Microbiology (2008), **154**, 2084–2095 DOI 10.1099/mic.0.2007/013581-0

Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea

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Ammonia-oxidizing archaea (AOA) have recently been found to be potentially important in nitrogen cycling in a variety of environments, such as terrestrial soils, wastewater treatment reactors, marine waters and sediments, and especially in estuaries, where high input of anthropogenic nitrogen is often experienced. The sedimentary AOA diversity, community structure and spatial distribution in the Changjiang Estuary and the adjacent East China Sea were studied. Multivariate statistical analysis indicated that the archaeal *amoA* genotype communities could be clustered according to sampling transects, and the station located in an estuarine mixing zone harboured a distinct AOA community. The distribution of AOA communities correlated significantly with the gradients of surface-water salinity and sediment sorting coefficient. The spatial distribution of putative soil-related AOA in certain sampling stations indicated a strong impact of the Changjiang freshwater discharge on the marine benthic microbial ecosystem. Besides freshwater, nutrients, organic matter and suspended particles, the Changjiang Diluted Water might also contribute to the transport of terrestrial archaea into the seawater and sediments along its flow path.

Received 24 September 2007
Revised 8 February 2008
Accepted 12 February 2008

INTRODUCTION

Microbial nitrification, the process of ammonia oxidation to nitrate via nitrite ($NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$), is a key component of the global nitrogen (N) biogeochemical cycle. Over geological timescales, the N cycle is thought to have affected the global biogeochemical cycle of carbon and hence the content of atmospheric CO_2 (Falkowski, 1997). On a local scale, microbial nitrification also contributes to the bioremediation of anthropogenic N eutrophication in estuarine and coastal environments (Francis *et al.*, 2005; Caffrey *et al.*, 2007), via coupled nitrification–denitrification or nitrification–anammox (anaerobic oxidation of ammonium) processes (Seitzinger, 1988; Coolen *et al.*, 2007; Lam *et al.*, 2007).

The recent discovery that some mesophilic archaea in the kingdom *Crenarchaeota* possess the potential for chemoautotrophic ammonia oxidation, the first and rate-limiting step in microbial nitrification, suggests an important role of archaea in the N cycle (Venter *et al.*, 2004; Konneke *et al.*, 2005; Treusch *et al.*, 2005). Crenarchaeota are ubiquitous and abundant in marine waters and sediments (DeLong, 1992; Vetriani *et al.*, 1999). Genomic and environmental microbiological studies indicated that the crenarchaeota microbiota could be chemoautotrophic (Wuchter *et al.*, 2003; Herndl *et al.*, 2005; Hallam *et al.*, 2006a, b; Ingalls *et al.*, 2006), heterotrophic (Ouverney & Fuhrman, 2000; Teira *et al.*, 2006) or mixotrophic. The crenarchaeotal ammonia mono-oxygenase gene (*amoA*) was found to be pervasive in the ocean (Francis *et al.*, 2005; Beman & Francis, 2006; Beman *et al.*, 2007; Lam *et al.*, 2007; Nakagawa *et al.*, 2007). Quantitative studies also

Abbreviations: AOA, ammonia-oxidizing archaea; CCA, canonical correspondence analysis; CDW, Changjiang Diluted Water; OrgN, organic nitrogen; OrgC, organic carbon; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; TWC, Taiwan Warm Current.

The GenBank/EMBL/DDBJ accession numbers for the archaeal *amoA* gene sequences determined in this study are EU025140 to EU025186.

Four supplementary figures are available with the online version of this paper.

indicated that ammonia-oxidizing archaea (AOA) appear to be more abundant than ammonia-oxidizing bacteria (Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Nakagawa *et al.*, 2007), and the potential estuarine nitrification rates increased as abundance of AOA *amoA* increased (Caffrey *et al.*, 2007), further suggesting the ecological importance of the AOA.

Although nitrification is of particular importance in estuarine and coastal sediments, and the ability to oxidize ammonia may be broadly distributed in the crenarchaeota (Nicol & Schleper, 2006; Caffrey *et al.*, 2007; Cavicchioli *et al.*, 2007; Francis *et al.*, 2007), only a few studies have actually examined the diversity and spatial distribution of the sedimentary AOA communities (Francis *et al.*, 2005; Beman & Francis, 2006). These studies have shown that diverse AOA phylotypes and distinct AOA communities exist in different marine environments both on a large geographical scale and in local estuarine gradients, demonstrating that their spatial distribution may be associated with environmental conditions (Francis *et al.*, 2005; Beman & Francis, 2006). However, these studies were limited to the temperate and subtropical coast of the East Pacific Ocean. The sedimentary diversity and spatial distribution of AOA are largely unknown in other coastal areas of the world oceans, including most large river estuaries.

The Changjiang River (historically called the Yangtze River) is the third-largest river in the world, with a huge water discharge of 9.24×10^{11} m^3 per year, equivalent to 1/50 of the water volume of the adjacent East China Sea (Yanagi, 1994), the largest continental marginal sea in the western Pacific. This river also delivers more than 7.5×10^{10} moles per year of N nutrients to the East China Sea (Zhu *et al.*, 2005). Thus, the estuary and shelf region has been the research focus of a series of international programmes, such as JGOFS (Joint Global Ocean Flux Study), GLOBEC (Global Ocean Ecosystems Dynamics), LOICZ (Land–Ocean Interactions in the Coastal Zone) and IMBER (Integrated Marine Biogeochemistry and Ecosystem Research), due to its importance in fishery, climate change and environmental issues, such as pollution, eutrophication, red tides and hypoxia (Li *et al.*, 2002; Li & Daler, 2004; Chai *et al.*, 2006). Being the interface of land, freshwater and marine environments, this region is extremely complicated and dynamic, due to the variability of freshwater input, currents and anthropogenic inputs, as well as the construction and operation of the Three Gorges Dam in the middle reaches of the Changjiang River (Zhang *et al.*, 1999; Jiao *et al.*, 2007). Micro-organisms may play important roles in this unique large river estuarine ecosystem, particularly in biogeochemical cycles and food webs. However, studies of microbial ecology started here only recently (Sekiguchi *et al.* 2002; Zhang & Jiao, 2007). It has been shown that marine crenarchaeota dominate the pelagic archaeal community, and 16S rRNA gene sequences related to autotrophic ammonia-oxidizing '*Candidatus* Nitrosopumilus maritimus' were detected in the estuarine area (Zeng *et al.*, 2007). Due to environmental heterogeneity and intense riverine input of anthropogenic N, we hypothesized that the Changjiang Estuary and the adjacent East China Sea could harbour diverse sedimentary AOA, with distinct community structures and diversity in response to specific estuarine, geochemical and eutrophication gradients. To test this hypothesis, a molecular study based on the archaeal *amoA* functional marker gene was conducted.

METHODS

Study area and sampling sites. Sediment samples were collected from the Changjiang Estuary and adjacent East China Sea during a cruise of the R/V 'Dong Fang Hong 2', 14–20 June, 2006. Two sampling transects and a total of seven sampling stations were chosen for the current AOA project (Fig. 1). Sediments were collected with a stainless steel 0.1 m^2 Gray O'Hara box corer and only undisturbed core samples with clear overlying water were used (Jonasson & Olausson, 1966). Replicate surface sediment subcore samples down to 5 cm depth for microbiological and environmental analyses were taken with sterile 60 ml syringes (luer end removed), homogenized and stored in airtight sterile plastic bags at −20 °C during the cruise and −80 °C after returning to the laboratory.

Environmental physico-chemical analyses. At each station, *in situ* measurements of the physico-chemical parameters of seawater (conductivity, density, depth, dissolved oxygen, pH, turbidity, salinity and temperature) were recorded at various water depths with a SeaBird model SBE9 conductivity-temperature-depth recorder (SeaBird Electronics) (Table 1). Other parameters were measured in the laboratory. A nutrient AutoAnalyser (QUAATRO; Bran + Luebbe) was used to measure pore-water dissolved N and P concentrations: nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N) and phosphate (PO_4-P). Sediment total organic carbon (OrgC) and nitrogen (OrgN) contents were measured with a PE2400 series II CHNS/O elemental analyser (Perkin Elmer). A Cilas 940L laser granulometer (Company Industrielle des Lasers) was used for sediment grain size analysis and median grain size, sorting coefficient, skewness and kurtosis were calculated (Table 1).

DNA extraction and archaeal *amoA* gene clone library analyses. DNA was extracted from 0.3 g sediment using a FastPrep DNA Extraction Kit for Soil and a FastPrep FP120 Cell Disrupter instrument (Qbiogene) (Francis *et al.*, 2005; Beman & Francis, 2006). Replicate DNA extractions from three subcore samples were pooled for each sampling station. Archaeal *amoA* fragments (~635 bp) were amplified in a PTC-200 thermal cycler (Bio-Rad) with published primers Arch-amoAF and Arch-amoAR and the corresponding PCR protocol (Francis *et al.*, 2005; Beman & Francis, 2006). PCR products from five reactions were pooled to minimize PCR bias, gel-purified, and ligated into pMD19-T simple vectors (Takara) according to the manufacturer's instructions. The hybrid vectors were used to transform *Escherichia coli* TOP10 competent cells prepared by using the calcium chloride protocol (Sambrook & Russell, 2001). Recombinants were selected by using X-Gal-IPTG Luria–Bertani (LB) indicator plates supplemented with 100 μg ampicillin ml^{-1}. Each clone library was constructed by random selection of approximately 100 white colonies from a single plate. A plasmid miniprep method was used for recombinant plasmid preparations (Dang & Lovell, 2000). Cloned *amoA* fragments were reamplified using primers M13-D (5′-AGGGTTTTCCCAGTCACGACG-3′) and RV-M (5′-GAGCGGATAACAATTTCACACA-

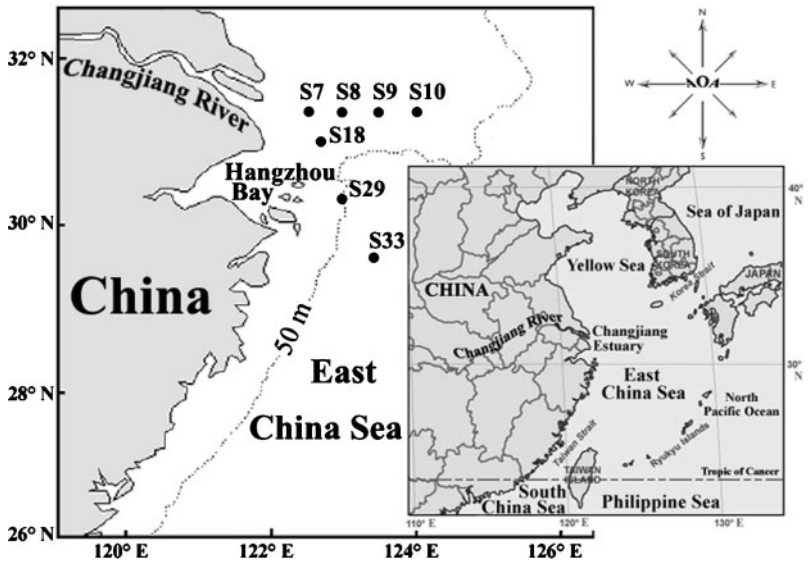


Fig. 1. Maps showing the sampling transects and stations in the Changjiang Estuary and the adjacent East China Sea.

GG-3′), flanking the insertion site of the vector. PCR products were screened for correct size and purity by 1 % agarose gel electrophoresis.

Amplimers with the correct size were digested using *Msp*I and *Hha*I (Fermentas). Restriction fragments were resolved by electrophoresis on 4 % agarose gels in 0.5 × TBE, and digitally photographed with an ImageMaster VDS imaging system (Pharmacia Biotech). The band patterns of the RFLP analysis were compared in order to identify redundant clones.

Clone vector primer RV-M was used for sequencing with an ABI 3770 automatic sequencer (Applied BioSystems). Several random clones from the same RFLP patterns resulted in identical *amoA* sequences; the genetic variation in each of the RFLP patterns was probably quite low. The resultant unique sequences were grouped into operational taxonomic units (OTUs) based on 95 % DNA sequence similarity calculated with the DOTUR program (Schloss & Handelsman, 2005), to facilitate comparison with other studies (Francis *et al.*, 2005; Beman & Francis, 2006; Park *et al.*, 2006; Beman *et al.*, 2007). The sequences of the putative archaeal *amoA* genes were translated into conceptual amino acid sequences using the BioEdit program (Hall, 1999), and the online BLAST program (Altschul *et al.*, 1997) from the GenBank database was used for retrieval of the closest matched sequences. AmoA sequences were aligned using the CLUSTAL_X program (Thompson *et al.*, 1994) and phylogenetic trees constructed using the PROTDIST and NEIGHBOR programs of the PHYLIP package (version 3.66) (Felsenstein, 1989).

Statistical analyses. The estimated coverage of the constructed archaeal *amoA* gene libraries was calculated as $C=[1-(n_1/N)]\times 100$, where n_1 is the number of unique (frequency=1) *amoA* RFLP genotypes or OTUs detected in a library and N is the total number of clones in the same library (Mullins *et al.*, 1995). This value approximated the probability that all the unique sequences present in a given sample were represented at least once in the library.

Indices of the *amoA* genotype diversity (Shannon–Wiener H and Simpson D) and evenness (J) were calculated with *amoA* RFLP genotypes or OTUs of the clone libraries (Brown & Bowman, 2001). Rarefaction analysis and two nonparametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao1 (S_{Chao1}), were calculated using the DOTUR program (Schloss & Handelsman, 2005; Beman & Francis, 2006). These diversity indices and richness estimators are useful statistical tools to compare the relative complexity of communities and to estimate the completeness of sampling.

The AOA community classification was determined with weighted UniFrac environmental clustering and principal coordinates analyses (PCoA) (Lozupone & Knight, 2005; Lozupone *et al.*, 2007). The online UniFrac program (http://bmf.colorado.edu/unifrac/index.psp) takes molecular evolutionary distances of the sequences and their environmental occurrences for microbial community similarity analyses, particularly suitable for sequence data. Correlations between AOA communities and environmental factors were analysed by canonical correspondence analysis (CCA) using the software Canoco (version 4.5, Microcomputer Power) (ter Braak & Šmilauer, 2002). High occurrence of zero entries (~64 %) in the species table (i.e. the OTU table) indicated that the unimodal CCA analysis was more suitable than the linear-model-based redundancy analysis (RDA) for our data (Lepš & Šmilauer, 2003). The percentage frequency data of the *amoA* OTUs were used as the species input, and the environmental variables entered into the CCA were normalized (i.e. adjusted for a mean of 0 and SD of 1 via Z transformation) (Magalhães *et al.*, 2007). Manual deselection of collinear environmental variables and forward selection with significance tests of Monte Carlo permutations were used to build the optimal models of the microbe–environment relationship (Lepš & Šmilauer, 2003). These multivariate statistical methods cope with major issues in microbial ecology, such as the distribution and relatedness of diversity and community structure with environmental variables or along an environmental gradient (Lozupone *et al.*, 2007; Magalhães *et al.*, 2007).

RESULTS

Environmental hydrochemical and geochemical settings

Changjiang River water entering the East China Sea in the flood (wet) season (usually from May to October) had low salinity, high temperature and high nutrient level, especially of N (~7.5×10^{10} moles nitrate per year) (Zhu *et al.*, 2005). Gradients of surface-water salinity, density and

Table 1. *In situ* environmental parameters of the sampling stations in the Changjiang Estuary and the adjacent East China Sea

Environmental factor	Station						
	S7	S8	S9	S10	S18	S29	S33
Latitude (°N)	31.3517	31.3277	31.3072	31.2967	31.0168	30.2507	29.4983
Longitude (°E)	122.4813	122.9297	123.4797	123.9747	122.6178	122.8357	123.197
Surface water							
Depth (m)	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Temperature (°C)	21.21	20.97	20.47	20.78	21.65	22.18	23.56
Conductivity (mS cm^{-1})	30.20	39.38	44.27	45.15	32.00	42.47	42.30
Salinity (PSU*)	20.4	27.4	31.7	32.1	21.5	29.1	28.0
Density (σ_t)	13.34	18.75	22.11	22.38	14.07	19.67	18.51
Dissolved oxygen (μM)	235.34	279.90	252.97	257.42	246.24	342.80	305.90
pH	8.22	8.46	8.44	8.39	8.27	8.63	8.55
Turbidity (FTU†)	12.03	0.97	0.66	0.37	3.95	1.78	0.38
Bottom water							
Depth (m)	19.0	41.0	45.0	49.0	20.0	26.0	60.0
Temperature (°C)	18.73	19.35	19.10	19.06	19.65	20.27	18.02
Conductivity (mS cm^{-1})	44.63	45.71	44.73	44.42	43.01	45.25	45.28
Salinity (PSU)	33.3	33.7	33.1	32.9	31.3	32.6	34.4
Density (σ_t)	23.80	23.94	23.53	23.37	22.01	22.86	24.83
Dissolved oxygen (μM)	149.53	173.53	186.23	218.87	171.55	192.92	183.52
pH	8.27	8.27	8.34	8.32	8.24	8.39	8.31
Turbidity (FTU)	37.33	12.29	4.33	2.41	122.13	16.72	2.33
Sediment pore-water							
Salinity (PSU)	30.0	32.0	ND	31.0	27.0	30.0	ND
pH	7.31	7.45	ND	7.8	7.41	7.32	ND
NH_4-N (μM)	550.54	704.63	ND	153.90	503.02	1808.07	ND
NO_2-N (μM)	0.86	6.45	ND	6.98	9.39	3.07	ND
NO_3-N (μM)	4.06	1.97	ND	3.33	8.38	6.40	ND
DIN‡ (μM)	555.46	713.05	ND	164.21	520.79	1817.54	ND
PO_4-P (μM)	2.26	6.54	ND	3.39	2.11	5.05	ND
N/P (DIN/PO_4-P)	245.78	109.03	ND	48.44	246.82	359.91	ND
Sediment							
OrgC (total organic C, %)	0.61	0.30	ND	0.21	0.64	0.27	ND
OrgN (total organic N, %)	0.08	0.03	ND	0.02	0.07	0.03	ND
Sand (%)	29.01	54.07	59.32	72.75	39.60	23.66	24.21
Silt (%)	57.67	34.10	30.17	20.69	44.64	52.73	48.33
Clay (%)	13.32	11.83	10.51	6.57	15.75	23.61	27.46
Median grain size (Ø)	5.09	3.86	2.92	2.91	4.98	6.32	6.51
Sorting coefficient	1.90	2.17	2.10	2.11	2.09	2.18	2.30
Skewness	1.79	2.18	2.29	2.38	1.86	1.11	0.37
Kurtosis	2.47	2.84	2.84	3.03	2.59	2.59	2.58

ND, Not determined.
*PSU, practical salinity units; 1 PSU ≈ 0.1 %.
†FTU, formazin turbidity units.
‡DIN, dissolved inorganic nitrogen.

conductivity along the two sampling transects indicated that strong freshwater runoff from the Changjiang River formed the characteristic Changjiang Diluted Water (CDW) in the East China Sea (Table 1). The northward intrusion of the Taiwan Warm Current (TWC) from the south along the 50 m isobath is another permanent feature of the East China Sea circulation in summer (Ichikawa & Beardsley, 2002; Chen *et al.*, 2003). The TWC saline water met with the CDW freshwater just off the Changjiang River mouth, forming salinity fronts around 122.5° E. Most of the fine particles carried by the CDW were deposited in this estuarine mixing zone, and were responsible for the creation of the estuarine maximum turbidity zone (Table 1). Surface-water salinity and turbidity gradients showed that part of the CDW flowed in the south-eastward branch along the coastline during our sampling time, in good

Table 2. Analyses of the archaeal *amoA* clone libraries constructed for the seven sedimentary sampling stations in the Changjiang Estuary and adjacent East China Sea

The calculations of the coverage, diversity indices and richness estimators are based on both RFLP genotypes (left of /) and OTUs (right of /).

Station	No. of clones	No. of RFLP genotypes	No. of OTUs	*C* (%)	*H*	1/*D*	*J*	S_{ACE}	S_{Chao1}
S7	87	24	16	86.2/96.6	3.891/3.562	11.839/10.421	0.849/0.890	41.7/17.5	57.0/17.5
S8	92	12	9	94.6/96.7	2.374/2.204	3.541/3.437	0.662/0.695	18.4/11.6	17.0/12.0
S9	90	9	6	95.6/98.9	1.984/1.858	2.998/2.967	0.626/0.719	14.0/6.4	15.0/6.0
S10	91	14	10	94.5/95.6	3.068/2.649	6.531/5.467	0.806/0.797	18.4/17.7	24.0/16.0
S18	90	16	12	90.0/95.6	2.707/2.550	4.495/4.339	0.677/0.711	35.6/15.1	28.0/14.0
S29	93	12	11	93.5/93.5	2.140/1.936	2.944/2.728	0.597/0.560	21.0/18.5	19.5/15.0
S33	94	14	10	93.6/94.7	3.021/2.453	6.683/4.660	0.793/0.738	29.4/33.8	21.5/20.0

accordance with previous findings in summer seasons (Chen *et al.*, 2003; Zhu *et al.*, 2005).

Due to the intrusion of the TWC, bottom water at the sampling stations usually had higher salinity than surface water (Table 1). The most significant gradients among the various bottom-water physico-chemical parameters were dissolved oxygen and turbidity. The S7 station was usually located inside the Changjiang Estuary seasonal oxygen minimum zone (Li *et al.*, 2002). Although the bottom water did not reach anoxic conditions during our sampling period, the lowest dissolved oxygen value did occur at station S7 and obvious gradients existed along both the sampling transects as expected (Table 1). The bottom-water turbidity maxima at stations S18 and S7 indicated that, besides surface-water particulate matter sedimentation, seafloor surface sediment resuspension occurs intensely in this area. This was consistent with the fact that seasonal upwelling occurred in the area in summer (Zhu, 2003; Zhu *et al.*, 2005), which might have a strong influence on the bottom-water physical and geochemical characteristics.

The sediment and pore-water geochemical parameters, especially NH_4-N (ranging from 153.90 μM to 1808.07 μM) and N/P (from 48.44 to 359.91) (Table 1), indicated significant eutrophication of most of our sampling stations. The highest values occurred at station S29 and the lowest at station S10.

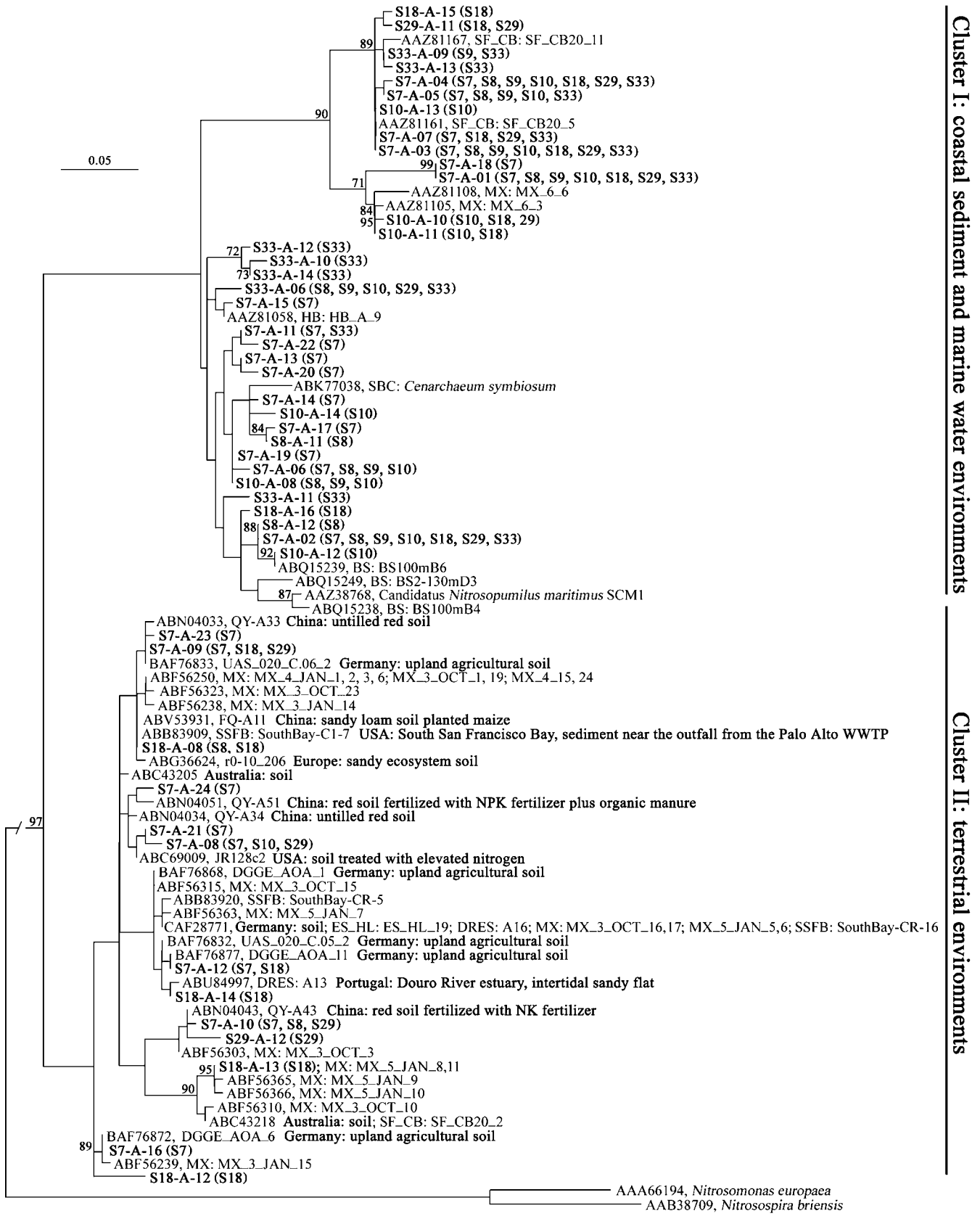
Diversity of the *amoA* libraries

From the seven archaeal *amoA* libraries constructed, 637 clones were screened, and 47 RFLP genotypes and 29 OTUs were identified. Sampling station S7 had the highest diversity of the archaeal *amoA* genotypes, and stations S9 and S29 had the lowest diversity, based on the values of the Shannon–Wiener, reciprocal of Simpson and evenness indices calculated with both the RFLP genotypes and the OTUs data, except for station S9's evenness index calculated with the OTUs data (Table 2). The estimated coverages of the clone libraries were quite high (Table 2), together with the rarefaction analyses (Supplementary Fig. S1, available with the online version of this paper), indicating that these libraries might have captured the majority of the archaeal *amoA* sequence types in the studied environments with the primers used. However, analyses of the richness estimators S_{ACE} and S_{Chao1} indicated that most of our sampling stations might have higher predicted archaeal *amoA* genotype diversity than recovered in the current study (Table 2).

AmoA sequence phylogenies

The 47 unique archaeal *amoA* gene sequences had 66.4–99.8 % sequence similarity among each other, and had various degrees of identity (87.8–99.7 %) to the closest matched GenBank sequences. After translation, the corresponding protein sequences had 74.9–100.0 %

Fig. 2 Phylogenetic tree constructed with the distance and neighbour-joining methods of the archaeal AmoA sequences translated from the cloned *amoA* gene sequences recovered from the Changjiang Estuary and the adjacent East China Sea. Partial archaeal AmoA sequences of 185 aligned amino acid positions were used for tree construction. The tree branch distances represent amino acid substitution rate, and the scale bar represents the expected number of changes per homologous position. Bacterial AmoA sequences from *Nitrosospira briensis* and *Nitrosomonas europaea* were used as the outgroup. Bootstrap values greater than 70 % of 100 resamplings are shown near nodes. Abbreviations: BS, Black Sea seawater; DRES, Douro River estuary, intertidal sandy flat (Portugal); ES_HL, Elkhorn Slough, Hudson's Landing, sediment (USA); HB, Huntington Beach, surf zone sediment (USA); MX, Bahia del Tobari, Gulf of California, estuarine sediments (Mexico); SBC, coast of Santa Barbara, marine sponge *Axinella mexicana* (USA); SF_CB, San Francisco Bay, Central Bay station 20, sediment (USA); SSFB, South San Francisco Bay, sediment (USA).


0.05
S18-A-15 (S18)
S29-A-11 (S18, S29)
AAZ81167, SF_CB: SF_CB20_11
S33-A-09 (S9, S33)
S33-A-13 (S33)
S7-A-04 (S7, S8, S9, S10, S18, S29, S33)
S7-A-05 (S7, S8, S9, S10, S33)
S10-A-13 (S10)
AAZ81161, SF_CB: SF_CB20_5
S7-A-07 (S7, S18, S29, S33)
S7-A-03 (S7, S8, S9, S10, S18, S29, S33)
S7-A-18 (S7)
S7-A-01 (S7, S8, S9, S10, S18, S29, S33)
AAZ81108, MX: MX_6_6
AAZ81105, MX: MX_6_3
S10-A-10 (S10, S18, 29)
S10-A-11 (S10, S18)
S33-A-12 (S33)
S33-A-10 (S33)
S33-A-14 (S33)
S33-A-06 (S8, S9, S10, S29, S33)
S7-A-15 (S7)
AAZ81058, HB: HB_A_9
S7-A-11 (S7, S33)
S7-A-22 (S7)
S7-A-13 (S7)
S7-A-20 (S7)
ABK77038, SBC: Cenarchaeum symbiosum
S7-A-14 (S7)
S10-A-14 (S10)
S7-A-17 (S7)
S8-A-11 (S8)
S7-A-19 (S7)
S7-A-06 (S7, S8, S9, S10)
S10-A-08 (S8, S9, S10)
S33-A-11 (S33)
S18-A-16 (S18)
S8-A-12 (S8)
S7-A-02 (S7, S8, S9, S10, S18, S29, S33)
S10-A-12 (S10)
ABQ15239, BS: BS100mB6
ABQ15249, BS: BS2-130mD3
AAZ38768, Candidatus Nitrosopumilus maritimus SCM1
ABQ15238, BS: BS100mB4
ABN04033, QY-A33 China: untilled red soil
S7-A-23 (S7)
S7-A-09 (S7, S18, S29)
BAF76833, UAS_020_C.06_2 Germany: upland agricultural soil
ABF56250, MX: MX_4_JAN_1, 2, 3, 6; MX_3_OCT_1, 19; MX_4_15, 24
ABF56323, MX: MX_3_OCT_23
ABF56238, MX: MX_3_JAN_14
ABV53931, FQ-A11 China: sandy loam soil planted maize
ABB83909, SSFB: SouthBay-C1-7 USA: South San Francisco Bay, sediment near the outfall from the Palo Alto WWTP
S18-A-08 (S8, S18)
ABG36624, r0-10_206 Europe: sandy ecosystem soil
ABC43205 Australia: soil
S7-A-24 (S7)
ABN04051, QY-A51 China: red soil fertilized with NPK fertilizer plus organic manure
ABN04034, QY-A34 China: untilled red soil
S7-A-21 (S7)
S7-A-08 (S7, S10, S29)
ABC69009, JR128c2 USA: soil treated with elevated nitrogen
BAF76868, DGGE_AOA_1 Germany: upland agricultural soil
ABF56315, MX: MX_3_OCT_15
ABB83920, SSFB: SouthBay-CR-5
ABF56363, MX: MX_5_JAN_7
CAF28771, Germany: soil; ES_HL: ES_HL_19; DRES: A16; MX: MX_3_OCT_16,17; MX_5_JAN_5,6; SSFB: SouthBay-CR-16
BAF76832, UAS_020_C.05_2 Germany: upland agricultural soil
BAF76877, DGGE_AOA_11 Germany: upland agricultural soil
S7-A-12 (S7, S18)
ABU84997, DRES: A13 Portugal: Douro River estuary, intertidal sandy flat
S18-A-14 (S18)
ABN04043, QY-A43 China: red soil fertilized with NK fertilizer
S7-A-10 (S7, S8, S29)
S29-A-12 (S29)
ABF56303, MX: MX_3_OCT_3
S18-A-13 (S18); MX: MX_5_JAN_8,11
ABF56365, MX: MX_5_JAN_9
ABF56366, MX: MX_5_JAN_10
ABF56310, MX: MX_3_OCT_10
ABC43218 Australia: soil; SF_CB: SF_CB20_2
BAF76872, DGGE_AOA_6 Germany: upland agricultural soil
S7-A-16 (S7)
ABF56239, MX: MX_3_JAN_15
S18-A-12 (S18)
AAA66194, Nitrosomonas europaea
AAB38709, Nitrosospira briensis
Cluster I: coastal sediment and marine water environments
Cluster II: terrestrial environments

sequence similarity among each other, and had quite high identities (95.2–100.0 %) to the closest matched GenBank sequences retrieved from a variety of terrestrial, estuarine, coastal and marine environments. Sequences that were related to soil or coastal sediment environments composed the majority (59.6 %) of our archaeal AmoA phylotypes, while the remaining sequences were related to marine water environments or the marine sponge *Axinella mexicana*.

The constructed phylogenetic tree showed that two AmoA sequence clusters could be identified based on 20 % sequence distance cutoff determined via the DOTUR program (Fig. 2). Sequences associated with coastal sediment (San Francisco Bay, Gulf of California and Huntington Beach) or marine water (Black Sea) environments composed cluster I (Francis *et al.*, 2005; Lam *et al.*, 2007), and sequences having their closest matches mainly from terrestrial environments (Chinese, American, Australian and German soils) composed cluster II (Treusch *et al.*, 2005; Leininger *et al.*, 2006; He *et al.*, 2007), although some estuarine sediment sequences were also affiliated within this cluster (Francis *et al.*, 2005; Beman & Francis, 2006; Park *et al.*, 2006). The putative soil-related sequences of cluster II in our clone libraries mainly occurred at stations S7, S8, S18, S29, and occasionally at station S10. Their relative abundance accounted for 33.3 %, 16.7 %, 37.5 %, 33.3 % and 7.1 % of the RFLP genotypes and 17.2 %, 9.8 %, 13.3 %, 4.3 % and 1.1 % of the clones in each of the corresponding libraries. The change of their relative abundances in the various libraries indicated that the AOA of these sequences might mainly occur in the area close to the Changjiang River mouth.

Eleven sequences (59 clones) were closely (95.2–96.7 % identity) related to the ammonia-oxidizing *Cenarchaeum symbiosum* AmoA sequence (Hallam *et al.*, 2006a). These sequences mainly occurred in sampling stations S7, S8, S9, S10 and S33. Five sequences (109 clones) were closely (94.3–96.2 % identity) related to the AmoA sequence of '*Candidatus* Nitrosopumilus maritimus' SCM1 (Konneke *et al.*, 2005), and they had wide distribution in the Changjiang Estuary and the adjacent East China Sea (Fig. 2).

Nearly 60 % of the AmoA sequences occurred in only one of the sampling stations. Four sequences, S7-A-01, S7-A-02, S7-A-03 and S7-A-04, which were related to Gulf of California and San Francisco Bay sediment and Black Sea water AmoA sequences, occurred in all the stations (Fig. 2). Some other sequences, such as S7-A-05, S33-A-06, S7-A-06 and S7-A-07, which were related to *Cenarchaeum symbiosum* or sedimentary archaeal AmoA sequences from San Francisco Bay and Huntington Beach, occurred in five or four of the sampling stations. These common sequences were also the most abundant ones (80.8 % of the 637 clones screened) in the libraries.

AOA community distribution in response to estuarine gradients

UniFrac environmental clustering of the archaeal *amoA* clone libraries showed clear grouping of the AOA communities in the Changjiang Estuary and the adjacent East China Sea (data not shown). The *amoA* genotype assemblages of stations S8, S9 and S10 on the transect perpendicular to the East China Sea coastline (the 'perpendicular transect' hereafter) were grouped together, those of stations S18, S29 and S33 on the transect aslant to the coastline (the 'slant transect' hereafter) were grouped together, and the *amoA* genotype assemblage of station S7, which was at the intersection of the two transects, formed the third group. PCoA showed similar clustering of the archaeal *amoA* genotype assemblages, and the first two principal coordinates (P1 and P2) explained 78.29 % of the total community variability (Fig. 3). Environmental clustering (data not shown) and PCoA (Supplementary Fig. S2) based on the archaeal AmoA protein sequences showed similar clustering of the sampling stations.

Spatial distribution of the sedimentary AOA communities in the Changjiang Estuary and the adjacent East China Sea may be influenced by river runoff and other related physico-chemical factors. CCA of the archaeal *amoA* genotype assemblages in response to seawater environmental variables confirmed this (Fig. 4a). The first two CCA axes explained 65.2 % of the total variance in the *amoA* genotypes composition and 71.6 % of the cumulative variance of the genotype–environment relationship. However, only surface-water salinity contributed significantly (P=0.015, F=1.92, 1000 Monte Carlo permutations) to the *amoA* genotype–environment relationship, and this factor alone provided 27.8 % of the total CCA explanatory power. The putative soil-related archaeal *amoA* OTUs had very restricted distribution in the CCA graph,

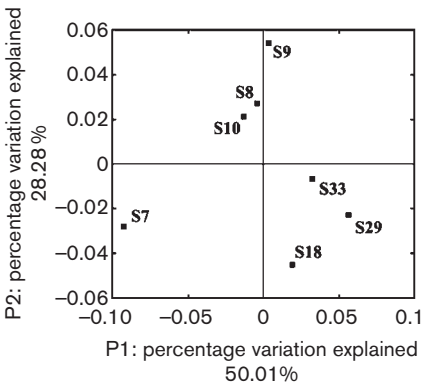


Fig. 3. PCoA of the sedimentary AOA communities with weighted UniFrac using the archaeal *amoA* sequences recovered from the Changjiang Estuary and adjacent East China Sea. Shown is the plot of the first two principal coordinate axes for PCoA and the distribution of the AOA communities (designated with the sampling station names) in response to these axes.

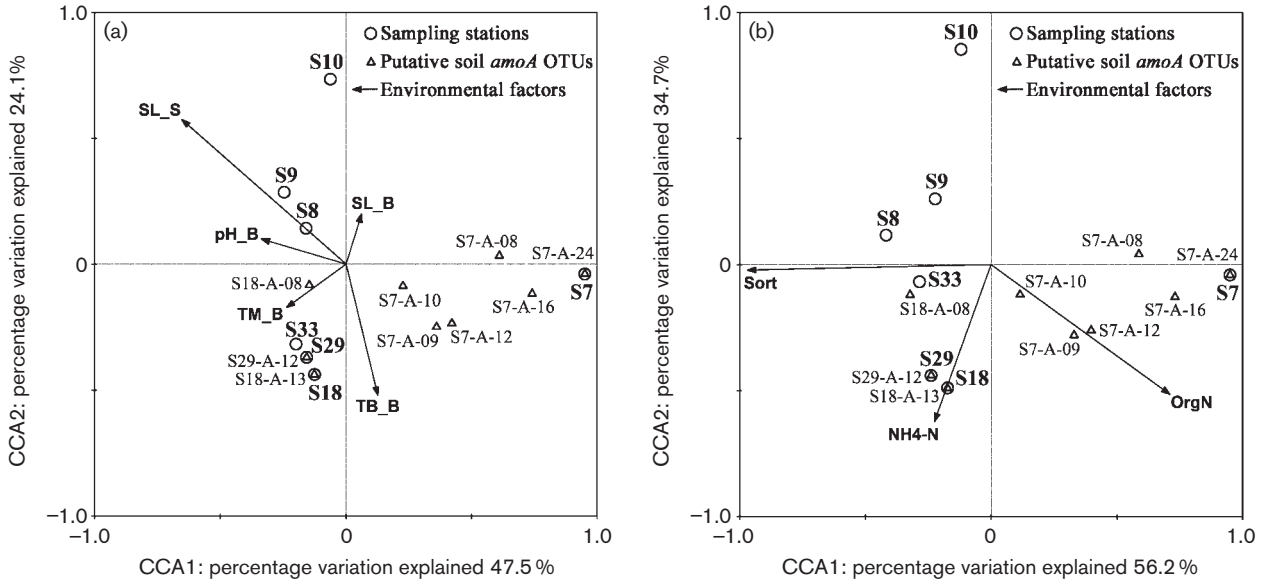


Fig. 4. CCA ordination plots for the first two dimensions of CCA of the relationship between the sedimentary AOA community compositions with (a) the hydrochemical parameters of the surface-water (_S) and bottom-water (_B) environments and (b) the sedimentological and pore-water geochemical parameters in the Changjiang Estuary and adjacent East China Sea. The optimal CCA models represented by the diagrams were produced with manual deselection of collinear variables and automatic forward selection via Monte Carlo permutation significance tests, and with scaling of scores focused on inter-species distances. The eigenvalues of the first two axes (CCA1 and CCA2) were 0.382 and 0.194, respectively, for (a), and 0.366 and 0.226, respectively, for (b). The species–environment correlations of the first two axes (CCA1 and CCA2) were 0.994 and 1.000, respectively, for (a), and 0.985 and 1.000, respectively, for (b). Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors). The distributions of the putative soil-AOA related OTUs in the CCA plots are shown; the other OTUs are not shown to avoid image cluttering. The raw 'CCA' analyses with all the measured environmental factors before model optimizations are shown in Supplementary Figs S3 and S4. Abbreviations: SL, salinity; Sort, sediment sorting coefficient; TB, turbidity; TM, temperature.

being mainly located on the negative half of the surface-water salinity vector (Fig. 4a). The distribution of these OTUs in response to low surface-water salinity indicated that the potential source of these putative soil-related AOA might be freshwater input from the Changjiang River. Although none of the other factors made a statistically significant contribution to the *amoA* genotype–environment relationship ($P>0.200$, 1000 Monte Carlo permutations), the combination of these environmental factors provided additionally 62.3 % of the total CCA explanatory power. Moreover, the bottom-water turbidity seemed to have a positive correlation with the distribution of the putative soil-related *amoA* OTUs.

AOA community distribution in response to sedimentological and geochemical gradients

The first two axes of the CCA of the sedimentary AOA communities versus geochemical and sedimentological factors explained 77.1 % of the total variance in the *amoA* genotypes composition and 90.9 % of the cumulative variance of the genotype–environment relationship (Fig. 4b). However, only the sediment sorting coefficient, a measure of the distribution or variability of particle sizes in the sediment, contributed significantly ($P=0.045$, $F=2.49$, 1000 Monte Carlo permutations) to the *amoA* genotype–environment relationship. This sedimentological factor alone provided 45.6 % of the total CCA explanatory power. Neither NH_4-N nor OrgN made a statistically significant contribution to the microbe–environment relationship ($P>0.25$, 1000 Monte Carlo permutations), although distinct spatial distribution of the AOA assemblages along their gradients could be identified (Fig. 4b). Moreover, the geochemical variable OrgN seemed to have a positive correlation with the distribution of the putative soil-related *amoA* OTUs (Fig. 4b).

DISCUSSION

Most of our putative archaeal *amoA* (especially AmoA) sequences had quite high similarity with known sequences from various soil environments or coastal and estuarine environments of the East Pacific Ocean (Fig. 2) (Francis *et al.*, 2005; Treusch *et al.*, 2005; Beman & Francis, 2006; Hallam *et al.*, 2006a; Leininger *et al.*, 2006; Park *et al.*, 2006; He *et al.*, 2007; Lam *et al.*, 2007). Comparison with the sediment archaeal *amoA* communities of the hypernutri-

fied subtropical Bahía del Tóbari Estuary (Beman & Francis, 2006) indicated that the Changjiang Estuary and the adjacent East China Sea harboured similar AOA diversity. The high sequence similarity between the two estuaries indicates that similar archaeal AOA communities might exist in similar estuarine environments across the Pacific Ocean, despite the great geographical distance.

The Changjiang Estuary is an important interface of the terrestrial and marine environments; it is also highly complex and dynamic. The CDW has been found to split into two branches upon entering the East China Sea near the Changjiang River mouth in summer. One flowed south-eastwards, and the other northeast-northwards (Chen *et al.*, 2003; Zhu *et al.*, 2005). The slow change of surface-water salinity along the slant transect indicated that part of the CDW flowed south-eastwards along the coastline during our sampling period. The sharp increase of surface-water salinity along the perpendicular transect indicated that the Changjiang freshwater runoff in the eastward direction was blocked by the offshore marine water or the northward intrusion of the TWC. Due to these differences, there were differences in the water physico-chemical properties between the two sampling transects. Surface water on the slant transect was more turbid than that on the perpendicular transect (not including station S7). This distinction could also be found in the difference of the sedimentary AOA assemblages (Figs 3 and 4; Supplementary Fig. S2).

The archaeal diversity in estuaries might be greater than that in the adjacent open oceans due to allochthonous terrestrial inputs, of which river water runoff might form the major source of particle-attached archaea in estuarine waters (Crump & Baross, 2000; Wells & Deming, 2003; Wells *et al.*, 2006). Some studies have indicated that the AOA communities in terrestrial environments are distinct from those in marine environments (Leininger *et al.*, 2006), while other studies have indicated that estuaries might harbour mixed populations of both soil and sediment AOA (Francis *et al.*, 2005; Beman & Francis, 2006). The deposition of microbes from the water column with freshwater input could potentially explain the existence of the putative soil-related archaeal AmoA sequences in the estuarine sedimentary environments. In the Changjiang estuarine area, besides freshwater, nutrients, organic matter and suspended particles, the CDW might also contribute to the transport of terrestrial micro-organisms into the seawater and sediments along its flow path. The distribution of terrestrial AOA in estuarine environments might be a common phenomenon based on AmoA phylogenetic analyses (Fig. 2) (Francis *et al.*, 2005; Beman & Francis, 2006), illustrating the potentially important effect of river freshwater on the coastal sedimentary AOA composition. Our results also indicate that the putative soil-related AOA in estuarine sedimentary environments might serve as a bioindicator or biotracer of riverine impact on the coastal benthic microbial ecosystem.

Station S7 had the highest diversity of archaeal *amoA* genotypes among all the sampling stations. This station received the strongest impact of freshwater discharge from the Changjiang River. It was also located in the estuarine mixing zone, and the consequential maximum-turbidity zone of the East China Sea. Particles could be a source of riverine archaea in estuaries (Crump & Baross, 2000; Wells & Deming, 2003; Wells *et al.*, 2006). Particles might also be a source of small organic compounds and potentially serve as a nutrient source for marine archaea, as studies have indicated that some marine crenarchaeota could utilize amino acids heterotrophically (Ouverney & Fuhrman, 2000; Teira *et al.*, 2006). The positive correlation of the S7 station *amoA* genotype assemblage with the sediment OrgN (Fig. 4b), although not statistically significant ($P>0.25$, 1000 Monte Carlo permutations), provides certain clues to the above reasoning. Furthermore, the distribution of the sedimentary archaeal *amoA* genotype assemblages also strongly correlated with surface-water turbidity (Supplementary Fig. S3). However, because of the collinearity between surface-water salinity and turbidity ($r=-0.814$), the exact contributions of these environmental factors could hardly be distinguished from each other. The edge effect of strong riverine–marine water interactions at the sharp estuarine salinity front around the S7 sampling station might provide a unique ecotone for the development and maintenance of a species-rich AOA assemblage (Chen *et al.*, 2003; Ries *et al.*, 2004; Zhu *et al.*, 2005).

Some 30.0 % of our sedimentary AmoA sequences had their closest match to sequences originally retrieved from marine water environments (Lam *et al.*, 2007). This indicates that sediment and seawater might share some common or similar AOA micro-organisms (Beman & Francis, 2006), or that exchange of AOA between sediment and seawater environments might occur in the Changjiang Estuary and East China Sea, via particle deposition, sediment resuspension or other mechanisms. A recent study indicated that the deep-sea sediments might harbour AOA communities distinct from those of the water columns of the deep oceans (Nakagawa *et al.*, 2007). Significant differences might exist between coastal and deep-sea environments in the effect of the water–sediment interactions on the sedimentary AOA communities. High rates of particle deposition and sediment resuspension in coastal waters, especially near large river estuaries, might provide stronger exchange between the water and sediment AOA communities.

Multivariate statistical analyses indicated that the spatial distribution of the sedimentary *amoA* genotype assemblages correlated significantly with the surface-water salinity and sediment sorting coefficient (Fig. 4a, b), indicating potential influences of Changjiang River freshwater runoff and sedimentological condition on the sedimentary AOA community. The influence of the sedimentological condition on the sedimentary AOA community could be complicated. Sedimentological conditions were mainly related to the *in situ* hydrological regime, such as currents, tides, waves, upwelling, lateral

transport, water mixing and exchange, and the intensity and dynamics of these activities. The correlation of the sediment sorting coefficient with archaeal *amoA* genotype assemblages could be related directly or indirectly to hydrological activities, via their impact on the sediment source, composition, organic matter content, pore-water redox, nutrient composition and concentration, and other physico-chemical, sedimentological or geochemical factors. Although we do not know the exact mechanism at present, our work is probably the first to show a direct correlation of a sedimentological factor with the distribution and structure of the sedimentary AOA community in an estuarine and continental shelf environment. However, because of the covariability of some of the environmental factors (Supplementary Figs S3 and S4), the exact influence and contribution of each environmental factor cannot be determined with certainty at present.

In summary, the diversity and spatial distribution of the sedimentary AOA have been studied in the Changjiang Estuary and adjacent East China Sea for the first time via analyses of the functional marker gene *amoA*. Our work indicated that this estuarine area might harbour similar sediment AOA communities to those of the East Pacific Ocean, and the transport of terrestrial AOA via river freshwater runoff might contribute to the composition and diversity of the estuarine sedimentary AOA communities.

ACKNOWLEDGEMENTS

The authors would like to thank Drs Christopher Francis and Michael Beman from Stanford University for their kind provision of information on the archaeal *amoA* primers. The authors would also like to express their appreciation to the three anonymous reviewers and the journal editor, Dr Werner Liesack. With their critiques and suggestions, the manuscript was improved greatly in scientific accuracy. This work was financially supported by the China National Natural Science Foundation grants 40576069 and 40576061, the Hi-Tech Research and Development Program of China grant 2007AA091903, the China Ocean Mineral Resources R&D Association grants DYXM-115-02-2-6 and DYXM-115-02-2-20, and the China National Key Basic Research Program from the Ministry of Science and Technology grant 2002CB412400.

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Edited by: W. Liesack