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ORIGINAL PAPER

Diversity and abundance of ammonia-oxidizing prokaryotes in sediments from the coastal Pearl River estuary to the South China Sea

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Abstract In the present study the diversity and abundance of nitrifying microbes including ammonia-oxidizing archaea (AOA) and betaproteobacteria (beta-AOB) were investigated, along with the physicochemical parameters potentially affecting them, in a transect of surface sediments from the coastal margin adjacent to the Pearl River estuary to the slope in the deep South China Sea. Nitrifying microbial diversity was determined by detecting the *amoA* (ammonia monooxygenase subunit A) gene. An obvious community structure shift for both AOA and beta-AOB from the coastal marginal areas to the slope in the deep-sea was detected, while the OTU numbers of AOA *amoA* were more stable than those of the beta-AOB. The OTUs of beta-AOB increased with the distance from the coastal margin areas to the slope in the deep-sea. Beta-AOB showed lower diversity with dominant strains in a polluted area but higher diversity without dominant strains in a clean area. Moreover, the diversity of beta-AOB was correlated with pH values, while no noticeable relationships were established between AOA and physicochemical parameters. Beta-AOB was more sensitive to transect environmental variability and might be a potential indicator for environmental changes. Additionally, the surface sediments surveyed in the South China Sea harboured diverse and distinct AOA and beta-AOB phylotypes different from other environments, suggesting the endemicity of some nitrifying prokaryotes in the South China Sea.

Keywords Ammonia-oxidizing archaea · Ammonia-oxidizing bacteria · South China Sea · Ammonia monooxygenase subunit A (*amoA*)

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Introduction

Nitrification, the microbial aerobic oxidation of ammonia to nitrate via nitrite plays an important role in the global nitrogen cycle and is the first and rate-limiting step. Besides two distinct monophyletic

groups of chemolithoautotrophic ammonia-oxidizing bacteria (AOB) (beta- and gamma-proteobacteria), a novel group of archaea (ammonia-oxidizing archaea, AOA) have been proposed recently to be involved in ammonia oxidation (Venter et al. 2004; Schleper et al. 2005). In estuarine and coastal sediments, microbial nitrification may be critical in the benthic nitrogen cycle by regulating nitrification and denitrification (Francis et al. 2005; Caffrey et al. 2007) and coupling with anammox (anaerobic ammonium oxidation) activity (Coolen et al. 2007; Lam et al. 2007). Studies have been conducted to investigate the community structure and abundance of nitrifying prokaryotes in estuaries (Francis et al. 2005; Beman and Francis 2006; Freitag et al. 2006; Bernhard et al. 2010; Wankel et al. 2011). Because estuaries and the intertidal sediments experience large fluctuations in hydrological activity, nutrient and chemical conditions, nitrifiers might respond to these changes, resulting in shifts of community structure and responses to the environmental changes. However, the intermediate area between marginal coasts to the deep-sea areas has not received enough attention, especially in the coastal area of the Western Pacific which is in the tropical and subtropical zones and is adjacent to several major estuaries of the region. For example, the Pearl River (Zhujiang), close to the northern part of the South China Sea (SCS), is the second largest river in China based on the discharge volume of water to the SCS through sub-estuaries (Huang et al. 2003). In the past two decades, the Pearl River estuary, one of the most complex estuarine systems in the world, has received a high loading of anthropogenic nutrients from one of the most rapidly developing areas of the world economy (Callahana et al. 2004), resulting in distinct differences between conditions in the Pearl River estuary and in the adjacent SCS ocean, e.g., an anthropogenic pollution gradient from extremely eutrophic in the Pearl River estuary to oligotrophic in the vast area of the northern SCS (Cai and Jiao 2008). Although Cai and Jiao (2008) recently surveyed the nitrate assimilation community in this area through amplifying the *nasA* gene, information about other microbes closely associated with the nitrogen cycles, e.g., nitrifying prokaryotes (AOA and AOB), is still scarce. Moreover, most former studies were confined to the temperate and subtropical coast of the East Pacific Ocean, while others focused on the estuarine area. The shift in microbial diversity from coastal area to the deep-sea has not been reported, especially in the western Pacific.

Selective species or lineages of nitrifying microbes including AOA and beta-AOB may be confined to a narrow habitat while others may tolerate to a much wider range of environmental conditions. For example, in Bahía del Tóbari, a hyper-nutrified estuary receiving substantial amounts of ammonium from agricultural runoff, bacterial *amoA* gene clone libraries were dominated by a few *Nitrosomonas*-like sequence types which were widely distributed, whereas AOA diversity showed significant variation in both richness and community composition (Beman and Francis 2006). In the Westerschelde estuary, the dominant beta-AOB groups were members of the genus *Nitrosomonas* (which were abundant in the brackish sites) and some lineages of the genus *Nitrosospira* (which were present at the marine sites) (Sahan and Muyzer 2008). Such information indicates there are significant differences in habitat requirement and potentially niche range between AOA and beta-AOB. To date, few factors have been confirmed to affect the microbial community structure of ammonia-oxidizing prokaryotes. Salinity has been shown to be an important factor to shape the community structure of nitrifying prokaryotes (Mosier and Francis 2008; Santoro et al. 2008). Besides this, the distinct seasonal variations of temperature, irradiance and river discharge also affect the microbial community structure of nitrifying microbes in estuaries. Moreover, the abundance of ammonia-oxidizing prokaryotes also changes with the gradient of some environmental parameters. For instance, Santoro et al. (2008) detected shifts in relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. Although salinity has been suggested to be an important factor to determine the diversity and abundance of AOA and beta-AOB (Mosier and Francis 2008; Santoro et al. 2008), no single factor is likely to shape the whole community structure and abundance of nitrifying microbes and it is possible that combined environmental factors might be collectively responsible for the diversity and abundance of nitrifying prokaryotes observed.

Surface sediments across a six-site transect along the continental shelf to the slope transect in the SCS with almost constant salinity were examined in this

study. The *amoA* gene was employed as a functional genetic marker to detect community structure and spatial distribution of AOA and beta-AOB and the *amoA* gene abundance was measured using quantitative PCR (q-PCR). Finally, the main potential physicochemical contributors to the diversity and abundance of AOA and beta-AOB were determined at this study site.

Materials and methods

Sampling and molecular experiments

Sampling was conducted aboard the R/V Shiyan 3 during a South China Sea Open Cruise in 2008. Six surface sedimentary samples along the coast to the slope of the SCS were selected (Supplementary Fig. S1). After the sediment cores were obtained on board, air exposed outer surfaces were removed using a sterilized stainless steel knife and the central core was placed into plastic bags and stored at −20°C immediately. The environmental parameters (pH, depth, temperature etc.) were monitored in situ and other chemical parameters (e.g., the concentrations of ammonium, nitrite and nitrate) in the sediments were measured in the laboratory using an autoanalyzer according to standard techniques (ASTM D3590-89B and D3867-99B) as described previously (Cao et al. 2011a, b; Li et al. 2011).

Two pairs of specific *amoA* PCR primers (Arch-amoAF: 5′-STAATGGTCTGGCTTAGACG-3′ and Arch-amoAR: 5′-GCGGCCATCCATCTGTATGT-3′ for AOA; amoA-1F: 5′-GGGGTTTCTACTGGTGGT-3′ and amoA-2R: 5′-CCCCTCKGSAAAGCCTTCTTC -3′ for beta-AOB) were employed to generate PCR products (635 bps and 491 bps for AOA and beta-AOB, respectively) from extracted total genomic DNA, as described previously (Cao et al. 2011a, b; Li et al. 2011). All PCR reactions (50 μl volume) were conducted using the following reagents: 1 μl template DNA (30–100 ng μl^{-1}), 20 pmol of each primer, 1.25 U of *Taq* polymerase, 200 μM each dNTP, 150 mM $MgCl_2$ and 1/10 volume of 10 × PCR buffer. A thermocycler program consisting of the following steps was used: 5 min at 94°C, then 35 cycles of 45 s at 94°C, then 45 s at annealing temperature (53°C for AOA and 55°C for beta-AOB) and 45 s extension at 72°C, with the last cycle run at 72°C for 7 min as the terminal extension. Purified PCR products were inserted into the T-vector (Takara, Hong Kong) for constructing clone libraries and then selected positive clones were sequenced using ABI 3730XL sequencer (ABI, Applied Biosystems). The abundance (copy numbers) of archaeal and bacterial *amoA* genes in the samples were determined with the specific primers above by quantitative PCR (q-PCR) using the same volume of each reaction mixture, including 1–10 ng template DNA, 0.2 mg/ml BSA, 200 nM of each primer and 12.5 μl of SYBR Premix (Takara, Hong Kong). The protocol used was according to Chen et al. (2008). All reactions were run in triplicate. A serial dilution of plasmids containing clone 704A-6 for AOA and clone 704B-6 for beta-AOB were separately used as standards.

Phylogenetic and statistical analyses

All of the *amoA* gene sequences determined were imported into MEGA 4.1 (Tamura et al. 2007) and translated into amino acid sequences to construct alignment files using Clustal X 1.81 in combination with the most similar AOA and beta-AOB deduced AmoA amino acid sequences retrieved from GenBank. Phylogenetic trees were constructed from a total of 103 deduced AOA AmoA protein sequences (59 sequences from this study) with the final length of 209 aa and 74 deduced beta-AOB AmoA protein sequences (35 sequences from this study) with the final length of 158 aa using neighbor-joining criterion in MEGA 4.1 (Tamura et al. 2007) with 1000 bootstrap tests.

Rarefaction analysis and two additional nonparametric richness estimators, Chao1, Simpson and the Shannon diversity index were performed in DOTUR based on a 5% gene sequence distance cutoff (Schloss and Handelsman 2006). Principal coordinates analyses (PCoA) and Jackknife Environment Clusters analyses were conducted using online software (UniFrac, http://bmf2.colorado.edu/unifrac/) for comparison of the microbial communities using phylogenetic information (Lozupone et al. 2006). Environment clusters trees were projected in MEGA 4.1 (Tamura et al. 2007). The relationships between physicochemical characteristics and those diversity parameters and abundance of *amoA* gene for AOA and beta-AOB were determined through calculation of Pearson moment correlations in Microsoft Excel.

Results

Environmental parameters

The environmental features of sediments at the six sites are shown in Supplementary Table S1. The seawater depth ranged from 40 m at the shallowest site 709 to 2370 m at the deepest site 702. The temperature was in clear contrast with the seawater depth and it decreased with the depth. The lowest surface sediment temperature was 2.9°C at site 702, whilst the highest was 21.3°C at site 709 with the shallowest depth. Salinity showed minimal fluctuation among all six sites. The pH value was the lowest at site 702 and the highest at site 708. The concentration of NH_4^+ peaked at site 707 and, except for this site, the concentration of NH_4^+ showed a decreasing trend from the continental shelf adjacent to Pearl River estuary discharge to the slope of the SCS. The concentration of NO_3^- was the highest at site 707, while NO_2^- was the highest at site 704.

Diversity and richness of *amoA* gene sequences in the surface sediments

Overall, a total of 88 and 27 OTUs based on *amoA* gene sequences were recovered for archaea and bacteria, respectively, based on a 5% *amoA* gene sequence distance cutoff. Within each clone library, the numbers of OTU did not vary much for AOA but they increased for AOB from the continental shelf to the slope in the deep-sea corresponding to an increase of depth, notably with the highest diversity of beta-AOB at site 702 (Table 1). The Chao1 diversity estimator, a conservative one for species richness, showed higher variability with beta-AOB than AOA (Table 1) at a 5% identity difference. The Shannon index, which takes into account both species richness and evenness, also showed a similar variability difference between AOA and beta-AOB at a 5% identity difference (Table 1). Both the Chao1 diversity estimator and the Shannon diversity index indicated that beta-AOB diversity was significantly correlated with pH values.

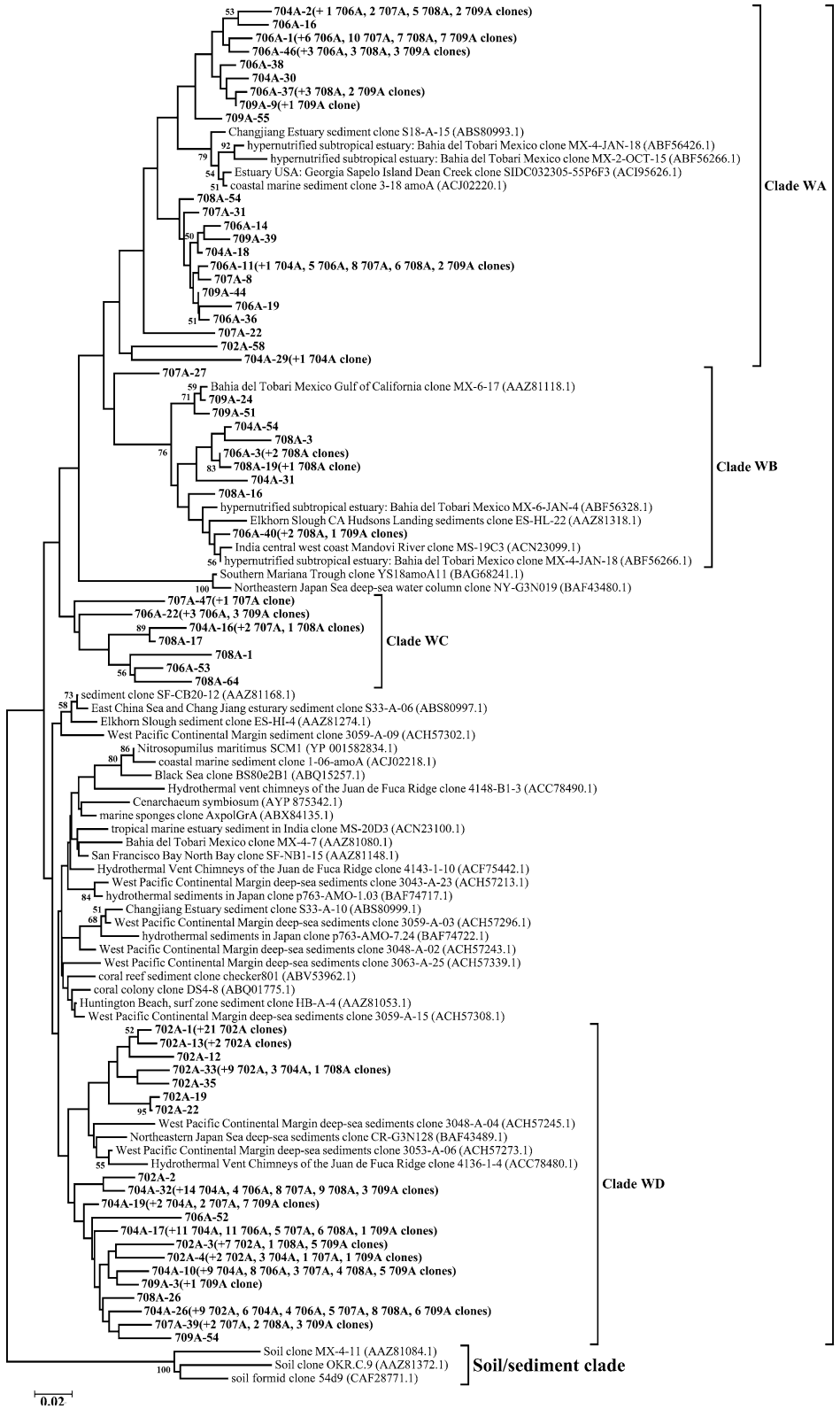
Fig. 1 Phylogenetic tree based on deduced archaeal AmoA protein sequences from sediment samples from a profile along the coastal shelf to the slope in South China Sea. The consensus phylogenetic tree was constructed using the neighbor-joining method. The numbers at the nodes are percentages represent of the credible values above 50 on the basis of 1000 times bootstrap test. Branch lengths were scaled by sequence differences. Sequences from this study are in *bold* and the information bracketed following the sequence names indicated other sequences obtained ▶

Phylogenetic diversity of deduced AmoA protein sequences in the surface sediments

The phylogenetic tree based on deduced archaeal AmoA protein indicated that the derived sequences were affiliated solely with sequences in marine water column/sediment clade and no sequences were associated with the soil/sediment clade detected in this phylogenetic tree (Fig. 1). However, a few subclades could be further delineated within the marine water/sediment clade (Fig. 1). Two subclades, Clades WA and WB, consisted of estuarine sequences from GenBank, e.g., Changjiang Estuary (Dang et al. 2008), subterranean estuary (Santoro et al. 2008), hyper-nutrified subtropical estuary (Beman and Francis 2006), Bahia del Tobari Mexico Gulf (Francis et al. 2005), Elkhorn Slough CA Hudsons Lan (Francis et al. 2005), tropical estuarine sediments on the central west coast of India (Singh et al. 2010). Except for a few sequences from site 704 and only

Table 1 Diversity and richness indices of the *amoA* gene sequences from the clone libraries

Sample	Clone number		OTUs		Simpson index		Shannon index		Chao1 index	
	AOA	AOB	AOA	AOB	AOA	AOB	AOA	AOB	AOA	AOB
702	60	39	19	11	0.11	0.12	2.50	2.15	22	12
704	61	41	23	9	0.07	0.24	2.80	1.63	34	19
706	54	41	19	5	0.08	0.40	2.61	1.05	21	5
707	52	44	18	4	0.09	0.43	2.57	0.94	20	4
708	68	31	20	2	0.07	0.94	2.66	0.14	29	2
709	55	30	19	5	0.05	0.29	2.77	1.34	21	5


704A-2(+ 1 706A, 2 707A, 5 708A, 2 709A clones)
706A-16
706A-1(+6 706A, 10 707A, 7 708A, 7 709A clones)
706A-46(+3 706A, 3 708A, 3 709A clones)
706A-38
704A-30
706A-37(+3 708A, 2 709A clones)
709A-9(+1 709A clone)
709A-55
Changjiang Estuary sediment clone S18-A-15 (ABS80993.1)
hypernutrified subtropical estuary: Bahia del Tobari Mexico clone MX-4-JAN-18 (ABF56426.1)
hypernutrified subtropical estuary: Bahia del Tobari Mexico clone MX-2-OCT-15 (ABF56266.1)
Estuary USA: Georgia Sapelo Island Dean Creek clone SIDC032305-55P6F3 (ACI95626.1)
coastal marine sediment clone 3-18 amoA (ACJ02220.1)
708A-54
707A-31
706A-14
709A-39
704A-18
706A-11(+1 704A, 5 706A, 8 707A, 6 708A, 2 709A clones)
707A-8
709A-44
706A-19
706A-36
707A-22
702A-58
704A-29(+1 704A clone)
Clade WA
707A-27
Bahia del Tobari Mexico Gulf of California clone MX-6-17 (AAZ81118.1)
709A-24
709A-51
704A-54
708A-3
706A-3(+2 708A clones)
708A-19(+1 708A clone)
704A-31
708A-16
hypernutrified subtropical estuary: Bahia del Tobari Mexico MX-6-JAN-4 (ABF56328.1)
Elkhorn Slough CA Hudsons Landing sediments clone ES-HL-22 (AAZ81318.1)
706A-40(+2 708A, 1 709A clones)
India central west coast Mandovi River clone MS-19C3 (ACN23099.1)
hypernutrified subtropical estuary: Bahia del Tobari Mexico clone MX-4-JAN-18 (ABF56266.1)
Clade WB
Southern Mariana Trough clone YS18amoA11 (BAG68241.1)
Northeastern Japan Sea deep-sea water column clone NY-G3N019 (BAF43480.1)
707A-47(+1 707A clone)
706A-22(+3 706A, 3 709A clones)
704A-16(+2 707A, 1 708A clones)
708A-17
708A-1
706A-53
708A-64
Clade WC
sediment clone SF-CB20-12 (AAZ81168.1)
East China Sea and Chang Jiang esturary sediment clone S33-A-06 (ABS80997.1)
Elkhorn Slough sediment clone ES-HI-4 (AAZ81274.1)
West Pacific Continental Margin sediment clone 3059-A-09 (ACH57302.1)
Nitrosopumilus maritimus SCM1 (YP 001582834.1)
coastal marine sediment clone 1-06-amoA (ACJ02218.1)
Black Sea clone BS80e2B1 (ABQ15257.1)
Hydrothermal vent chimneys of the Juan de Fuca Ridge clone 4148-B1-3 (ACC78490.1)
Cenarchaeum symbiosum (AYP 875342.1)
marine sponges clone AxpolGrA (ABX84135.1)
tropical marine estuary sediment in India clone MS-20D3 (ACN23100.1)
Bahia del Tobari Mexico clone MX-4-7 (AAZ81080.1)
San Francisco Bay North Bay clone SF-NB1-15 (AAZ81148.1)
Hydrothermal Vent Chimneys of the Juan de Fuca Ridge clone 4143-1-10 (ACF75442.1)
West Pacific Continental Margin deep-sea sediments clone 3043-A-23 (ACH57213.1)
hydrothermal sediments in Japan clone p763-AMO-1.03 (BAF74717.1)
Changjiang Estuary sediment clone S33-A-10 (ABS80999.1)
West Pacific Continental Margin deep-sea sediments clone 3059-A-03 (ACH57296.1)
hydrothermal sediments in Japan clone p763-AMO-7.24 (BAF74722.1)
West Pacific Continental Margin deep-sea sediments clone 3048-A-02 (ACH57243.1)
West Pacific Continental Margin deep-sea sediments clone 3063-A-25 (ACH57339.1)
coral reef sediment clone checker801 (ABV53962.1)
coral colony clone DS4-8 (ABQ01775.1)
Huntington Beach, surf zone sediment clone HB-A-4 (AAZ81053.1)
West Pacific Continental Margin deep-sea sediments clone 3059-A-15 (ACH57308.1)
702A-1(+21 702A clones)
702A-13(+2 702A clones)
702A-12
702A-33(+9 702A, 3 704A, 1 708A clones)
702A-35
702A-19
702A-22
West Pacific Continental Margin deep-sea sediments clone 3048-A-04 (ACH57245.1)
Northeastern Japan Sea deep-sea sediments clone CR-G3N128 (BAF43489.1)
West Pacific Continental Margin deep-sea sediments clone 3053-A-06 (ACH57273.1)
Hydrothermal Vent Chimneys of the Juan de Fuca Ridge clone 4136-1-4 (ACC78480.1)
702A-2
704A-32(+14 704A, 4 706A, 8 707A, 9 708A, 3 709A clones)
704A-19(+2 704A, 2 707A, 7 709A clones)
706A-52
704A-17(+11 704A, 11 706A, 5 707A, 6 708A, 1 709A clones)
702A-3(+7 702A, 1 708A, 5 709A clones)
702A-4(+2 702A, 3 704A, 1 707A, 1 709A clones)
704A-10(+9 704A, 8 706A, 3 707A, 4 708A, 5 709A clones)
709A-3(+1 709A clone)
708A-26
704A-26(+9 702A, 6 704A, 4 706A, 5 707A, 8 708A, 6 709A clones)
707A-39(+2 707A, 2 708A, 3 709A clones)
709A-54
Clade WD
Soil clone MX-4-11 (AAZ81084.1)
Soil clone OKR.C.9 (AAZ81372.1)
soil formid clone 54d9 (CAF28771.1)
Soil/sediment clade
Marine water column/sediment clade
0.02

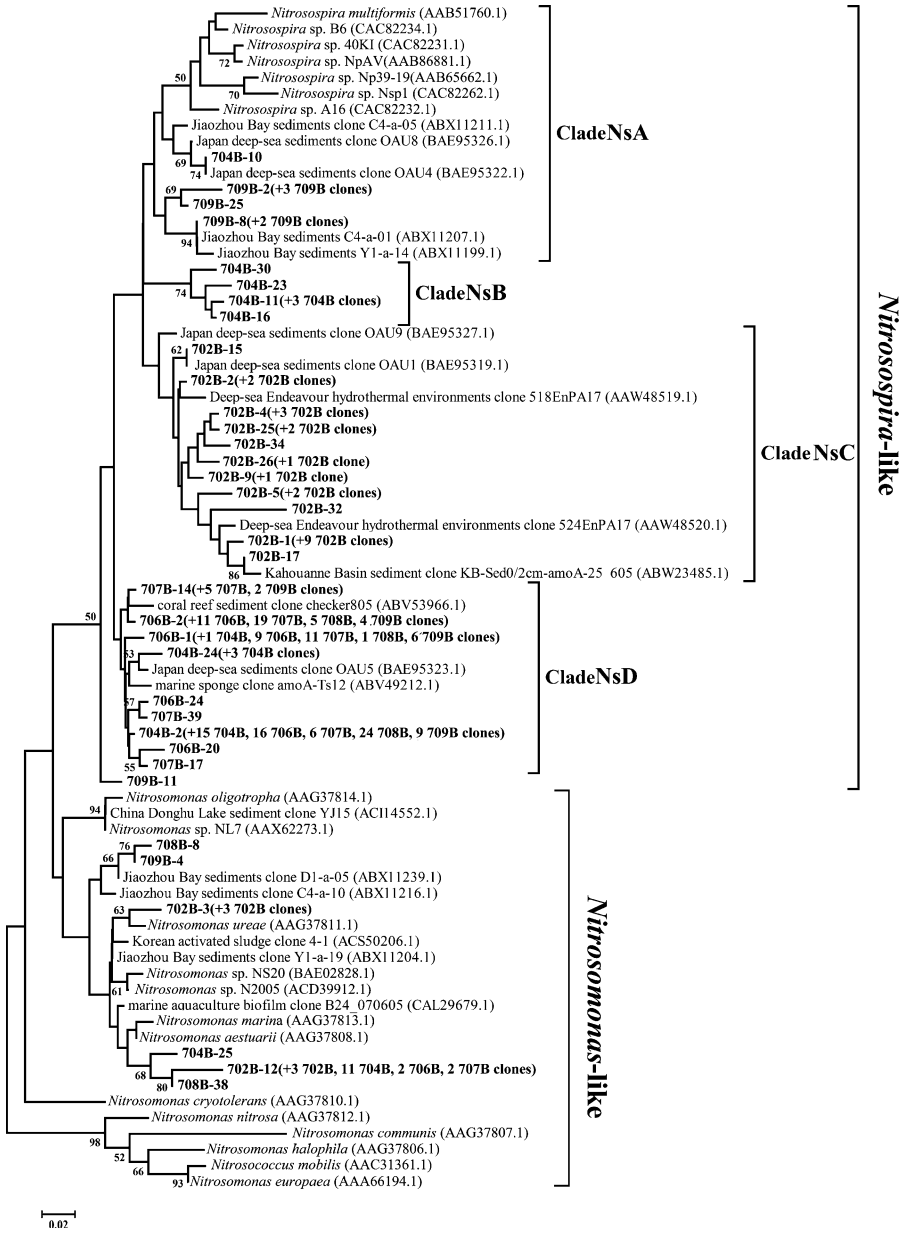


Fig. 2 Phylogenetic tree constructed from deduced bacterial AmoA protein sequences retrieved from sediment samples from a profile along the coastal shelf to the slope in South China Sea based on the neighbor-joining criterion. The numbers near the nodes represent of the credible values (>50) calculated from 1000 times bootstrap test. Branch lengths correspond to the amino acid differences. Sequences from this study are in *bold* and the information bracketed following the sequence names indicated other sequences obtained

one from site 702, most sequences affiliated with these two subclades were from sites 706, 707, 708 and 709, which are coastal samples close to the estuary. Clade WD contains sequences from deep-sea sediments, e.g., the tropical West Pacific Continental Margin sediment (Dang et al. 2009), Northeastern Japan Sea deep-sea sediments (Nakagawa et al. 2007) and hydrothermal vent chimneys of the Juan de Fuca Ridge (Wang et al. 2009). Most of the sequences affiliated with this subclade are from sites 702 and 704. Only a few sequences from sites 706, 707, 708, 709 clustered together and formed Clade WC, sharing <92% identity with others retrieved from GenBank at the deduced amino acid level, notably 708A-1 sharing <85% identity with sequences from hydrothermal vent of Juan de Fuca Ridge (the main endeavour segment, Mothra field) (Wang et al. 2009) and from Changjiang estuary, e.g., S7-A-19, S8-A-10 (Dang et al. 2008). It is clear that phylogenetically dominant lineages form a transitory distribution pattern along the transect from the estuarine-related site 709 to the deep-sea related site 702 in this study.

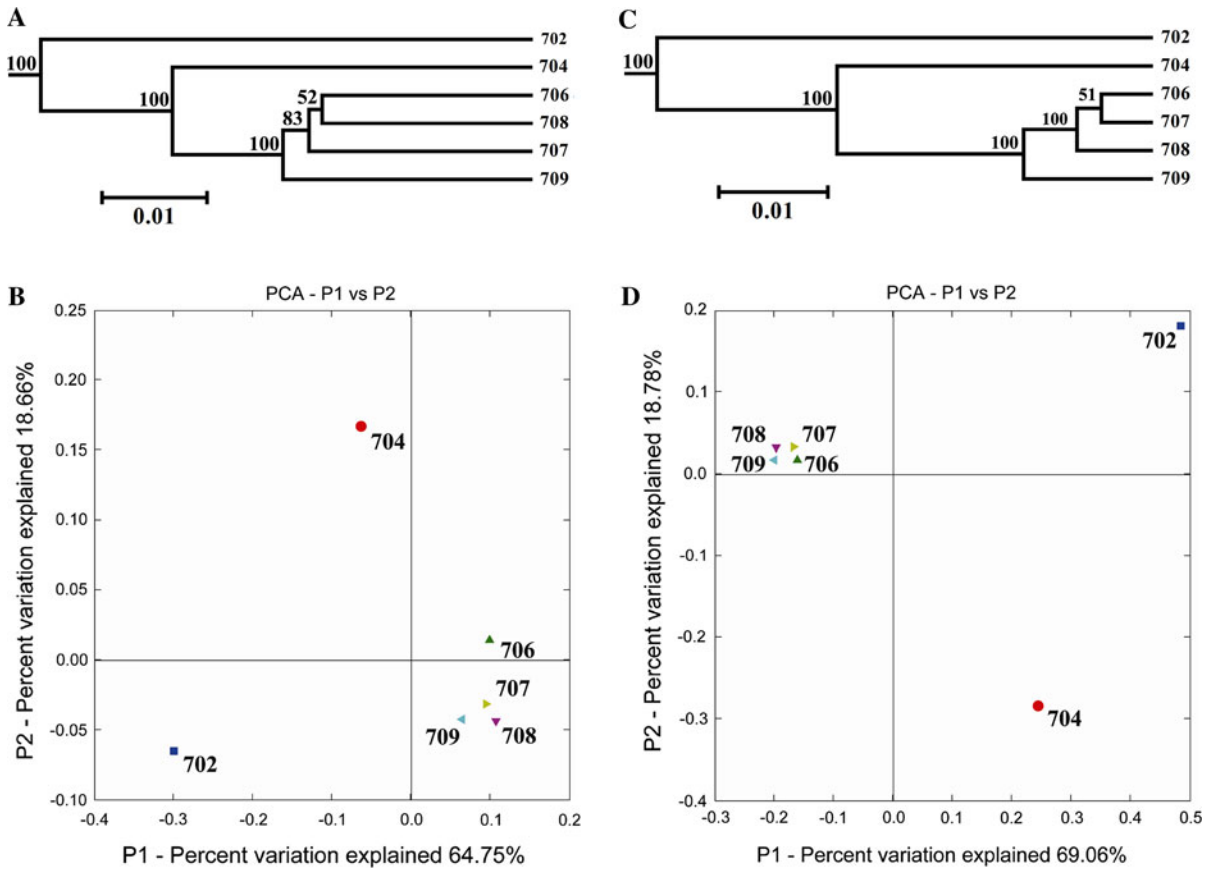


Fig. 3 Jackknife Environment Clusters (UPGMA algorithm with 100 replicates, Jackknife supporting test) and Principal coordinate analysis (PCoA) analyses for *amoA* gene assemblages of AOA and beta-AOB obtained from the online UniFrac software. **a** and **c** represent of the Jackknife Environment Clusters trees separately for AOA and beta-AOB. **b** and **d** show the PCoA separately for AOA and beta-AOB

Two distinct clades, of *Nitrosospira*-like and *Nitrosomonas*-like sequences, were present in the deduced bacterial AmoA protein phylogenetic tree (Fig. 2). Most of them were affiliated to *Nitrosospira*-like lineages (199 out of 226), while only 27 sequences grouped with *Nitrosomonas*-like lineages. In the *Nitrosomonas*-like clade, two sequences were clustered with those from sediments of Jiaozhou Bay (Dang et al. 2010) while others closely related to some pure cultures, including *Nitrosomonas ureae*, *Nitrosomonas marina* and *Nitrosomonas aestuarii*. The *Nitrosospira*-like lineages were the main inhabitants of this environment and showed higher diversity with four subclades. Sequences in Clade NsA were grouped with those from Jiaozhou Bay (Dang et al. 2010) and from deep-sea sediments in Japan (Hayashi et al. 2007) and was comprised mostly by sequences from site 709, with only one sequence from site 704. Another subclade, Clade NsC, exclusively contained sequences from site 702 grouped with sequences from deep-sea sediments in Japan (Hayashi et al. 2007), Kahouanne Basin sediment (Meyer and Kuever 2008) and deep-sea hydrothermal environments (unpublished) and thus showed deep-sea characteristics. Besides these two subclades, sequences from site 704 comprised of Clade NsB and formed a trifurcation with Clade NsA and Clade NsC as the intermediate branch of deep-sea sediment sequences and estuarine ones. The fourth subclade was comprised of sequences from all sites except for site 702 and grouped with sequences from Jiaozhou Bay (Dang et al. 2010), deep-sea hydrothermal environments (unpublished), marine sponges (Bayer et al. 2008), coral reef sediment in Hawaii of USA (unpublished).

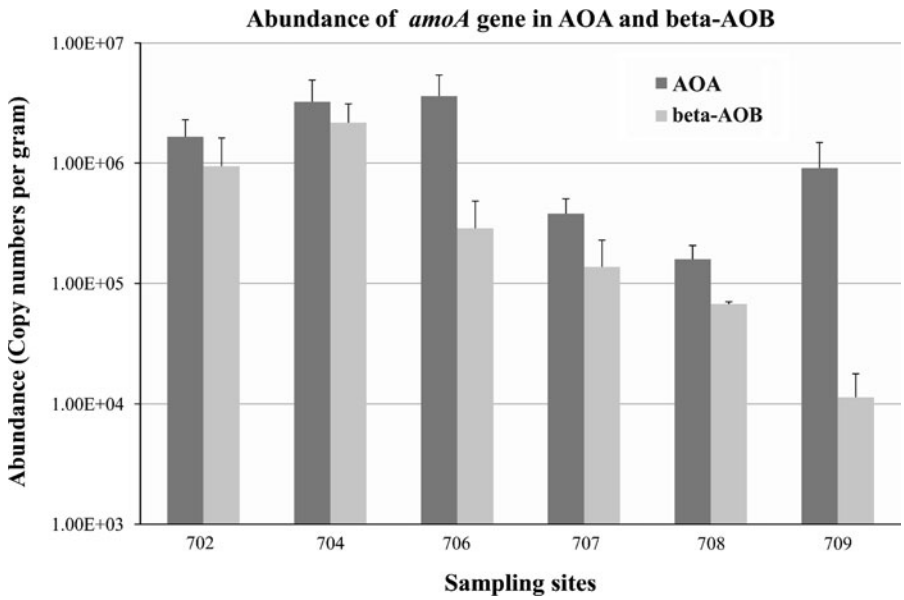


Fig. 4 Abundance of *amoA* gene for AOA and beta-AOB observed at the sampling sites. The measurements were performed in triplicate. *Error bars* represent standard deviations of triplicate analyses

Table 2 Statistical analysis of physicochemical parameters and the diversity and abundance of nitrifying prokaryotes in this study

Parameter	Pearson moment correlation[a]									
	OUT		Shannon Index		Simpson Index		Chao		Abundance	
	AOA	AOB	AOA	AOB	AOA	AOB	AOA	AOB	AOA	AOB
Depth	−0.15	0.76	−0.62	0.70	0.77	−0.50	−0.18	0.36	0.03	0.25
T	−0.14	−0.88*	0.56	−0.76	−0.78	0.56	−0.08	−0.62	−0.21	−0.54
pH	−0.19	−0.95*	0.12	−0.96*	−0.40	0.84*	0.01	−0.70	−0.33	−0.55
NH_4^+	−0.52	−0.42	−0.19	−0.26	0.05	0.07	−0.47	−0.41	−0.49	−0.41
NO_2^-	0.77	−0.42	0.34	0.27	0.10	−0.25	0.72	0.80	0.40	0.88*
$NO_3^- + NO_2^-$	0.41	0.39	0.08	0.34	0.29	−0.39	0.34	0.63	0.22	0.67
$NH_4^+/(NO_2^- + NO_3^-)$[b]	−0.49	−0.64	0.30	−0.40	−0.65	0.25	−0.46	−0.68	−0.57	−0.73

[a] Pearson moment correlation (r) calculated based on the following equation: $r = \frac{n(\sum XY)-(\sum X)(\sum Y)}{\sqrt{\left[n\sum X^2-(\sum X)^2\right]\left[n\sum Y^2-(\sum Y)^2\right]}}$. Asterisks indicates P of <0.05, could be as significant determined by Excel function TDIST. The t value deduced from the following equation: $t = X\sqrt{\frac{n-2}{(1-r^2)}}$. n is the number of sampling sites

[b] Molar ratios between ammonium and oxidized inorganic species (nitrate plus nitrite)

This subclade should be considered as a costal or estuarine lineage since it lacks any sequence from site 702, the only deep-sea sample.

Community classification of AmoA protein sequences in the gradient of sediments

Both PCoA and the Jackknife Environment Cluster analyses were employed to detect the significant differences in the *amoA* assemblages (Lozupone et al. 2007). Not only for AOA, but also for beta-AOB, sites 702 and 704 separated from other sites with larger discrepancy, while the other four sites grouped together with higher support values, whilst 709 was separated from the other three sites (Fig. 3).

Abundance of *amoA* gene sequences

The abundance of *amoA* gene sequences derived from AOA was from 1.59×10^5 to 3.63×10^6/gram

sediment (wet weight) and from 1.14×10^4 to 2.17×10^6/gram sediment (wet weight) for beta-AOB at different sites, without large differences within samples. The highest abundance identified was more than two orders than the lowest one in beta-AOB (the SD shown in the Fig. 4). Generally, the archaeal *amoA* gene abundance was higher than bacteria at the same sites. However, the AOA/beta-AOB abundance ratio was not high, except at site 709 which showed a notably high value (ca. 80). Moreover, the *amoA* gene abundance for AOA and beta-AOB showed a gradient from coastal samples to the deep-sea, excluding the archaeal *amoA* abundance at site 709.

Relationships between diversity and abundance of *amoA* sequences and physicochemical parameters

Temperature and pH values were indicated to be potentially important contributors to the diversity of bacterial *amoA* gene abundance, while the concentration of nitrite was related to the abundance of bacterial *amoA* gene in the present investigated area (Table 2). However, no factor was shown to contribute significantly to the diversity or abundance of the archaeal *amoA* genes in this study area (Table 2).

Discussion

A clear shift of both AOA and beta-AOB distribution from the marginal coast to the slope in the deep-sea was evident based on the diversity and phylogenetic analyses. Although the diversity indices of AOA were stable among all samples, differences of archaeal *amoA* gene assemblages could also be distinguished. For example, sites 706, 707, 708 and 709 were grouped together in the UniFrac analyses and shared some terrestrial characteristics, while site 702 possessed obvious deep-sea marine features. Most of the deep-sea AOA lineages were from sites 702 and 704, while most sequences from other sites formed other subclades close to the estuarial lineages, indicating the niche specificity along the transect of this study. For the beta-AOB, community changes were also obvious, not only judged by the diversity index but also based on the phylogenetic analyses. Most of the sequences were grouped into the *Nitrosospira*-like clade, while only a few sequences were clustered with the *Nitrosomonas*-like clade. This type of distribution was also detected in Changjiang estuary and the adjacent East China Sea through analyzing the AOA *amoA* gene, indicating a strong impact of the Changjiang freshwater discharge on the marine benthic microbial ecosystem (Dang et al. 2008). Here, from the coastal area to the slope in the deep-sea, environmental factors may be the driving force to shape the observed distribution pattern for these two types of nitrifying microbes.

Based on correlation analyses, temperature and pH values are proposed to be potential contributors to the diversity of bacterial *amoA* genes, while the concentration of nitrite was related to the abundance of bacterial *amoA* genes (Table 2). However, no noticeable factor could be identified for the diversity or abundance of archaeal *amoA* genes (Table 2). It was found that pH value was positively correlated with the diversity of beta-AOB, likely because pH is an important factor affecting the concentration of ammonia which is the substrate of ammonia monooxygenase. For the temperature effect, it is more likely to be associated with other factors such as depth, even though temperature was proposed to be the driving force to affect the beta-AOB community (Sahan and Muyzer 2008; Tourna et al. 2008; Urakawa et al. 2008). Altogether, this information could provide insights to understand the differences of ammonia monooxygenase between AOA and beta-AOB.

Though substantial numbers of sequences for AOA were retrieved from each sediment sample, there was no sequences sharing highest identity with those of the soil/sediment clade, as the sequences were only grouped into the marine water column/sediment clade. The similar observation that soil/sediment clade lineages were not retrieved was also reported at deep-sea hydrothermal fields of the Southern Mariana Trough (Kato et al. 2009). One possible explanation is that a bias of our PCR primers resulted in the mismatch with the potential AOA lineages inhabiting in this environment. Other gene markers should be employed to eliminate this possibility, such as the genes for the 16S rRNA, *nir*K (nitrite reductase) (Bartossek et al. 2010), *hcd* (putative 4-hydroxybutyrate-CoA dehydratase) (Zhang et al. 2010; Offre et al. 2011), *accA* (acetyl-CoA carboxylase) or *hbd* (4-hydroxybutyryl-CoA dehydratase) (Yakimov et al. 2011). However, it is

plausible that the actual distribution of the AOA were retrieved here. Given the results, there should be some parameters that shape the specific distribution of AOA. On the other hand, some lineages present here might have higher adaptive ability to this special environment than others but further studies are needed to clarify this.

Although the soil/sediment clade was absent in the phylogenetic tree of the archaeal AmoA deduced protein sequences present, two subclades shared much higher identity with those retrieved from marine and estuarine environments than those typically of terrestrial environment represent by soils and hot springs. These two subclades are separately represented by deep-sea related and estuarine sequences and also show some endemic lineages. Most of the archaeal AmoA protein sequences in the deep-sea related subclade (Clade WD) from the present study grouped with those retrieved from the Western Pacific Ocean, e.g., the Deep-Ocean of Northeastern Japan Sea (Nakagawa et al. 2007), especially the tropical West Pacific Continental Margin (Dang et al. 2009) with high identities at the protein level, indicating these lineages might tolerate to a much wider range of environmental conditions in West Pacific or that these environments share some similarity. In addition, a few sequences shared larger genetic distances (with only about 85% identity) with deep-sea and estuary related subclades, for instance sequences 707A-47, 706A-53, 708A-1, 708A-64, 707A-15, 708A-17, 704A-16, 707A-43. This indicates the endemicity of these AOA lineages in the SCS. In another type of estuarine subclades, most of the sequences were clustered with those from estuaries (Beman and Francis 2006; Dang et al. 2008; Santoro et al. 2008) while most of the sequences still separated from these retrieved from GenBank, especially in Clade WA, indicating the endemicity of the lineages recovered here.

In the phylogenetic tree of bacterial deduced AmoA amino acid sequences, most sequences were grouped in the genus *Nitrosospira*-like clade, while only 27 sequences were affiliated to the *Nitrosomonas*-like clade. Moreover, all of the beta-AOB sequences were grouped with those from estuaries, bays or marine sediments, suggesting these are typical marine or estuarine features. In polluted terrestrial environments (away from estuarine regions), the dominant beta-AOB generally is the genus *Nitrosomonas* (Dang et al. 2010). It seems that *Nitrosospira*-like lineages may be more adaptable than *Nitrosomonas* in estuarine areas. Beta-AOB communities at sampling sites with a strong marine influence were dominated by *Nitrosospira* cluster-like sequences and those with a strong freshwater influence were dominated by *Nitrosomonas oligotropha*-like sequences (Freitag et al. 2006). All of the beta-AOB lineages presented here showed large distances from *Nitrosomonas oligotropha* like lineages (Fig. 2).

The presence of ammonia-oxidizing prokaryotes in the SCS continental margin suggests they may play important roles from the continental shelf to the slope in the deep-sea because the former is adjacent to the Pearl River estuary, which receives a high loading of anthropogenic nutrients in the river discharge. The community changes of AOA and beta-AOB detected in the present study should advance our understanding about the contributors to the nitrogen transformation process in this area. Studies have shown that the archaeal diversity in estuaries might be greater than that in the adjacent open oceans due to terrestrial inputs (Llyd and Jody 2003). For the AOA lineages, some studies have proposed that those in terrestrial environments are distinct from those in marine environments while other studies have indicated that estuaries might harbor mixed populations from soil/sediment and marine water column/sediment AOA lineages (Dang et al. 2008; Mosier and Francis 2008). The deposition of microbes from the freshwater input could potentially explain the existence of the putative soil-related AOA lineages in the estuarine sedimentary environments. However, in the current study, no obvious soil/sediment-related AOA lineages were present, only estuary and deep-sea related lineages, indicating this area was not strongly affected by the Pearl River discharge. Another study on the total inorganic nitrogen concentration in this area indicated that the sampling sites had reduced inorganic nitrogen influence from the Pearl River estuary (Li et al. 2007) and this is consistent with the absence of soil/sediment-related archaeal *amoA* observed. However, the community structure change of ammonia-oxidizing beta-AOB along the transect could reflect environmental conditions in this area.

In summary, community structure shifts of AOA and beta-AOB were detected from the coastal marginal area to the slope in the deep-sea. The OTU

numbers of *amoA* in AOA were much more stable than in beta-AOB. Moreover, the diversity of beta-AOB was potentially correlated with pH values, while no obvious relationships between AOA and physicochemical parameters were detected, indicating beta-AOB responded more variably to transect changes in this environment, as shown with the diversity and richness indices. Thus beta-AOB might be a indicator for environmental changes. Spatial patterns of these nitrifying microbes and some physicochemical parameters that potentially influence their diversity and distribution are proposed. Besides these, endemic AOA and beta-AOB phylotypes existed in our research area.

The GenBank accession numbers for the *amoA* gene sequences reported in this study are JF923943 to JF924293 and JF924294 to JF924519 for AOA and AOB, respectively.

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