

Community shift of ammonia-oxidizing bacteria along an anthropogenic pollution gradient from the Pearl River Delta to the South China Sea

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Abstract The phylogenetic diversity and abundance of ammonia-oxidizing beta-proteobacteria (beta-AOB) was analyzed along an anthropogenic pollution gradient from the coastal Pearl River Delta to the South China Sea using the ammonia monooxygenase subunit A (*amoA*) gene. Along the gradient from coastal to the open ocean, the phylogenetic diversity of the dominant genus changed from *Nitrosomonas* to *Nitrospira*, indicating the niche specificity by these two genera as both salinity and anthropogenic influence were major factors involved. The diversity of bacterial *amoA* gene was also variable along the gradient, with the highest in the deep-sea sediments, followed by the marshes sediments and the lowest in the coastal areas. Within the *Nitrosomonas*-related clade, four distinct lineages were identified including a putative new one (A5-16) from the different sites over the large geographical area. In the *Nitrospira*-related clade, the habitat-specific lineages to the deep-sea and coastal sediments were identified. This study also provides strong

support that *Nitrosomonas* genus, especially *Nitrosomonas oligotropha* lineage (6a) could be a potential bio-indicator species for pollution or freshwater/wastewater input into coastal environments. A suite of statistical analyses used showed that water depth and temperature were major factors shaping the community structure of beta-AOB in this study area.

Keywords Ammonia monooxygenase α -subunit (*amoA*) gene · Ammonia-oxidizing bacteria (AOB) · South China Sea · Anthropogenic pollution gradient

Introduction

Nitrification has been known as an important step in the nitrogen (N) biogeochemical cycle because of the oxidation from ammonia to nitrate via nitrite. Nitrification also affects the global climate change through the emission of greenhouse gases nitrous oxide (N₂O) and nitric oxide (NO) (Falkowski 1997; Casciotti et al. 2003; Shaw et al. 2006; Gruber and Galloway 2008). Nitrification could detoxify the excess ammonia in the anthropogenic affected estuarine and coastal ecosystems (Camargo and Alonso 2006). Nitrification is also responsible for the formation of the huge nitrate reservoir in the deep sea (Galloway et al. 2004; Lam et al. 2007; Klotz and Stein 2008). Ammonia-oxidizing bacteria (AOB) and the recently discovered ammonia-oxidizing archaea (AOA) (Venter et al. 2004; Schleper et al. 2005) which are affiliated to *Thaumarchaeota* (Brochier-Armanet et al. 2008; Spang et al. 2010) are involved in the nitrification process. AOA have been surveyed in a broad range of environments based on the culture-independent molecular methods (Francis et al. 2005; Prosser and Nicol 2008; Schleper and Nicol 2010).

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Though AOA were proposed as the dominant group to be responsible for the aerobic nitrification in a wide range of environments (Prosser and Nicol 2008; Schleper and Nicol 2010), the relative contributions of this group to the nitrification is still in debate and AOB remains a more robust model system for microbial ecology investigation (Kowalchuk and Stephen 2001; Horz et al. 2004; Ward 2005). AOB taxonomically falls into two monophyletic groups, beta- and gamma-proteobacteria. Beta-AOB mainly consists of two genera, *Nitrosomonas* and *Nitrosospira* in which *Nitrosomonas* genus could be further divided into several lineages (Purkhold et al. 2000, 2003). Three newly defined clusters affiliated to *Nitrosospira* genus and one belonged to *Nitrosomonas* has been proposed recently (Dang et al. 2010). Although gamma-AOB may have a much wide distribution (Ward and O'Mullan 2002), gamma-proteobacteria such as *Nitrosococcus oceani* are minor members of the marine sediment ammonia-oxidizing communities (Nold et al. 2000) with lower diversity in most marine environments analyzed (O'Mullan and Ward 2005) or no positive results (Ward et al. 2000).

In the estuarial, coastal and deep-sea ecosystems, beta-AOB are ecologically significance based on numerous studies through culture-independent molecular detection of ammonia monooxygenase subunit A gene (*amoA*) which could provide reliable information about the beta-AOB community structure and their response to environmental changes (de Bie et al. 2001; Bollmann and Laanbroek 2002; Caffrey et al. 2003, 2007; Francis et al. 2003; Bernhard et al. 2005, 2007; O'Mullan and Ward 2005; Beman and Francis 2006; Freitag et al. 2006; Hunter et al. 2006; Urakawa et al. 2006a, b; Nakagawa et al. 2007; Mosier and Francis 2008; Sahan and Muyzer 2008; Santoro et al. 2008; Dang et al. 2010; Wankel et al. 2011). Nevertheless, the partial information from different ecological niches could not guarantee how the beta-AOB community structure, abundance, and distribution respond to the environmental change on a larger scale (Kowalchuk and Stephen 2001; Freitag et al. 2006; Urakawa et al. 2006a, 2006b). On the other hand, although long-standing efforts to explore the driving forces including salinity, pH, ammonium, and O₂ concentrations on selecting differential ecophysiological lineages of beta-AOB, a thorough understanding of these relationships is not available (Bernhard et al. 2007; Dang et al. 2010), especially in the Pearl River Estuary which is a complex dynamic environments impacted strongly by anthropogenic activities and also the most rapidly developing activity of the world economy in the past more than two decades, and the South China Sea (SCS) (Huang et al. 2003; Dai et al. 2008; Harrison et al. 2008; Zhou et al. 2011). Recently, studies on the nitrifying bacterial in the Pearl River Estuary and the SCS provided an opportunity to comprehensively review the beta-AOB

community structures along a gradient of surface sediments from the Pearl River Estuary, coastal to deep-sea sites (Cao et al. 2011e; Jin et al. 2011; Li et al. 2011) because surface sediments is a major site for nitrification due to a relatively high beta-AOB abundance and activity (Satoh et al. 2007). In addition, another study conducted in Jiaozhou Bay of North China indicated that the sediment beta-AOB community could serve as bio-tracers or bio-indicators to reflect the continental influence (Dang et al. 2010). The beta-AOB in the surface sediments from the Pearl River Estuary to the SCS could be employed as a model for this test.

The phylogenetic diversity of beta-AOB was surveyed and the abundance of both groups of ammonia oxidizers including AOA and beta-AOB was also evaluated in the northern part of the SCS with a deep rhombus-shaped basin in the eastern part employed *amoA* gene as a functional genetic biomarker. Comparative analyses of beta-AOB community structures were also conducted with those from the Pearl River Estuary and the Mai Po Nature Reserve marshes of Hong Kong. Along the gradient formed by the surface sediments from wetland, estuary to the coastal and deep sea, the community structure changes of beta-AOB were detected and dominated lineages were identified so as to draw a general view about the spatial distributions of beta-AOB in this area under increasing pressure of anthropogenic influences.

Materials and methods

Sampling and molecular experiments

Seven surface sedimentary samples were collected on the SCS Open Cruise in July 2008. When sediment cores were retrieved on board, air-exposed outer surfaces of the sediment were discarded and the central core was taken with sterile stainless steel knife and put into plastic bags for storage at -20°C immediately. All samples from the SCS could be divided into two groups, shallow and deep-sea ones from the coastal areas to the deep sea as shown in Fig. S1 and Table S1 in the **ESM**. The detailed descriptions of the sampling sites involved in this study are also available elsewhere (Cao et al. 2011a, b, c, d, e). Additional samples from this large area covering the anthropogenic pollution gradient were reported in previous studies and also included in the current analysis to expand our dataset (Cao et al. 2011a, b, c, d, e; Jin et al. 2011; Li et al. 2011).

Bacterial *amoA* gene sequences were amplified using the primer set (*amoA*-1F: 5'-GGGGTTTCTACTGGTGGT-3' and *amoA*-2R: 5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al. 1997) through polymerase chain reaction (PCR) amplification of the total genomic DNA extracted from sediment samples as described earlier (Cao et al.

2011a, b, c, d, e; Li et al. 2011). In addition to the bacterial *amoA* gene sequences retrieved from the seven samples in the present study, more *amoA* gene sequences from a number of other studies over the same large area were also downloaded from the GenBank and used in the analyses (Cao et al. 2011b, e; Jin et al. 2011; Li et al. 2011). The abundance (gene copy numbers) of archaeal and bacterial *amoA* genes were determined with specific primers (Arch-amoAF: 5'-STAATGGTCTGGCTTAGACG-3' and Arch-amoAR: 5'-GCGGCCATCCATCTGTATGT-3' for AOA; amoA-1F and amoA-2R for AOB) (Rotthauwe et al. 1997; Francis et al. 2005) by quantitative PCR (Q-PCR) in 25- μ l reaction volume including 1 μ l template DNA, 0.2 mg/ml BSA, 200 nM of each primer, and 12.5 μ l of SYBR Premix (Takara, Hong Kong). The protocol used was described previously by Chen et al. (2008). All reactions were run in triplicate. A serial dilution of plasmids containing clone 704BA-6 for AOA and clone 704BB-6 for AOB were separately used as standards in Q-PCR assays (Cao et al. 2011b, d).

Phylogenetic and statistical analyses in the surface sediments from SCS

The bacterial *amoA* gene sequences from this study were firstly evaluated by the rarefaction analysis, and richness estimators (Chao1 and Shannon) and diversity index (Simpson) were simultaneously calculated using DOTUR (Schloss and Handelsman 2006) prior to other analyses. To keep the congruence between different studies, the distance cut-off was set at 5% for operational taxonomic units (OTUs) definition in DOTUR (Schloss and Handelsman 2006).

Bacterial *amoA* gene sequences recovered from the present study and the relevant ones retrieved from GenBank including those from the Mai Po Nature Reserve marshes and the Pearl River Estuary were transferred into MEGA 5.0 (Tamura et al. 2011) and merged for editing and further analyses (Cao et al. 2011e; Jin et al. 2011; Li et al. 2011). All sequences were aligned by Clustal X 1.81 (Higgins et al. 1997) and the phylogenetic analysis was conducted in MEGA 5.0 (Tamura et al. 2011). Phylogenetic tree was constructed based on the neighbor-joining criterion and credible values for every node were evaluated with bootstrap method via 1,000 replicates in MEGA 5.0 (Tamura et al. 2011).

Principal coordinates analyses (PCoA), Jackknife Environment Clusters analyses were conducted in an online software UniFrac (<http://bmf2.colorado.edu/unifrac/index.psp>) (Lozupone et al. 2006) which employs the genetic distances to evaluate the community similarity based on the gene sequences data. The bacterial *amoA* gene sequence communities from the SCS surface sediments and other

former studies were classified on the basis of the environmental variables. The environment clusters tree was projected in MEGA 5.0 (Tamura et al. 2011). Canonical correspondence analysis (CCA) was executed in CANOCO 4.5 to determine the correlations between bacterial *amoA* gene sequence community compositions from northern SCS and physicochemical parameters (ter Braak and Smilauer 2002). In addition, Pearson moment correlations were also evaluated with Microsoft Excel to relate the physicochemical factors with the diversity and richness indices of bacterial *amoA* genes.

Nucleotide sequences accession numbers

The bacterial *amoA* gene sequences retrieved in this study from the seven surface sediment samples were deposited in the GenBank with the accession numbers JN575094–JN575260.

Results

Environmental conditions in the study areas

Eutrophication is a widely known environmental issue in the Pearl River Delta and the adjacent coastal areas of the northern SCS because of rapid economic growth driven by manufacturing for the world and also urbanization, resulting in a high load of dissolved inorganic nitrogen (Huang et al. 2003; Dai et al. 2008; Harrison et al. 2008; Zhou et al. 2011). Mai Po Nature Reserve marshes in Hong Kong receive a large quantity of wastewater from the adjacent areas with highly contaminated sediments showing high contents of inorganic and organic nitrogen and heavy metals (Cheung et al. 2003; Liang and Wong 2003; Laboratory of Environmental Toxicology 2009) and an apparent decline of infauna diversity over the last 7 years (Shen et al., 2010). In this study, four types of ecosystem sediments were identified based on the environmental conditions, namely marshes, estuarine, coastal and deep-sea sediments. The salinity was on an average of $27.8 \pm 7.1\text{‰}$ in the estuarine coastal plume, $32.9 \pm 1.3\text{‰}$ on the continental shelf, and $33.5 \pm 0.8\text{‰}$ in the open ocean (He et al. 2009). However, salinity in the Mai Po Nature Reserve was highly variable seasonally and lower ($\sim 17\text{‰}$) than the other areas mentioned above (Cao et al. 2011e; Li et al. 2011). Generally, the salinity increases from the marshes to the estuarine and the open sea. In contrast, organic contaminants and heavy metals, e.g., mercury (Hg) in the sediments displayed a decreasing trend from the Pearl River Delta estuary to the open sea (Shi et al. 2010). High nitrification rates were also observed in the Pearl River Estuary and the adjacent areas indicating the importance of nitrifying microbes (Dai et al. 2008).

Sediments of the SCS surveyed in this study could be divided into two groups, shallow and deep-sea ones because temperature and water depth are evidently clustered into two groups. Besides, other environmental factors also showed variable trend (Table S1 in the Electronic supplemental materials (ESM)) and previous studies (Cao et al. 2011b, c), for instance the decrease of ammonium concentrations from the Pearl River Delta areas to the SCS (Dai et al. 2008; Cao et al. 2011b, c; Jin et al. 2011) (Table S1 in the ESM). On the other hand, within the seven sites from this study, site CF5 exhibited extremely high concentration of inorganic nitrogen, especially NH_4^+ , followed by site 201 with the second highest (Table S1 in the ESM).

Molecular diversity of bacterial *amoA* gene sequences

Higher diversities of bacterial *amoA* gene sequences were observed in deep-sea sites 08CF7 and 525 while lower ones in coastal sites with shallow overlying water, sites 201, 510, and 708 based on the indices for samples from the SCS (Table 1) (Cao et al. 2011b, c). Within each clone library, the number of OTU recovered was variable from 2 to 13 based on rarefaction analysis (Fig. 1) (Cao et al. 2011b, c). A total of 351 sequences from the SCS (including others from the previous studies) (Cao et al. 2011b) were combined for analyses, and 26 OTUs were identified based on 5% cutoff for nucleotide sequences. For the Pearl River Estuary, the diversity of bacterial *amoA* genes was difficult to be evaluated and compared with others because only one clone library containing all

samples was constructed with 26 sequences but with obvious diversity information (Jin et al. 2011). In the Mai Po Nature Reserve marsh sediments, the diversity indices were slightly higher than in the coastal areas, but lower than in the deep-sea sediments of the SCS (Cao et al. 2011e; Li et al. 2011).

Phylogenetic diversity of the beta-AOB *amoA* gene

Two larger clades separately grouped with known sequences of beta-AOB in the *Nitrosomonas* and *Nitrosospira* genera based on the phylogenetic analysis of *amoA* gene sequences (Fig. 2). Most of the sequences from the Mai Po Nature Reserve marshes were in the *Nitrosomonas*-like clade. In contrast, a few sequences from the Pearl River Estuary, coastal and deep-sea of the Northern SCS (all in cluster 6a) were also grouped into this clade. As in the former studies, three lineages, *Nitrosomonas* sp. Nm143 (cluster 143), *Nitrosomonas oligotropha* (cluster 6a), and *Nitrosomonas marina* (cluster 6b) were delineated in the current phylogeny (Purkhold et al. 2003; Dang et al. 2010). Another new cluster, tentatively named cluster A5-16 from Jiaozhou Bay is also present in this clade (Dang et al. 2010; Jin et al. 2011). Two sequences from the Pearl River Estuary sediments were grouped into this cluster, which appeared at the basal position and clearly separated from the *Nitrosomonas europaea*, *Nitrosomonas communis*, and *Nitrosomonas cryotolerans* lineages (Dang et al. 2010).

In the *Nitrosospira*-like clade (Fig. 2b), except for one sequence from site 407 which was affiliated to the cultured

Table 1 Bacterial *amoA* gene diversity and richness indices from the surface sediments in the northern South China Sea

Sample name	Clone numbers	OTUs (95%)	Diversity and richness		
			Simpson	Shannon	Chao1
407	32	7	0.29	1.31	5
525	24	10	0.19	1.61	6
CF5	27	8	0.39	1.02	4
08CF7	44	13	0.67	0.82	15.5
425	23	8	0.25	1.44	9
510	20	4	0.64	0.71	4.5
201	21	4	0.48	0.90	5
702 ^a	39	11	0.12	2.15	12
704 ^a	41	9	0.24	1.63	19
706 ^a	41	5	0.40	1.05	5
707 ^a	44	4	0.43	0.94	4
708 ^a	31	2	0.94	0.14	2
709 ^a	30	5	0.29	1.34	5
Total	351	26	0.15	2.26	30.2

^aThe samples cited from the reference Cao et al. (2011b)

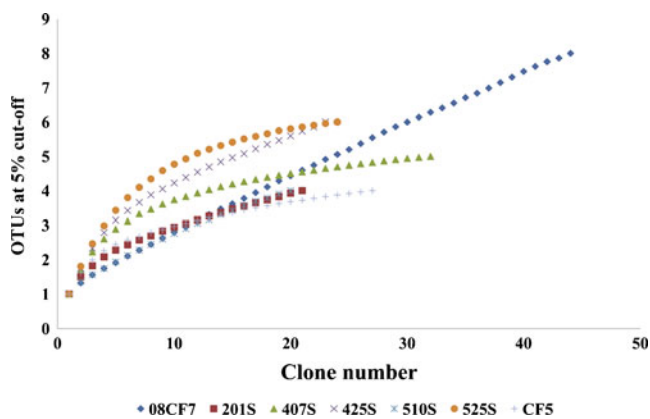


Fig. 1 Rarefaction curves of bacterial *amoA* gene sequences retrieved from the northern South China Sea with 5% distance cut-off

Nitrosospira sp. ka3 (Aakra et al. 1999), others were not grouped within any cultured beta-AOB *amoA* gene sequences cluster, similar to those retrieved before from various estuarine, coastal, and deep-sea sediments (Bernhard et al. 2007; Hayashi et al. 2007; Kim et al. 2008; Dang et al. 2010). Three tentatively named clusters 13, 14 and 15 were evident in this study based on the nomenclature by Avrahami and Conrad (2003) and Dang et al. (2010). Cluster 13 was constituted by *amoA* gene sequences from coastal marine environments as proposed in the previous study, e.g., the sequences from sites 201, 510, 704, 706, 707, 708, and 709 (Bernhard et al. 2007; Kim et al. 2008; Dang et al. 2010). Cluster 14 was mainly comprised of environmental *amoA* gene sequences from deep-sea marine sediments (Hayashi et al. 2007; Dang et al. 2010). On the other hand, cluster 15 with another synonym as “*Nitrosospira*-like cluster B” was composed by the *amoA* gene sequences mainly from estuarine and coastal sediments (Francis et al. 2003; Kim et al. 2008; Dang et al. 2010).

Community analyses of the beta-AOB assemblages

A few groups were identified from the UniFrac environment clustering analysis based on phylogenetic diversity of bacterial *amoA* gene sequences (Fig. 3a). Samples from the Mai Po Nature Reserve wetland and the Pearl River Estuary were distinguished separately. In the wetland assemblage, sequences from sample D-May separated from others, consistent with our previous analysis (Cao et al. 2011e). The coastal sites in the SCS were divided into two sub-classes: one comprised by the sequences from sites 704 and 425 and another constituted by sites 510, 706, 707, 708, and 709. The deep-sea sites in the SCS formed one distinct class. Similar classification was revealed in the UniFrac PCoA analysis (Fig. 3b). The first principal coordinate (P1), which explained 60.63% of the total community variability, unambiguously distinguished

the *amoA* assemblages of wetland from those of the Pearl River Estuary and the SCS.

An all-environment UniFrac significance test indicated a significant difference ($P=0.002$) among the all *amoA* assemblages in the current study. Pairwise UniFrac significance test indicated that the *amoA* assemblage of site 407 was marginally significantly different from 702 ($P=0.02$), 510 from 707 ($P=0.03$), 510 from 707 to 709 ($P=0.03$), and A-NOV from D-NOV ($P=0.04$). Those of sites CF5 with 525 ($P=0.09$) and 706 with 707 ($P=0.06$), were of different community composition.

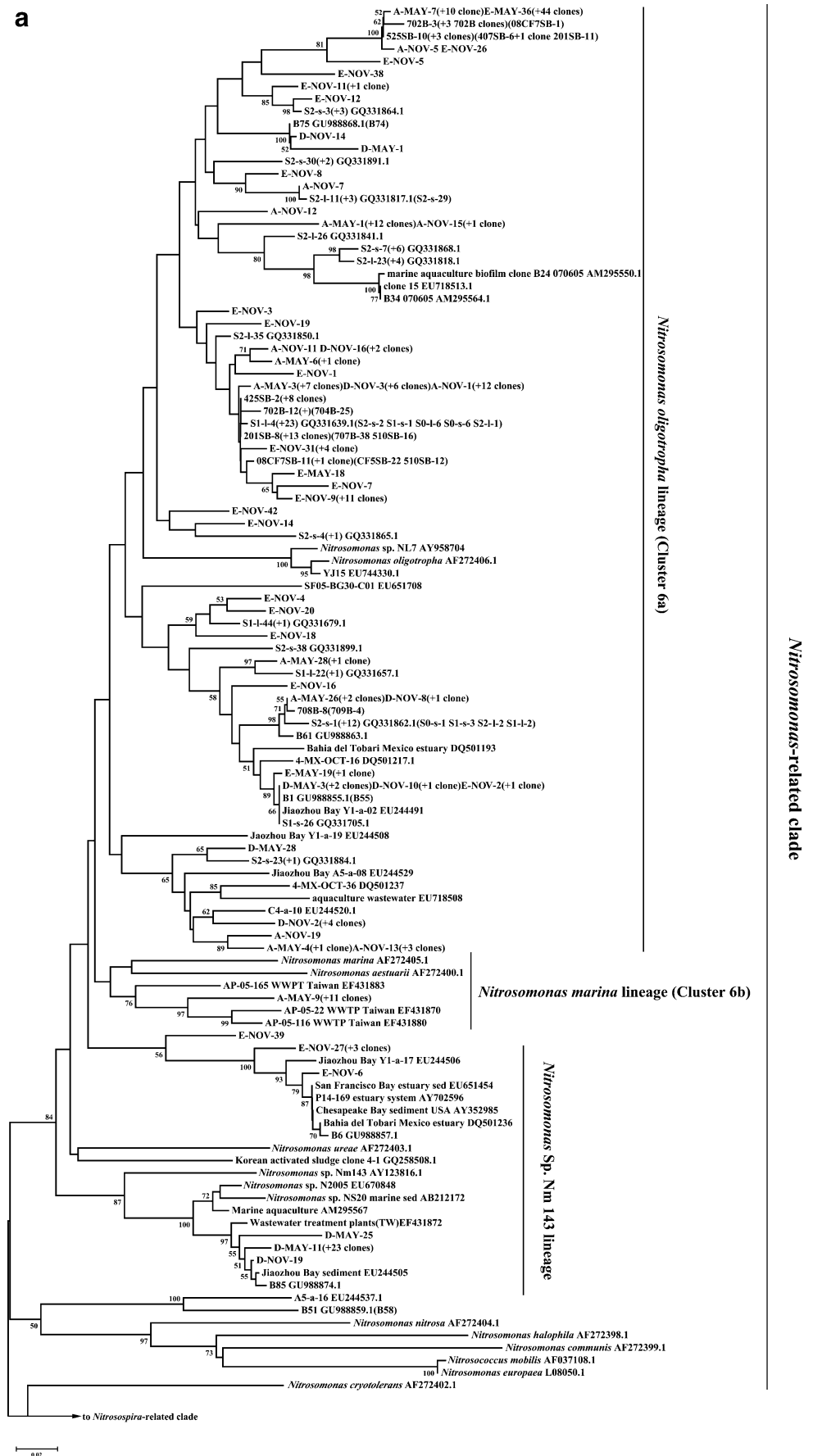
Environmental factors to shape the distribution of the beta-AOB

Pearson moment analyses provided clues to the environmental parameters that shape the spatial distribution of beta-AOB in the northern SCS. Both depth and temperature showed weaker relationships with the diversity of bacterial *amoA* genes (Table S2 in the [ESM](#)). The concentration of nitrite was correlated with the diversity of bacterial *amoA* genes. Correlations of the bacterial *amoA* assemblages with environmental parameters analyzed by CCA indicated that depth and temperature via the CCA axis 1 separated all the samples into two groups, namely shallow and deep-sea sediments groups, consistent with the UniFrac community classification. In addition, the concentration of inorganic nitrogen could contribute to the distribution of the deep-sea sediments, 08CF7, 525 and CF5, while the concentration of nitrite separated 201, 704, and 707 (Fig. 4).

Bacterial and archaeal quantification analyses of the *amoA* gene

Beta-AOB *amoA* gene ranged from 4.24×10^4 to 1.99×10^6 copies per gram of sediment (wet weight), and the highest one was only more than two orders than the lowest one (Fig. 5). The AOA *amoA* gene abundance ranged from 1.6×10^5 to 2.45×10^8 copies/g of sediment (wet weight), the highest and the lowest differed by 3-fold (Fig. 5). The ratios of AOA/beta-AOB *amoA* gene copy numbers ranged from 2.69 to 2.65×10^3 . The PCR amplification efficiencies were 91–95% (average, 93%) for archaeal *amoA* and 88–92% (average, 90%) for beta-proteobacterial *amoA*. Correlation coefficient (R^2) for both assays on average were 0.98 ± 0.01 (standard deviation). Generally, the archaeal *amoA* gene abundance was higher than bacteria at each site. The Q-PCR results showed a heterogeneous distribution of the sediment archaeal and bacterial *amoA* gene abundance and the ratio of them among the sampling sites of the SCS, in that the sites CF5 and 425 showed higher gene abundance than the others. The ratio was higher at site 425.

Fig. 2 Phylogenetic tree split into *Nitrosomonas*- (a) and *Nitrospira*-related (b) ones constructed based on bacterial *amoA* gene sequences from the various surface sediments in the Mai Po Nature Reserve marshes sediments, the Pearl River Estuary and the northern South China Sea sediments with most similar sequences from GenBank through the neighbor-joining (NJ) method. Numbers at the nodes represent the significant bootstrap values (>50) via 1,000 replicates test. Branch lengths correspond to sequence differences as indicated by the scale bar (2% nucleotide substitution)



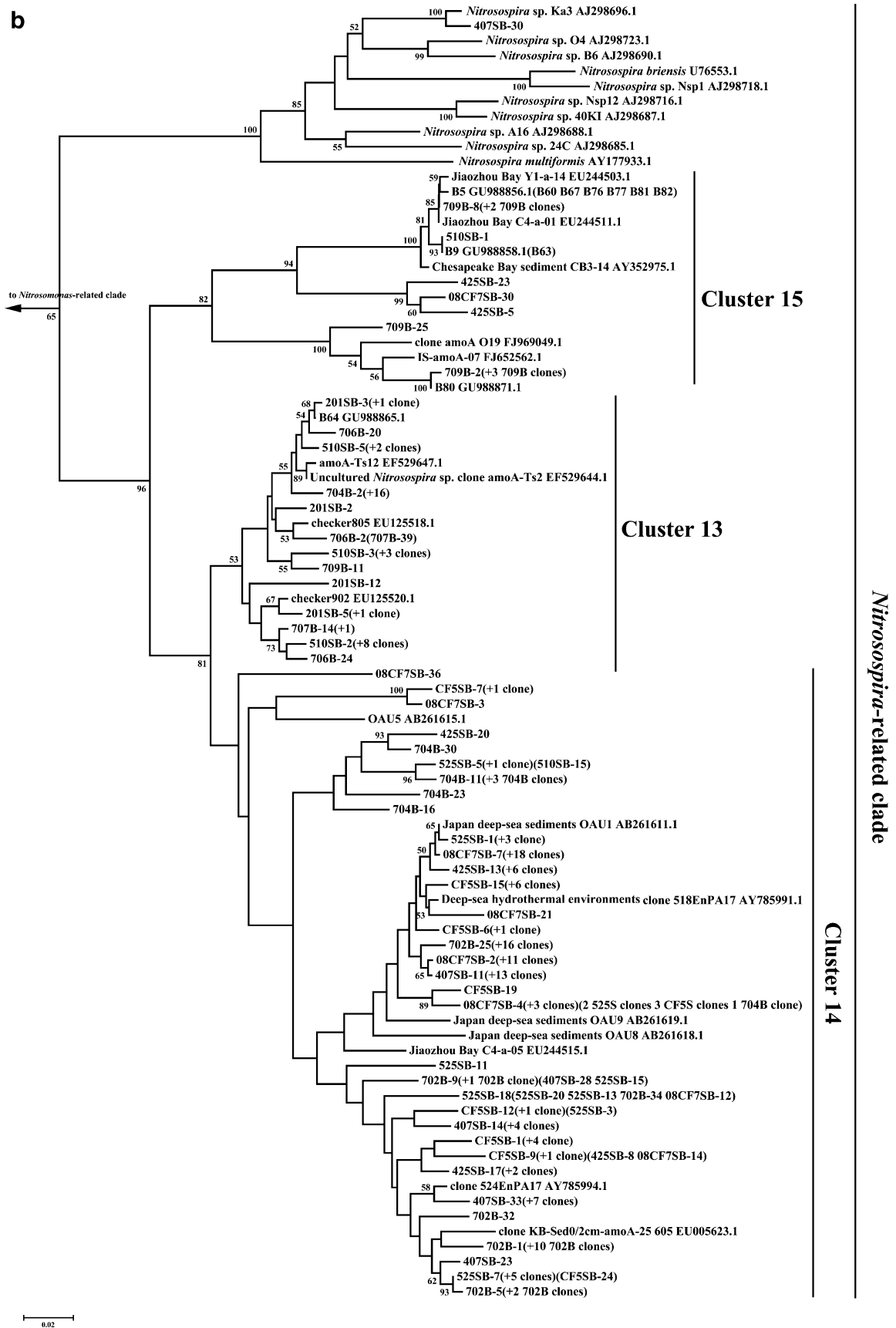


Fig. 2 (continued)

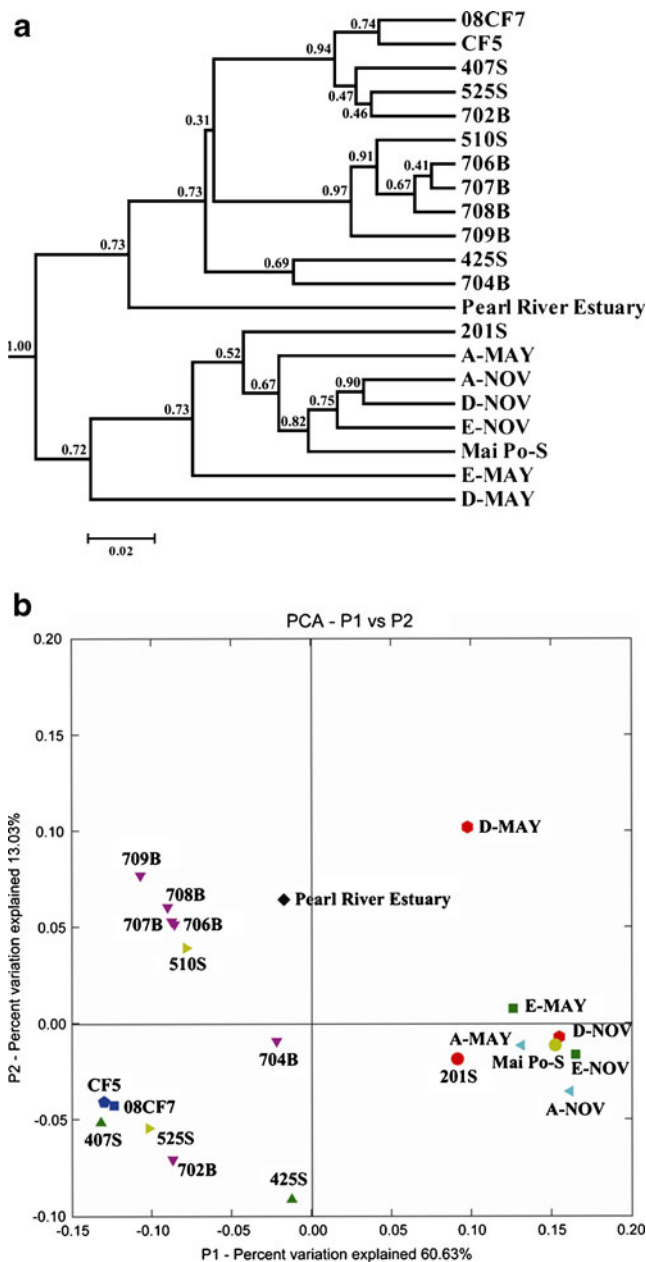


Fig. 3 PCoA (a) and hierarchical clustering analysis (UPGMA algorithm with 100 replicates Jackknife supporting test) (b) for the bacterial *amoA* gene sequences from the Mai Po Nature Reserve marshes sediments, the Pearl River Estuary and the northern South China Sea sediments on the basis of the online software UniFrac

Discussion

Beta-AOB phylogenetic diversity along the pollution gradient

In the surface sediments from the wetland in the Pearl River Delta to the coastal and deep sea in the SCS, the phylogenetic diversity of beta-AOB delineated by *amoA*

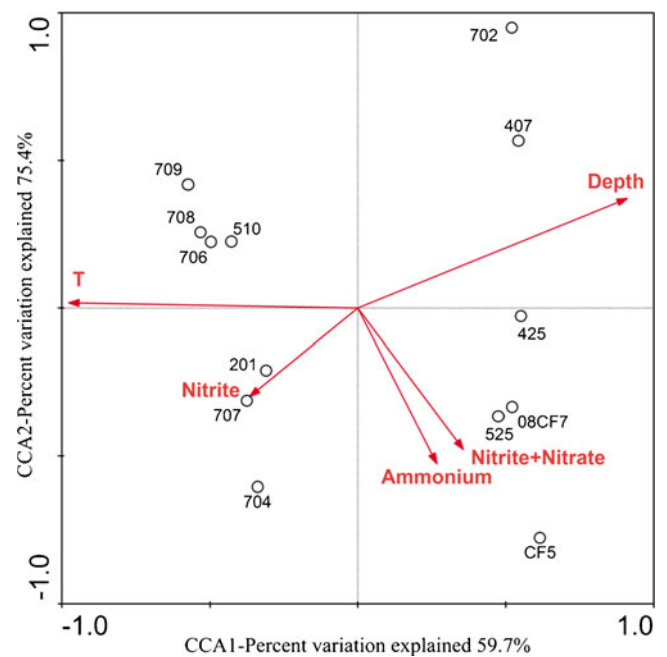


Fig. 4 CCA ordination plots for the environmental factors and the ammonia-oxidizing bacterial lineages representing by *amoA* gene sequences analyzing from the CANOCO software

gene sequences was spatially distributed from the dominance by *Nitrosomonas*- to *Nitrospira*-related clades supported by the UniFrac analyses. Most sequences from the SCS, both in coastal and deep-sea sediments, were affiliated to *Nitrospira*. All *amoA* gene sequences from two previous studies at the Mai Po Nature Reserve wetland were also exclusively clustered into the *Nitrosomonas*-related clade (Cao et al. 2011e; Li et al. 2011). In each clade, a few specific beta-proteobacterial *amoA* gene lineages were identified and each lineage potentially occupied a distinct habitat through adaptation. And a few lineages identified before were delineated again with much more similar sequences from the SCS this time.

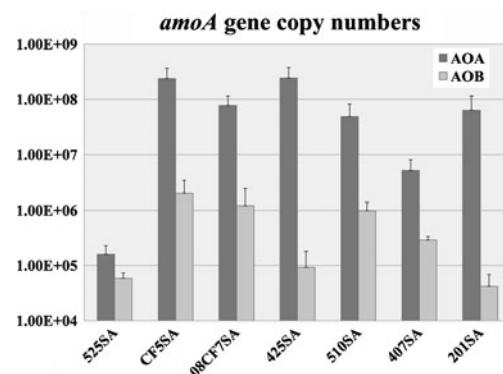


Fig. 5 Relative abundance of archaeal and beta-proteobacterial *amoA* gene. The error bar represents the standard deviation of triplicate Q-PCR reactions

The lineage designated as cluster A5-16, because of one specific OTU A5-a-16 identified here, was clustered with another two clones, B51 and B58 (shared about 90% identity with clone A5-a-16) retrieved from the Pearl River Estuary (Dang et al. 2010; Jin et al. 2011). And CCA analysis in a previous study suggested that beta-AOB in this lineage might participate in the in situ nitrification in sediments (Dang et al. 2010). The *amoA* gene sequences from this lineage shared large genetic distance with others (0.21 to 0.37, percent nucleotide divergent, data not shown) indicating the novelty of this lineage and they were grouped with a few cultured AOB stains, e.g., *N. europaea* and *N. communis* which had been detected in anthropogenic influenced areas with heavy metals and $\text{NH}_4^+\text{-N}$ before (Fig. 2a) (Stein et al. 2007 and references therein). These three sequences diverged from the close relatives with a few non-synonymous substitutes, indicating the possible specific adaptation of beta-AOB for these habitats and these two sequences from the Pearl River Estuary have only four non-synonymous substitutes from the OTU A5-a-16, resulting from the large geographical distance (Fig. S2 in the [ESM](#)). As the OTU A5-a-16 occurred at the location with high discharge from the adjacent river and the wastewater treatment plants (WWTPs) (Dai et al. 2008), the Pearl River Estuary experienced strong anthropogenic impact. In contrast, this lineage was not retrieved from the highly polluted Mai Po Nature Reserve wetland (Cao et al. 2011e; Li et al. 2011). This could be resulted from the distinctiveness between wetland with vegetations and estuarine sediments or the lower abundance of this lineage in the environments (Dang et al. 2010; Jin et al. 2011). Up to date, this lineage has only been detected in the estuarine environments and may represent a novel lineage to be further investigated, especially in pure culture study.

The *N. oligotropha* lineage (Cluster 6a designated before) (Purkhold et al. 2003) was proposed as the major member in freshwater environments (Stehr et al. 1995; Koops and Pommerening-Röser 2001; Kowalchuk and Stephen 2001), wastewaters and activated sludges, biofilm reactors, and biofilters (Purkhold et al. 2003; Limpiyakorn et al. 2005; Lydmark et al. 2007) as the largest lineage in the *Nitrosomonas*-related clade (Fig. 2a). Most of the sequences from the Mai Po Nature Reserve wetland belonged to this lineage indicating the contributions from the adjacent terrestrial runoff, freshwater river or non-treated waste discharges as shown in other studies (Nicolaisen and Ramsing 2002; Caffrey et al. 2003) and directly discharges from WWTPs in Tokyo Bay (Urakawa et al., 2006b) and in other geographical locations (de Bie et al. 2001; Cébron et al. 2003, 2004; Nakamura et al. 2006). The environmental factors which could affect the distribution of these lineages have been investigated before, for instance, the affiliation with biofilms or benthic infaunal burrow walls as well as

support for the survival or preservation of these lineages within the highly dynamic estuarine ecosystems with tidal or seasonal salinity fluctuation (Magalhães et al. 2005; Satoh et al. 2007). Salinity usually has inhibitory effects on estuarine sediment nitrification (Rysgaard et al. 1999; Bernhard et al. 2007), probably due to the low salt tolerance of the nitrifiers, particularly the *N. oligotropha* lineage (Stehr et al. 1995). As discussed above, this lineage was detected in the estuarine or coastal environments with low salinity (Stehr et al. 1995; de Bie et al. 2001; Ottosen et al. 2001; Bollmann and Laanbroek 2002; Cébron et al. 2003, 2004; Francis et al. 2003; Bernhard et al. 2005; Magalhães et al. 2005; Nakamura et al. 2006; Satoh et al. 2007; Dang et al. 2010). These findings provided stronger evidence to support that freshwater flush or wastewater discharge should be the main factor shaping the freshwater beta-AOB community in brackish and marine ecosystems. The dominant distribution of these beta-AOB originated from freshwater in the coastal environments could provide an effective means to track the original sources with the microbial composition analysis (Cébron et al. 2003, 2004; Urakawa et al. 2006a, 2006b). As the *N. oligotropha* lineage is usually associated with WWTP effluents or rivers, environmental *N. oligotropha* clones might be potentially served as bio-indicators of pollution in coastal environments. In the present study, some sequences from the coastal sediments, sites 201 and 510, were also grouped into this lineage (Fig. 2a). Collectively, this study provides additional new evidence on the use of this lineage as the bio-indicators for pollution monitoring and tracking in coastal environments from the Pearl River Delta and the SCS.

The three newly defined *amoA* gene sequence lineages of the *Nitrosospira* clade (Dang et al. 2010) contained only environmental sequences from estuarine, coastal, and deep-sea sediments, and these sequences and clusters may represent novel beta-AOB species and lineages (Avrahami and Conrad 2003; Bernhard et al. 2007; Hayashi et al. 2007; Kim et al. 2008; Dang et al. 2010). Clusters 13 was constituted by *amoA* gene sequences from coastal marine environments as proposed in the previous study, e.g., the sequences from sites 201, 510, 704, 706, 707, 708, and 709 (Bernhard et al. 2007; Kim et al. 2008; Dang et al. 2010). Cluster 14 is mainly comprised of environmental *amoA* gene sequences from deep-sea marine environments (Hayashi et al. 2007; Meyer and Kuever 2008; Dang et al. 2010). As proposed in the former study, sequences from this lineage were related to high salinity niche (Dang et al. 2010). However, in contrast with other lineages shown in the phylogenetic tree, salinity could not be the sole factor, and other factors, including depth, pressure, oxygen and temperature could contribute collectively to the distributions of this lineage in deep-sea and separated them from the others. Cluster 15 with another synonym as “*Nitrosospira*-like cluster B” in another study was composed of the *amoA* gene

sequences mainly from estuarine and coastal sediment environments, especially estuaries (Francis et al. 2003; Kim et al. 2008; Dang et al. 2010; Jin et al. 2011). Based on the above analysis, the clusters 13 and 15 sequences could occupy similar niches and both clusters should have the higher adaptability to a wide range of coastal environments as shown by *Nitrosomonas* sp. Nm143 lineage (Freitag et al. 2006) supported by another study showing no significant correlation with any environmental factors (Dang et al. 2010).

Community structure change of beta-AOB and environmental factors to shape beta-AOB

The beta-AOB diversity decreased with the increase in salinity in some estuaries (de Bie et al. 2001; Francis et al. 2003; Cébron et al. 2004; Urakawa et al. 2006a, 2006b; Ward et al. 2007). However, from the wetland to the open sea in this study, the diversity of beta-AOB was higher in the deep sea of the SCS than in the coastal sediments of the SCS, wetland and estuary, indicating different beta-AOB assemblages in the deep-sea. On the other hand, the community of beta-AOB from mainly *Nitrosomonas* to *Nitrospira* has been proposed to be affected by the salinity in estuarine and coastal environments (Freitag et al. 2006). However, other studies provided a different conclusion about the salinity effect on the community structure of beta-AOB (Bernhard et al. 2007; Dang et al. 2010). In particular at the Jiaozhou Bay of Northern China, the microbe-environment CCA analyses indicated that salinity contributed very little to the distribution and classification of the sediment *amoA* sequence assemblages (Dang et al. 2010). Current study provides a strong evidence on a much large scale with continuity from the marshes to coastal and then deep-sea to delineate salinity as a factor driving the spatial distribution of beta-AOB from *Nitrosomonas*- to *Nitrospira*-related lineages along the pollution gradient. Other studies also indicated the distinctions of beta-AOB community compositions between freshwater and marine ecosystems (Bollmann and Laanbroek 2002; Coci et al. 2005; Grommen et al. 2005). Microbes flushed from rivers and WWTPs with nutrients could be the main contributors of beta-AOB to the Mai Po Nature Reserve marshes indicating *Nitrosomonas*-related lineages are the typical beta-AOB in this ecosystem. In this ecosystem, salinity is lower than coastal and the open sea of the SCS for most of the time in a year (Cao et al. 2011b, c, e; He et al. 2009; Jin et al. 2011; Li et al. 2011). Beta-AOB community in the Pearl River Estuary showed a mixture of these two genera (Fig. 2a, b) (Jin et al. 2011) while in the SCS, the *Nitrospira*-related lineages are dominant indicating beta-AOB community change along the gradient could be a candidate bio-indicator for environmental pollution and source tracking (Kowalchuk and Stephen 2001; Dang et al. 2010; Cao et al. 2011d).

Although the abundance of AOA was higher than that of beta-AOB in the sampling sites of this study as reported in many other studies, the potential ecological role of AOA still needs further activity confirmation, and the community transition change of beta-AOB along the gradient was apparently a major focus in this study. Sedimentological parameters may influence the bacterial and archaeal community structures responsible for N cycle (Dang et al. 2008, 2009; Jackson and Weeks 2008). In the SCS, based on the CCA analysis, depth and temperature separated all samples into two groups: deep-sea and cold as one, shallow and warm as another indicating the water depth and resulting temperature, and hydro-pressure could shape the community structure of beta-AOB. In addition, NH_4^+ and NO_2^- could be correlated to the samples from the deep-sea in the SCS (Fig. 4). Relationships between the diversity of beta-AOB and depth, temperature and nitrite were weak (Table S2 in the [ESM](#)). The oxygen concentration and pressure in the deep-sea sediments should select the beta-AOB to adapt to the unique habitats, resulting in the distinctness of the beta-AOB from others. Although the oxic sediment layers from the SCS sampling stations were known to exhibit higher nitrification activity (Urakawa et al. 2006a, 2006b), the oxygen still could be one limiting factor in the deep-sea sediments. Additionally, at site 201, the most bacterial *amoA* gene sequences (14 of 21 clones) were grouped into *N. oligotropha* lineage indicating the impact from the terrestrial environments. This is consistent with the information on ammonia-oxidizing archaea from this site in which the obvious soil/sediment clade AOA was dominant (Cao et al. 2011c). The highest abundance of beta-AOB existed in the site CF5 where the highest concentration of ammonium was also detected, indicating ammonium as the substrate for nitrification is a controlling factor for the diversity of beta-AOB. Taken all together, sedimentological conditions related to the hydrological regime, for instance, currents, tides, upwelling could affect the sediment composition, inorganic N concentration, redox, pH values, and temperature, so as to shape the microbial community, specifically the beta-AOB assemblages. However, relationships between them still need to be explored in the future studies (Dang et al. 2009, 2010). On the other hand, most bacterial *amoA* gene sequences from deep-sea sites were represented in the cluster 14 and also grouped with sequences from hydrothermal plum (Lam et al. 2004) without the basal position in the phylogenetic history as that in the AOA (de la Torre et al. 2008; Hatzenpichler et al. 2008; Prosser and Nicol 2008; Zhang et al. 2008; Kato et al. 2009; Dang et al. 2010; Cao et al. 2011c), indicating these two groups of nitrifying microorganisms experienced different evolutionary history. As oxygen limited condition in the deep-sea sediments, the relationships between the aerobic ammonia oxidizers, including AOA and AOB and anaerobic

ammonium oxidizing (anammox) bacteria should be much more interesting for the further studies and the anammox bacteria might have a higher contribution to the overall nitrogen cycle.

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