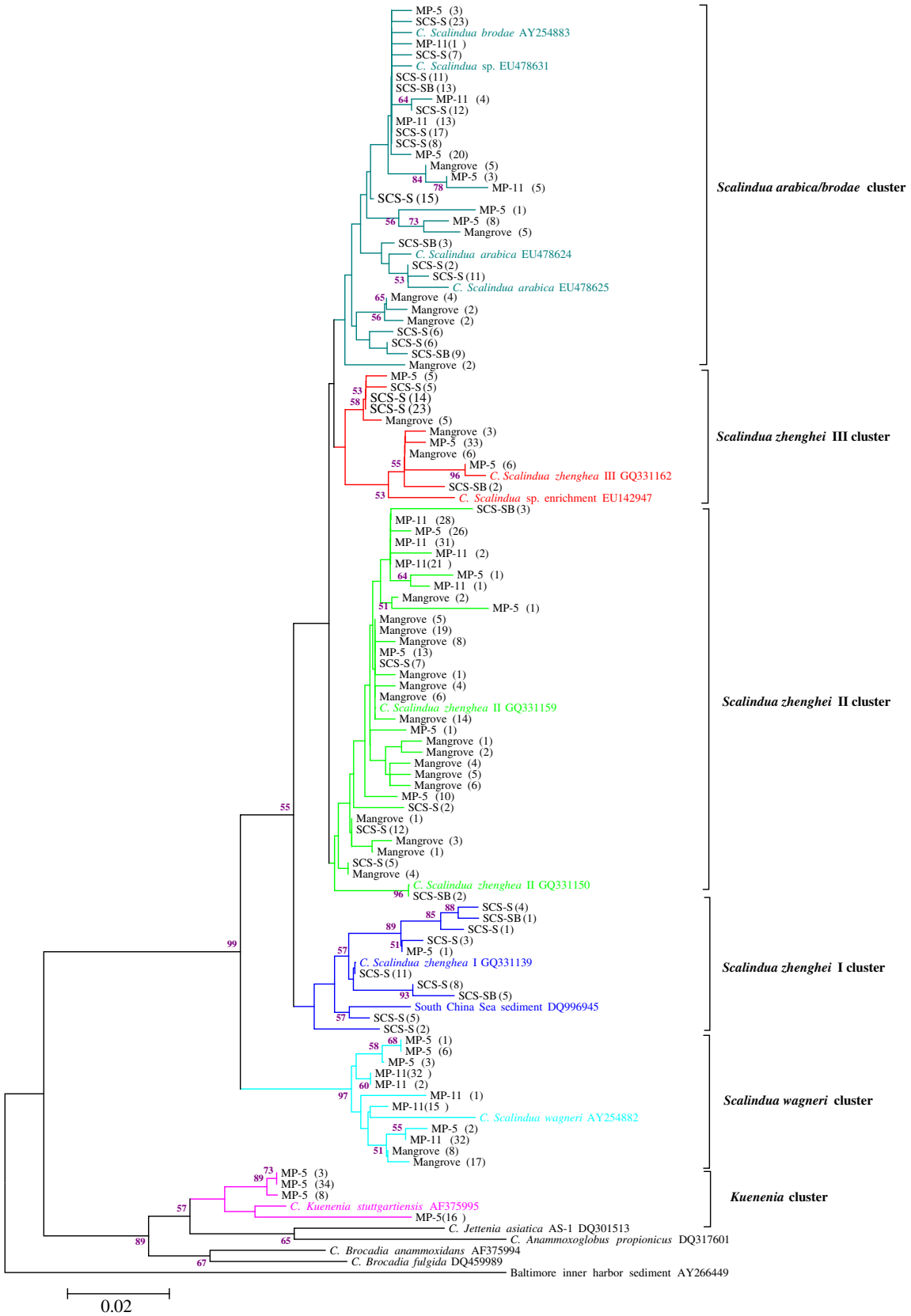

Fig. 2 Phylogenetic relationships of anammox bacteria represented by 16S rRNA gene sequences

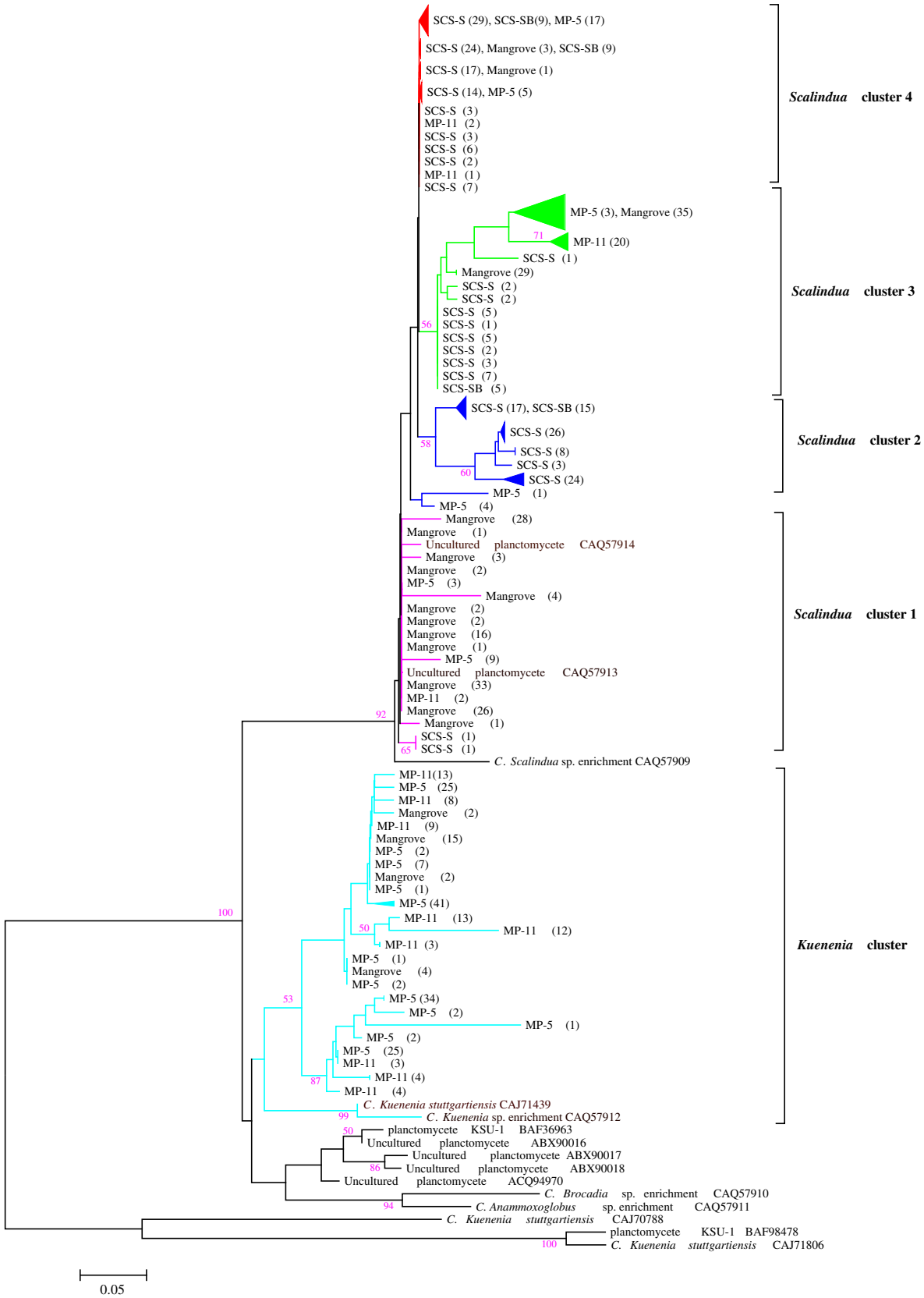

Fig. 3 Phylogenetic relationships of anammox bacteria represented by HZO sequences

Discussion

In this study, the dynamics of anammox bacteria community structure in five group sediment samples collected from three ecosystems located in the PRD were evaluated, where these sediment samples form an anthropogenic/terrestrial input gradient. Through the evaluations of two molecular biomarkers (16S rRNA gene and HZO), we found that the proportion of anammox bacteria affiliated with genera of *Kuenenia*/*Brocadia* would increase along with the increasing anthropogenic or terrestrial inputs, while the proportions of *S. arabica*/*brodae* (or the *hzo Scalindua* cluster 4) decrease at the same gradient. Thus, it is clearly showed that the anammox bacteria community compositions shift from the high anthropogenic/terrestrial input environments (MP-5) to the low anthropogenic/terrestrial inputs and pristine environments (SCS-SS and SCS-SB). Furthermore, higher diversity of anammox bacteria (genus level) was also found in the high anthropogenic/terrestrial-influenced environments though anammox bacteria might also have a very high microdiversity within the same genus in the low anthropogenic/terrestrial inputs or pristine environments (SCS-SS). In addition, similar results found in the previous study (Wang et al. 2012) and several other estuarine ecosystems from USA (Dale et al. 2009), Japan, and UK were also provided with strong evidence to confirm the shifting of anammox bacteria community structures along with the anthropogenic/terrestrial input gradient (Fig. 4).

To explain how anammox bacteria community structures verify in these terrestrial and marine interacted zones, several possibilities should be considered. If we assume that all anammox bacteria retrieved from these sediment samples are endogenous, the variation of anammox bacteria community structures might be due to the different responses of different anammox bacteria to the environmental conditions. According to previous study, different species of anammox bacteria in different genera show different affinity for ammonium and nitrite and also have quite different tolerances

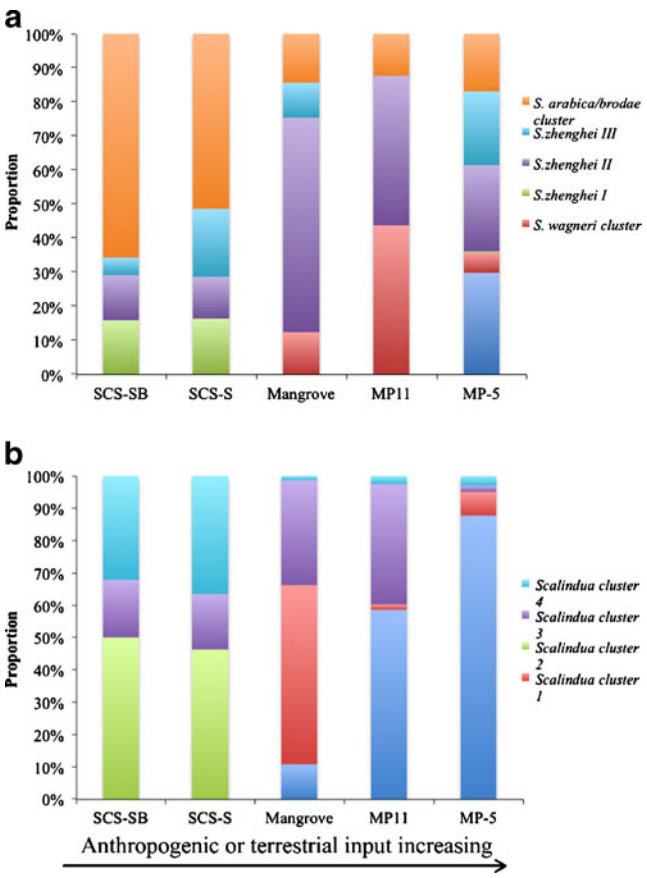

Fig. 4 Shifting of anammox bacteria community compositions from estuary to the SCS open ocean based on the analyses of 16S rRNA (**a**) and *hzo* (**b**) genes

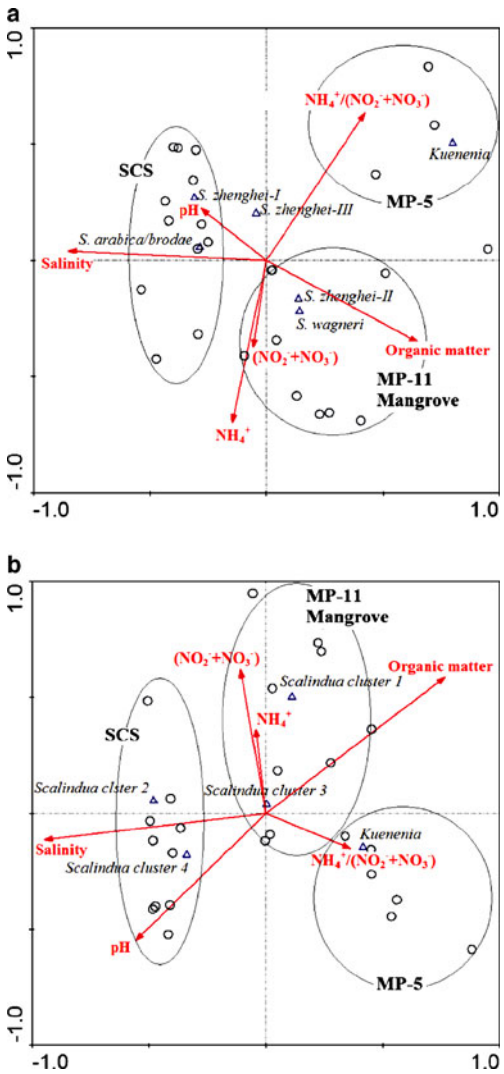

Fig. 5 CCA ordination plots for the physicochemical parameters. Anammox bacteria groups (*triangle*) represented by 16S rRNA (**a**) and HZO (**b**) sequences and their sampling locations (*circle*). Correlations between environmental variables and CCA axes are represented by the *length and angle of arrows* (environmental factors)

on nitrite, dissolved oxygen, phosphate, and salinity (Oshiki et al. 2011). Thus, the rapid changing of physicochemical conditions in estuarine ecosystems would shape different anammox bacteria community structures, which is also discussed in several published reports. On the other hand, the anammox bacteria recovered from different sediment samples in the estuarine ecosystems might also be exogenous, such as through the wastewater or terrestrial inputs. Previous studies have demonstrated that *Scalindua* anammox bacteria are the dominant group in marine environments (Schmid et al. 2007; Woebken et al. 2008), while *Brocadia* and *Kuenenia* anammox bacteria are usually found in engineered and terrestrial systems (Jetten et al. 2005; Jetten et al. 2009). Therefore, higher proportion of *Brocadia* or *Kuenenia* anammox bacteria would be detected in the sediment samples with higher anthropogenic/terrestrial inputs but lower proportions or even absence of *Brocadia* or *Kuenenia* anammox bacteria could be detected in marine ecosystems with less anthropogenic/terrestrial inputs. Furthermore, it might be more reasonable to explain the variation of anammox bacteria in estuarine ecosystems by combing these two processes, where some *Brocadia* and *Kuenenia* anammox bacteria discovered in estuaries might originate from wastewater or terrestrial inputs, and these exogenous anammox bacteria, together with the endogenous anammox bacteria, show different responses on the changing environmental factors. However, no matter which process really causes the changing of anammox bacteria in estuaries, it is still reasonable to use the variation of anammox bacteria community structures as a bio-indicator to monitor the anthropogenic/terrestrial inputs of marine environments.

To further confirm our hypothesis, the correlations between anammox bacteria diversity distribution and environmental factors are also analyzed in the PRD sediment samples (Fig. 5). It is clearly showed that salinity strongly affects the community structures and distribution of anammox bacteria in the PRD sediments, especially the group of *S. arabica*/*brodae*. However, the significant influences of organic matters, concentrations of NH_4^+ and ($NO_2^- + NO_3^-$), and their ratio on the anammox bacteria community and distribution further provide evidence on the hypothesis by using the variation of anammox bacteria community structures as a bio-indicator to monitor the anthropogenic/terrestrial inputs of marine environments.

In summary, we have evaluated the community structure and their variation in sediment samples collected from the PRD area, and the correlations between environmental factors and the community composition and distribution were also analyzed in this study. Combining the results in the PRD area and that in several estuarine ecosystems, it clearly demonstrated that the variation of anammox bacteria community structures could serve as a bio-indicator to monitor the anthropogenic/terrestrial inputs of marine environments.

Acknowledgments This research was supported by a PhD studentship (M.L.), Environmental and Conservation Fund grant no. 15/2011(J-DG), South China Sea Open Cruise by R/V Shiyan 3, and South China Sea Institute of Oceanology, CAS. We thank Jessie Lai for general laboratory support at The University of Hong Kong during this study.

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